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Quinolones Induce Partial or Total Loss of Pathogenicity Islands in Uropathogenic *Escherichia coli* by SOS-Dependent or -Independent Pathways, Respectively

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***Escherichia coli* is the most common microorganism causing urinary tract infections. Quinolone-resistant *E. coli* strains have fewer virulence factors than quinolone-susceptible strains. Several urovirulence genes are located in pathogenicity islands (PAIs). We investigated the capacity of quinolones to induce loss of virulence factors such as hemolysin, cytotoxic necrotizing factor 1, P fimbriae, and autotransporter Sat included in PAIs in three uropathogenic *E. coli* strains. In a multistep selection, all strains lost hemolytic capacity at between 1 and 4 passages when they were incubated with subinhibitory concentrations of ciprofloxacin, showing a partial or total loss of the PAI containing the *hly* (hemolysin) and *cnf-1* (cytotoxic necrotizing factor 1) genes. RecA$^-$ mutants were obtained from the two *E. coli* strains with partial or total loss of the PAI. The inactivation of the RecA protein affected only the partial loss of the PAI induced by quinolones. No spontaneous loss of PAIs was observed on incubation in the absence of quinolones in either the wild-type or mutant *E. coli* strains. Quinolones induce partial or total loss of PAIs in vitro in uropathogenic *E. coli* by SOS-dependent or -independent pathways, respectively.**

Escherichia coli is by far the most common cause of urinary tract infections. Uropathogenic *E. coli* (UPEC) strains possess several virulence determinants that allow them to colonize the urinary tract, avoid host defenses, and cause damage to the uroepithelium, which may, in some cases, lead to passage of the bacterium into the bloodstream (3).

Several genes encoding urovirulence factors such as hemolysin (*hly* gene), cytotoxic necrotizing factor type 1 (*cnf-1* gene), P-pili F13 (*pap* genes), S-family adhesions (*sfa* gene), aerobactin (*aer* gene), yersiniabactin (*fyu* gene), some capsule factors (K5), and the autotransporter toxin Sat1 (*sat1* gene) are located in the chromosome and/or plasmids forming clusters named pathogenicity islands (PAIs). These PAIs have been defined for three strains of UPEC, strains 536 (2), J96 (17), and CFT073 (11), and contain several genes encoding virulence factors which may be useful to analyze the presence of PAIs. Some genes, such as the *hly* and *cnf1* genes (PAI-I), the *hly* and *pap* genes (PAI-II), the *sfa* gene (PAI-III), and the *fyu* gene (PAI-IV), are used as PAI markers. Some PAIs, such as PAI-I$_{536}$ and PAI-II$_{536}$ of strain 536, are unstable regions and can be spontaneously released from the chromosome. The *sat* gene has also been shown to be located in PAI-II of the *E. coli* CFT073 strain (5).

Several studies have demonstrated that quinolone-resistant *E. coli* strains have fewer virulence factors than quinolone-susceptible strains (13, 19). These virulence genes include those located in PAI (19). Thus, the aim of this work was to study the capacity of quinolones to induce the loss of virulence factors located in PAIs in UPEC.

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MATERIALS AND METHODS

Bacterial strains. Three UPEC clinical isolates (reference numbers HC14366, HC109, and HC359), belonging to phylotype B2, collected from urine samples in the Hospital Clinic of Barcelona were selected for this study.

MICs. The MICs of ciprofloxacin were determined by Etest (AB Biodisk, Solna, Sweden) following the manufacturer's recommendations. *E. coli* ATCC 25922 and ATCC 35218 were used as the control strains.

Determination of mutations in the quinolone resistance-determining region. The quinolone resistance-determining regions of the *gyrA*, *gyrB*, *parC*, and *parE* genes were amplified by PCR and DNA was sequenced as described in reference 18.

Induction of PAI loss. The selection of nonhemolytic strains was performed by serial passages with ciprofloxacin concentrations of 2 doubling dilutions below and 2 doubling dilutions above the MIC in Luria-Bertani broth in microtiter plates, incubated at 37°C for 24 h. A sample of the well showing growth at the highest quinolone concentration was diluted 1:10^4, and 1 ml of each dilution was spread onto large Columbia blood agar plates (bioMérieux, Spain) and incubated at 37°C for 24 h. An aliquot of the same culture was simultaneously used to prepare a 0.5 McFarland standard dilution and 50 μl was again cultured into the next passage containing each diluted drug. Except when it is mentioned, daily subculturing was done until a nonhemolytic-variant colony was detected in the plates. To test a possible spontaneous loss of PAIs, the wild-type strains were also incubated in antimicrobial-free broth culture and submitted to the same procedure. The assay was done in triplicate to test reproducibility.

PCR procedures. All nonhemolytic colonies found in the blood agar plates were tested by PCR to confirm the loss of the *hlyA* gene, using gene-specific primers (19). The presence of other PAI-related genes, such as *cnf1*, *sat1*, *fyu*, *papA*, *papC*, *papG*, *papEF*, and *sfa* (10), was also determined by PCR. The *hra* gene, encoding a heat-resistant agglutinin, was also tested by PCR using the primers described in reference 9.

PFGE analysis. Some nonhemolytic colonies and the wild-type strains of each case were analyzed by pulsed-field gel electrophoresis (PFGE) with the endonuclease XbaI. DNA bands were transferred by Southern blot to nitrocellulose membranes (Amersham, Barcelona, Spain) and hybridized with *hlyA*-, *cnf1*-, *sfa*-, *papA*-, *sat*- and *hra*-specific probes to determine the location of these genes in the wild-type strain and to confirm their loss in the nonhemolytic derived strains. The probes were synthesized and labeled by PCR using gene-specific primers and the PCR digoxigenin probe synthesis kit (Roche Applied Biosciences, Penzberg, Germany). Membrane developing was performed by colorimetric reaction using 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) reagents (Roche Applied Biosciences, Penzberg, Germany).

TABLE 1. Features of uropathogenic *Escherichia coli* wild-type strains and their derivatives[a,c]

Strain	PAI-related genes	MIC of Cip for wt strain	Expt no. (loss rate), without Cip	Expt no. (passage/ loss rate), with Cip[b]	MIC of Cip for derivatives	VFs lost
HC14366	*hly, cnf1, papA, -C, -EF, -G, fyu, sat1, hra*	0.012	1 ($<10^{-5}$) 2 ($<10^{-5}$) 3 ($<10^{-5}$)	1 ($1/1 \times 10^{-3}$) 2 ($2/2 \times 10^{-3}$) 3 ($4/5 \times 10^{-3}$)	0.12 0.032 0.016	*hly, cnf1, sat1* *hly, cnf1, sat1* *hly, cnf1, sat1*
HC109	*hly, cnf1, papA, -C, -EF, -G, sfa, iroN, fyu, hra*	0.008	1 ($<10^{-5}$) 2 ($<10^{-5}$) 3 ($<10^{-5}$)	1 ($4/1 \times 10^{-3}$) 2 ($1/7 \times 10^{-4}$) 2 ($1/4.8 \times 10^{-4}$) 3 ($1/2.6 \times 10^{-4}$)	0.016 0.032 0.032	*hly, cnf1, hra* *hly, cnf1, hra* *hly, cnf1, hra*
HC359	*hly, cnf1, papA, -C, -EF, -G, sfa, iroN, fyu, hra*	0.006	1 ($<10^{-5}$) 2 ($<10^{-5}$) 3 ($<10^{-5}$)	1 ($4/1 \times 10^{-4}$)* 2 ($2/4 \times 10^{-4}$)* 3 ($4/2.8 \times 10^{-4}$)*	0.19 0.06 0.19	*hly, cnf1, hra* *hly, cnf1, hra* *hly, cnf1, hra*
HC14366-*recA* mutant	*hly, cnf1, papA, -C, -EF, -G, fyu, sat1, hra*	0.012	1 ($<10^{-5}$) 2 ($<10^{-5}$) 3 ($<10^{-5}$)	1 ($<10^{-5}$) 2 ($<10^{-5}$) 3 ($<10^{-5}$)	0.016 0.016 0.016	
HC109-*recA* mutant	*hly, cnf1, papA, -C, -EF, -G, sfa, iroN, fyu, hra*	0.008	1 ($<10^{-5}$) 2 ($<10^{-5}$) 3 ($<10^{-5}$)	1 ($2/5 \times 10^{-4}$) 2 ($2/1 \times 10^{-4}$) 3 ($2/3 \times 10^{-4}$)	0.064 0.032 0.032	*hly, cnf1, hra* *hly, cnf1, hra* *hly, cnf1, hra*

[a] Cip, ciprofloxacin; wt, wild type; VFs, virulence factors.
[b] *, isolates showing a mutation in the Ser-83 codon of the *gyrA* gene.
[c] MICs are in mg/liter.

Knockout construction. The *recA* gene was amplified by PCR using the primers recA-K1 (AAAAGATCTGACGATTAAAAATC) and recA-K2 (CCCGGATCCATGGCTATCGACG), which contained a BglII and BamHI cut point, respectively. The fragment obtained was cut with these two endonucleases and cloned into the pUK21 vector. The cassette that encoded a tetracycline resistance gene contained within the plasmid pHP45Ω-Tc (4) was introduced into the *recA* gene, and the colonies which presented this construction were selected. Finally, the construction was cloned into the suicide vector pIVET8 (12) and introduced by transformation into the HC14366 and HC109 wild-type *E. coli* strains. The ampicillin- and tetracycline-resistant colonies were tested to confirm the presence of the plasmid. The positive colonies were grown in Luria-Bertani broth supplemented with tetracycline to force the second crossover. The tetracycline-resistant but ampicillin-susceptible colonies were analyzed by PCR to confirm the loss of the pIVET8 plasmid and the presence of the *recA* knockout gene.

RESULTS

The three strains included in this study lost some PAI-associated genes when exposed to subinhibitory concentrations of ciprofloxacin (Table 1). All three strains belonged to the B2 phylotype and were hemolytic and quinolone susceptible. Wild-type strains showed MICs of ciprofloxacin ranging from 0.006 to 0.012 mg/liter.

The three strains lost the hemolytic capacity between passages 1 and 4 in the presence of ciprofloxacin (Table 1). All the nonhemolytic colonies growing on the Columbia blood agar plates seeded with cultures exposed to subinhibitory concentrations of ciprofloxacin were studied. The loss rate was between 1×10^{-4} and 5×10^{-3}. The MICs of ciprofloxacin for the nonhemolytic isolates were determined by Etest and the values are shown in Table 1. Only the isolates with a MIC of ciprofloxacin of ≥0.06 mg/liter presented a mutation in the codon for Ser-83 of the *gyrA* gene. An increase in the number of nonhemolytic colonies was found in the next passage of the procedure (1×10^{-3} in passage 1 and 8.1×10^{-3} in passage 2 for the HC14366 strain; 4×10^{-4} in passage 2 and 5.6×10^{-3} in passage 3 for the HC359 strain).

To determine the frequency at which a loss of hemolytic activity could happen in the absence of quinolone exposure, the wild-type strains were submitted to the same procedure using antimicrobial-free culture medium. No colonies without hemolytic capacity were found after 15 passages in the three different experiments, with the frequency being $<10^{-5}$.

Two procedures were undertaken to determine whether the loss of hemolytic activity was due to a partial or total loss of PAIs: (i) PCR using specific primers for the PAI-associated genes and (ii) hybridization of PFGE profiles using gene-specific probes obtained by PCR. In the first approach, the nonhemolytic strains derived from the incubation of the HC14366, HC359, and HC109 strains with ciprofloxacin lacked both the *hlyA* and *cnf1* genes; HC14366 derivatives also lost the *sat1* gene. Five HC109 nonhemolytic derivatives obtained in the presence of ciprofloxacin were positive for the *hlyA* gene but did not show hemolytic capacity in the blood agar plates (Table 1). All three *E. coli* wild-type strains showed the *hra* gene, which was not present in the HC109 and HC359 nonhemolytic derivatives, whereas the HC14366 nonhemolytic derivative strains did not lose this gene.

PFGE was carried out using the endonuclease XbaI. Clear differences were observed between the HC14366 wild-type strains and their derivatives, which lost a fragment of about 130 kb (Fig. 1A). The DNA bands were transferred onto nitrocellulose membranes by Southern blotting and hybridized with *hly*, *cnf*, *sfa*, *papA*, *sat*, and *hra* gene probes. The following observations were derived from this analysis. In the HC14366 wild-type strain, the *hly*, *cnf*, and *hra* probes hybridized in the same XbaI fragment of about 400 kb and the *sat* probe hybridized in a fragment of 130 kb (Fig. 1B and D) while the non-

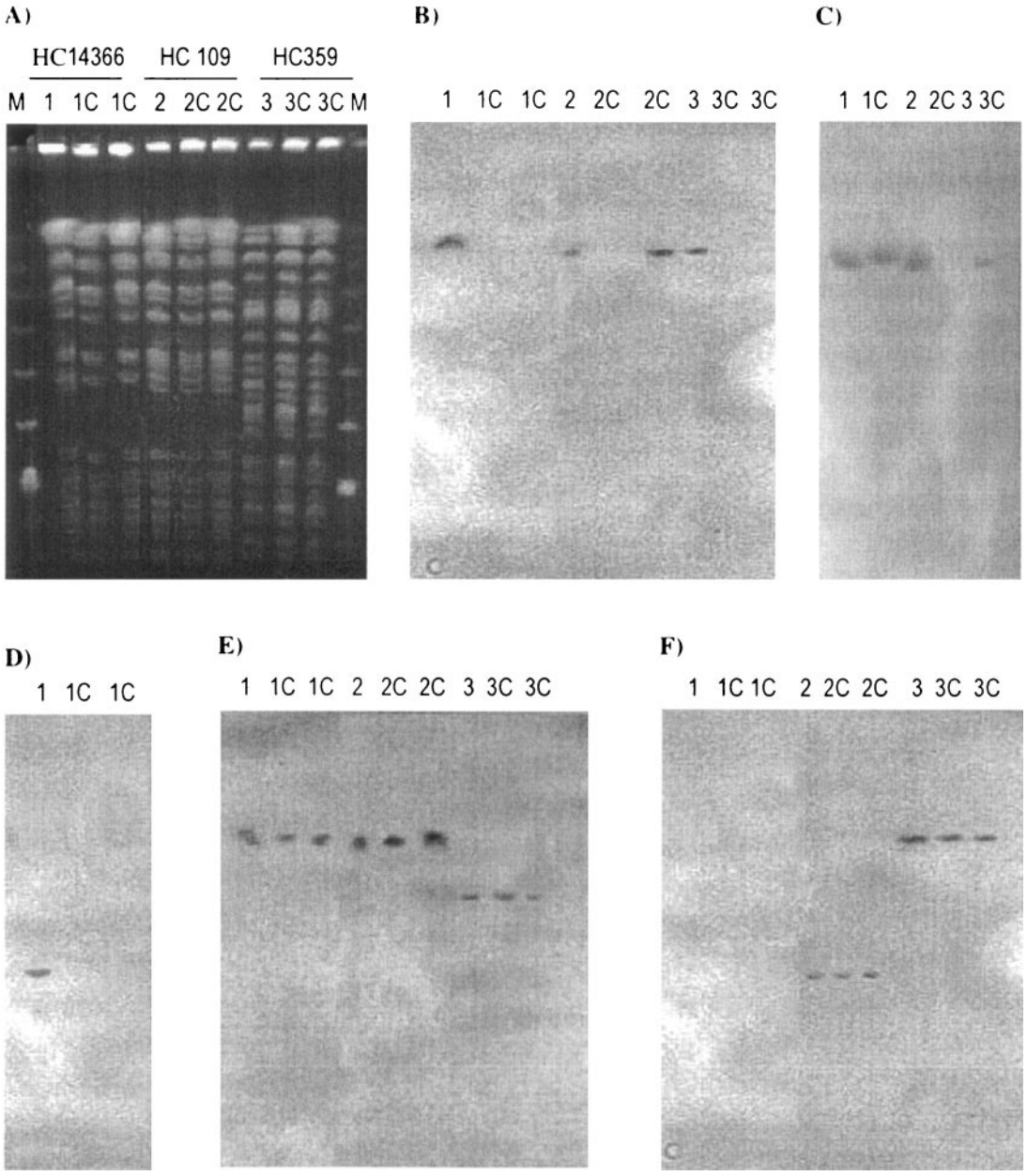

FIG. 1. PFGE with XbaI of wild-type strains and their derivatives. (A) PFGE profiles are shown. For all panels: lane 1, HC14366 wild-type strain; lanes 1C, nonhemolytic derivatives from the HC14366 strain selected with ciprofloxacin; lane 2, HC109 wild-type strain; lanes 2C, nonhemolytic derivatives from the HC109 strain; lane 3, HC359 wild-type strain; lanes 3C, nonhemolytic derivatives from the HC359 strain. Lane M, molecular size marker. Also shown are hybridization of PFGE profiles with the *hlyA* and *cnf1* probes (B), *hra* probe (C), *sat1* probe (D), *papA* probe (E), and *sfa* probe (F).

hemolytic strains derived from ciprofloxacin lacked the hybridization signal for the *hly*, *cnf*, and *sat* probes, except for the *hra* gene probe (Fig. 1C). The 130-kb fragment containing the *sat* gene did not appear in the nonhemolytic derivatives from this strain. To confirm this result, this fragment was purified from the agarose gel and used as a DNA template in a PCR with the *sat* gene-specific primers. The PCR product obtained was sequenced, confirming the location of the *sat* gene in this DNA fragment. In the HC109 and HC359 wild-type strains, the *hly*, *cnf*, and *hra* probes hybridized with a DNA fragment of 380 kb. The HC359 and some HC109 derivatives did not present a hybridization signal using the *hly* and *cnf* (Fig. 1B) and *hra* (Fig. 1C) probes. The *papA* probe hybridized with a fragment of about 400 kb in the HC14366 wild-type strain and its derivatives, with a fragment of about 225 kb in the HC359 wild-type strain and its derivatives, and with a fragment of about 380 kb in the HC109 wild-type strain and its derivatives (Fig. 1E). Finally, the *sfa* probe hybridized with DNA fragments of about 125 and 380 kb in the HC109 wild type and derivatives and in the HC359 wild type and derivatives, respectively (Fig. 1F).

recA knockout mutants were obtained from the HC14366 and HC109 *E. coli* strains. When the *recA* mutant of the HC14366 *E. coli* strain was submitted to subinhibitory ciprofloxacin concentrations, no hemolysin activity was lost after 15 passages. However, when the *recA* mutant of the HC109 *E. coli* strain was submitted to the same procedure, hemolysin activity was lost after two passages, at a rate of 1×10^{-4} to 5×10^{-4} (Table 1). The presence of the *hra*, *hly*, and *cnf-1* genes was determined in these hemolysin-negative HC109 derivatives by PCR, indicating that this derivative lost the three genes.

DISCUSSION

PAIs represent distinct large chromosomal regions that contribute to the evolution of bacterial pathogens (7). Several studies had been made to determine the frequency and the mechanisms involved in the instability and spontaneous loss of PAIs. Middendorf et al. (14) recently reported that the frequency of loss depends on the PAI, finding values of 2×10^{-6}, 2×10^{-5}, 5×10^{-5}, and 1×10^{-6} for PAI-I_{536}, PAI-II I_{536}, PAI-III_{536}, and PAI-V_{536}, respectively, when the strains were grown in LB medium at 37°C, with PAI-IV_{536} being apparently stable under these circumstances. These authors suggested that the stability of PAI-IV was due to the absence of direct repeats flanking this PAI. In three replicate experiments we were unable to confirm the spontaneous loss of PAIs during growth in drug-free Luria-Bertani broth.

Middendorf et al. (14) also analyzed the possible deletion of PAIs in the *E. coli* 536 strain induced by different environmental conditions, such as temperature, osmotic stress, nutrient-limiting conditions, or growth in artificial urine. They found that only low temperature and high cell density increased loss of PAI-II_{536}, while the deletion rates of PAI-I and PAI-V were not affected.

It is well known that resistance to quinolones in *E. coli* is an increasing problem in Spain and other countries (13). We previously observed (19) that quinolone-resistant UPEC strains express fewer virulence factors than quinolone-susceptible strains, and we have recently found that this phenomenon might be particularly frequent among strains of the B2 phylotype (8). Among the virulence factors, hemolysin and cytotoxic necrotizing factor were more frequently found in quinolone-susceptible than in quinolone-resistant *E. coli* isolates (19). These virulence factors are located in one or two PAIs, depending on the strain. Herein, we found that quinolones increase the frequency of loss of some PAIs in phylotype B2 *E. coli* strains. The loss of PAI-I was observed, whereas the loss of PAI-IV was not. Spontaneous loss of hemolytic capacity was not observed in any of the three tested strains in the absence of quinolone exposure. After quinolone exposure, some derivatives from the HC109 strain had lost the capacity to produce hemolysis in blood agar but they had not lost the *hly* gene. This fact could be in accordance with the results obtained by Hacker et al. (6), who found two types of hemolysin-negative mutants, one that did not show external and internal hemolysin activity (type I, Hly^{ext-}/Hly^{int-}) and another that presented internal hemolysin but not external expression (type II, Hly^{ext-}/Hly^{int+}). The type II mutants presented a mutation in the *hlyB* gene, which was involved in the transportation of hemolysin toxin out of the cell. These findings may explain the phenomenon observed in some HC109 nonhemolytic strains obtained in our study; however, the possibility of mutations in other components of the hemolysin operon is not discarded. Although showing a slight increase with respect to the wild-type strain, the MICs of ciprofloxacin for the nonhemolytic derivatives remained below 1 mg/liter. This is clinically important, since ciprofloxacin may decrease bacterial virulence without selection of resistant populations.

We did not observe a loss of PAI-III (*sfa*). However, several studies have demonstrated that an entire PAI deletion (type I deletion) or that of a portion in the center of PAI-III including the *sacB* insertion site (type II deletion) may occur (14).

The *hra* gene, which was located in the extreme of PAI-II in the J96 UPEC strain, was used as a marker to determine the total or partial loss of the PAI. In the present study, we observed the following two phenomena. In two of the strains, HC109 and HC359, the *hra* gene was not presented by its nonhemolytic derivatives, indicating the total loss of the PAI. However, the nonhemolytic derivatives from the HC14366 strain did present this gene, indicating a partial loss of the PAI.

The deletion processes may play a role in the adaptation of UPEC during certain stages of infection. Therefore, the genetic flexibility of pathogenic microbes may create selective advantages over other, less flexible organisms and may result finally in proper replication in host organisms or other ecological niches (1).

Further studies were performed to elucidate the mechanism involved in the induction of the loss of PAIs by quinolones. The role of the SOS system was analyzed because it is well known that quinolones induce the SOS system response (DNA repair mechanism) (15), which could favor the splitting of bacteriophages or related sequences from the chromosome. Shaikh and Tarr (16) demonstrated that quinolones promote the excision of stx_2 bacteriophages as well as complete and truncated stx_1 bacteriophages. In our study, in the *recA* mutant of the HC14366 *E. coli* strain, no loss of hemolysin was observed after incubation with subinhibitory concentrations of ciprofloxacin for 15 passages, suggesting that a complete SOS system is needed for quinolones to induce the partial loss of this PAI. On the other hand, in the *recA* mutant of the HC109 *E. coli* strain, total loss of the PAI induced by quinolones was observed, suggesting an SOS-independent pathway to induce this loss.

In conclusion, these findings suggest that subinhibitory concentrations of quinolones induce the partial or total in vitro loss of PAIs in UPEC strains. This partial or total loss of PAIs induced by quinolones can be observed to occur by an SOS-dependent or -independent pathway, respectively. Quinolone resistance is not necessary for *E. coli* strains to lose PAIs; however, quinolone-resistant *E. coli* strains have likely been in previous contact with these antimicrobial agents, thus favoring the loss of the PAI.

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