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Macrolide Resistance in *Helicobacter pylori*: Rapid Detection of Point Mutations and Assays of Macrolide Binding to Ribosomes

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Resistance of *Helicobacter pylori* to macrolides is a major cause of failure of eradication therapies. Single base substitutions in the *H. pylori* 23S rRNA genes have been associated with macrolide resistance in the United States. Our goal was to extend this work to European strains, to determine the consequence of this mutation on erythromycin binding to *H. pylori* ribosomes, and to find a quick method to detect the mutation. Seven pairs of *H. pylori* strains were used, the parent strain being naturally susceptible to macrolides and the second strain having acquired an in vivo resistance during a treatment regimen that included clarithromycin. The identity of the strains was confirmed by random amplified polymorphic DNA testing with two different primers, indicating that resistance was the result of the selection of variants of the infecting strain. All resistant strains were found to have point mutations at position 2143 (three cases) or 2144 (four cases) but never on the opposite DNA fragment of domain V of the 23S rRNA gene. The mutation was A→G in all cases except one (A→C) at position 2143. Using *Bsa*I and *Bbs*I restriction enzymes on the amplified products, we confirmed the mutations of A→G at positions 2144 and 2143, respectively. Macrolide binding was tested on purified ribosomes isolated from four pairs of strains with [^{14}C]erythromycin. Erythromycin binding increased in a dose-dependent manner for the susceptible strain but not for the resistant one. In conclusion we suggest that the limited disruption of the peptidyltransferase loop conformation, caused by a point mutation, reduces drug binding and consequently confers resistance to macrolides. Finally, the macrolide resistance could be detected without sequencing by performing restriction fragment length polymorphism with appropriate restriction enzymes.

Helicobacter pylori, a gram-negative bacterium which colonizes the human stomach, has been the focus of important research in recent years. *H. pylori* infection induces chronic gastritis (16) which can lead to duodenal and gastric ulcers (19) as well as to malignancies such as gastric carcinoma and lymphoma (9). The importance of eradication of the bacteria and the resulting cure of peptic ulcer disease was confirmed at a Consensus Conference organized by the National Institutes of Health in 1994 (23). More recently a European Consensus report recommended a triple therapy, associating a potent antisecretory drug with two antibiotics, as the first-choice treatment (8).

Clarithromycin is a component of the most widely used regimens. However, macrolide resistance in *H. pylori* has been shown to occur at different rates (1 to 10%) in different countries (17) and is an important cause of the failure of these regimens. Moreover, *H. pylori* mutants resistant to macrolides are easily obtained by in vitro selection (12). Macrolides inhibit protein synthesis, stimulating dissociation of peptidyl-tRNA from the ribosome during the elongation reaction (21).

Macrolide resistance is due to several mechanisms including lack of macrolide binding to the ribosome target and, to a lesser extent, macrolide inactivation by enzymes, impermeability of the bacterial membrane, and active drug efflux (35). This last mechanism is apparently of importance in *Streptococcus pyogenes* and *Streptococcus pneumoniae* (4, 26, 30). The target modification usually involves a posttranscriptional modification or a mutation situated in the peptidyltransferase domain (domain V) of the 23S rRNA (14), while mutations of the ribosomal proteins have also been described (35). More recently, macrolide resistance in *Escherichia coli* (27, 34), *Mycoplasma pneumoniae* (15), *Mycobacterium intracellulare* (20), and *Mycobacterium avium* (22) has also been linked to a point mutation in the 23S rRNA. This mutation (A→G at positions 2143 or 2144, formerly labelled 2058 and 2059, respectively) was found for the first time by Versalovic et al. in *H. pylori* strains isolated in the United States (33).

The aim of this study was to test the consequences of these mutations on macrolide binding to *H. pylori* ribosomes and to develop a method which would be easier than sequencing to detect these mutations.

MATERIALS AND METHODS

Bacterial strains. The strains were isolated from patients consulting at St. André Hospital, Bordeaux, France, who suffered from duodenal ulcer disease or nonulcer dyspepsia. Gastric biopsies were ground and then cultured on Wilkins Chalgren agar (Oxoid, Basingstoke, United Kingdom) enriched with 10% human blood and containing vancomycin (10 mg/liter), cefsulodin (2 mg/liter), trimethoprim (5 mg/liter), and cycloheximide (Acti-Dione; 100 mg/liter) and on pylori agar (bioMérieux, Marcy-l'Etoile, France). Plates were incubated for 3 to 7 days in a microaerobic atmosphere at 37°C. Identification at the species level was based on morphology and the presence of oxidase, catalase, and urease. Seven pairs of *H. pylori* strains, in addition to reference strain CIP 101260 from the Collection de l'Institut Pasteur, were included in this study. For each pair of strains, the second strain was isolated at a follow-up visit after an unsuccessful treatment that included clarithromycin.

Determination of MICs. MICs of clarithromycin were determined by the standard agar dilution technique. Briefly, a suspension of each strain (approximately 10^9 CFU/ml) was inoculated, with a Steers apparatus (28), onto plates containing Wilkins Chalgren agar enriched with 10% sheep blood and Polyvitex, as well as clarithromycin at concentrations ranging from 0.0035 to 128 mg/liter. Incubation was performed in a microaerobic atmosphere for 48 h at 37°C.

The strains were also screened for resistance to other macrolides including

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TABLE 1. Agar dilution MICs of clarithromycin and mutations of *H. pylori* isolates investigated

Patient or strain	Date of specimen collection (day/mo/yr)	Isolate	Clarithromycin MIC (μg/ml)	23S rRNA domain V
Ia	02/07/92	594S	0.06	Wild type
Ib	23/10/92	677R	32	2144A→G[a]
IIa	07/09/92	638S	0.06	Wild type
IIb	19/10/92	675R	32	2144A→G
IIIa	14/09/92	646S	0.06	Wild type
IIIb	04/11/92	683R	16	2143A→G
IVa	30/04/93	764S	0.06	Wild type
IVb	11/06/93	782R	64	2144A→G
Va	02/08/93	803S	0.06	Wild type
Vb	05/10/95	825R	>128	2143A→C
VIa	22/11/93	848S	0.03	Wild type
VIb	02/01/95	1060R	>128	2143A→G
VIIa	12/12/94	1054S	0.125	Wild type
VIIb	16/01/95	1069R	16	2144A→G
CIP 101260				Wild type

[a] 2144A→G, A→G mutation at position 2144.

14-carbon ring (erythromycin, roxithromycin), 15-carbon ring (azithromycin), and 16-carbon ring (spiramycin) compounds and related compounds; lincosamides (lincomycin, clindamycin); and streptogramins (pristinamycin), as well as tetracycline and chloramphenicol. The two components of streptogramins were tested together. Susceptibility testing was performed by using the E test (AB Biodisk, Solwa, Sweden) except for testing of spiramycin, lincomycin, and pristinamycin for which only disks (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) were used.

Random amplified polymorphic DNA (RAPD) testing. Genomic DNA was isolated from lysed *H. pylori* cells following phenol-chloroform extraction and ethanol precipitation as described previously (13). Amplification was carried out in a 25-μl volume containing 1 μg of *H. pylori* genomic DNA, 67 mM Tris-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 1.5 mM $MgCl_2$, a 0.4 mM concentration of deoxynucleoside triphosphate mixture, a 5 μM concentration of primer, 1 U of *Taq* DNA polymerase (Eurobio, Les Ulis, France), and sterilized water. Each reaction mixture was overlaid with 50 μl of mineral oil. Two primers were used: 3881 (5′-AAC GCG CAA C-3′) (1) and OPH8 (5′-GAA ACA CCC C-3′) (Bioprobe Systems, Montreuil, France). A Perkin-Elmer (Foster City, Calif.) 480 thermal cycler was used for amplification. The cycling program was composed of 1 cycle at 94°C for 10 min; 40 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 1 min; and a final incubation at 72°C for 10 min. Then, 20-μl aliquots of PCR products were separated by electrophoresis in 1.5% agarose gels containing 1 μg of ethidium bromide per ml, and the gel was photographed under UV light. The DNA of ΦX174 *Hae*III was used as a size marker in all gels.

Detection of mutations. (i) PCR amplification. Two pairs of primers were used to amplify two fragments of the peptidyltransferase region of the 23S rRNA. The sequences of the primers were based on the published sequence of the 23S ribosomal DNA gene of *H. pylori* (GenBank accession no., U27270). A primer extending from position 1820 to 1839 (5′-CCA CAG CGA TGT GGT CTC AG-3′) and a reverse primer from position 2244 to 2225 (5′-CTC CAT AAG AGC CAA AGC CC-3′) were used to amplify a fragment (A) of 425 bp. A primer from position 2415 to 2433 (5′-GCA CAA GCC AGC CTG ACT G-3′) and a reverse primer from position 2828 to 2810 (5′-AGC AGT TAT CAC ATC CGT G-3′) were chosen to amplify a fragment (B) of 414 bp. PCR amplification of DNA was performed in a final volume of 100 μl containing 1 μg of *H. pylori* genomic DNA, 67 mM Tris-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 1.5 mM $MgCl_2$, a 0.2 mM concentration of deoxynucleoside triphosphate mixture, 1 μM concentrations of primers, and 2 U of *Taq* DNA polymerase. Each reaction mixture was overlaid with 50 μl of mineral oil. The cycling program was 1 cycle at 94°C for 10 min; 30 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min; and a final elongation step at 72°C for 10 min. The two reactions resulted in a single fragment of the expected size.

(ii) Sequencing of domain V of the 23S rRNA gene. Before sequencing, PCR products (200 to 300 μl from each strain) were prepared by purification and concentration with Wizard PCR Prep columns (Promega, Madison, Wis.) and were resuspended in 50 μl of H_2O. The same primers used for PCR amplification of fragments A and B were used for sequencing. Sequencing was performed on the two strands of each amplicon with an automated DNA sequencer and the *Taq* DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer).

(iii) PCR-RFLP. We took advantage of the occurrence of restriction sites for specific enzymes to perform PCR-restriction fragment length polymorphism (RFLP) as a rapid method of detection of the mutation.

The method used to detect the mutations without sequencing involved restriction of the PCR products. Ten microliters of the fragment A amplicon (425 bp) were treated with enzyme *Bsa*I or *Bbs*I (New England Biolabs, Beverly, Mass.). The fragments were incubated for 24 h at 56°C for *Bsa*I and at 37°C for *Bbs*I in order to detect the restriction site occurring when the mutation was A→G at position 2144 or at position 2143, respectively.

Ribosome isolation and erythromycin binding assays. Studies of the binding of radiolabelled antibiotics to ribosomes, isolated from four pairs of strains by the filter binding method, were carried out as described by Goldman et al. (11) and Doucet-Populaire et al. (6) with slight modifications. Briefly, cells were grown on Wilkins Chalgren blood agar, suspended in brucella broth, centrifuged (4000 × *g* for 15 min), and washed in phosphate-buffered saline and in buffer (10 mM Tris-HCl, 4 mM $MgCl_2$, 10 mM NH_4Cl, 100 mM KCl, pH 7.2). Cell lysis was performed by sonication (five cycles of 60 s each with a 60% active cycle) with a Soniprep 150 sonicator (Bioblock, Vanves, France) at 20 kHz, and ribosome extraction was performed by differential centrifugation (30,000 × *g* for 30 min at 4°C followed by centrifugation of the supernatant at 100,000 × *g* for 30 min). The binding of [*N-methyl*-^{14}C]erythromycin (200 nM) to increasing concentrations of 70 S ribosomes (optical density, 1, 2, 3, and 4) was performed as described by Doucet-Populaire et al. (6).

RESULTS

MIC determination. The isolate obtained from each of the seven patients prior to treatment was susceptible to all antibiotics tested (MIC, 0.03 to 0.125 mg/liter). The clarithromycin MIC for the second isolate obtained from each patient after an unsuccessful treatment with a regimen including clarithromycin exhibited a marked increase (to >2 mg/liter) (Table 1). Cross-resistance to the other macrolides and lincosamides screened by E test and disk diffusion was found. In contrast, the isolates remained susceptible to pristinamycin with one exception (isolate 825). All the strains were susceptible to tetracycline and chloramphenicol.

RAPD fingerprinting. The profiles obtained were similar for each pair of strains but different from one pair to the other (Fig. 1), indicating the persistance of the same strain despite the treatment received.

Detection of mutations. The complete sequence of the peptidyltransferase region of the 23S rRNA gene was determined for each isolate by sequencing 800 nucleotides (fragments A and B). A mutation in domain V of the 23S rRNA was detected in all isolates which were resistant to macrolides and lincosamides but in none of the parent strains which were susceptible to the agents (Table 1). Mutations were found in positions which are homologous to positions 2058 and 2059 of *E. coli*. They were transition mutations (A→G at position 2144 [four cases] and at position 2143 [two cases]) and transversion mutation A→C at position 2143 [one case]).

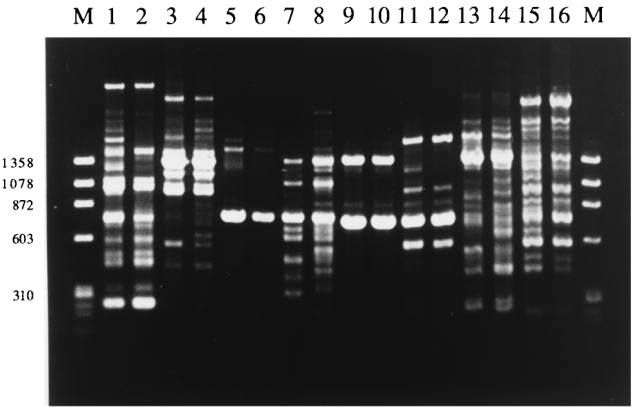

FIG. 1. RAPD patterns (primer 3881) of seven pairs of macrolide-susceptible (S) pretreatment and macrolide-resistant (R) posttreatment *H. pylori* isolates. Lanes: 1, 594S; 2, 677R; 3, 638S; 4, 675R; 5, 646S; 6, 683R; 7, 764S; 8, 782R; 9, 803S; 10, 825R; 11, 848S; 12, 1060R; 13, 1054S; 14, 1069R; 15 and 16, CIP 101260; M, DNA marker (ΦX 174 *Hae*III).

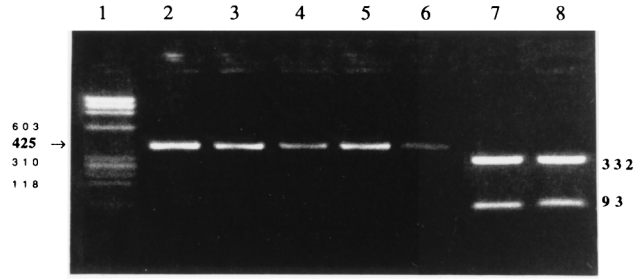

FIG. 2. Restriction profiles of fragment A treated with *Bbs*I for three pairs of *H. pylori* strains. Lanes: 1, DNA marker (ΦX174 *Hae*III); 2, amplicon of CIP 101260 strains; 3, 803; 4, 646; 5, 848 (susceptible wild-type strain); 6, 825 (resistant mutant [A→C at position 2143] strain); 7, 683; 8, 1060 (resistant mutant [A→G at position 2143] strain).

It was possible to detect the A→G transition mutations at positions 2144 and 2143 by restriction with *Bsa*I and *Bbs*I, respectively, as expected from the determined sequence (Fig. 2). However, this strategy failed to detect the transversion A→C.

Binding of [*N-methyl*-^{14}C]erythromycin to *H. pylori* ribosomes. Figure 3 presents the results obtained for the four pairs of strains tested. There was an increased binding of [*N-methyl*-^{14}C]erythromycin when the quantity of ribosomes of the susceptible parent strain increased, while virtually no binding was observed with ribosomes from the derived resistant strains.

DISCUSSION

Following the recognition of the important pathogenic role of *H. pylori* infection in the development of gastroduodenal diseases, there has been a continuous search for improved eradication therapy. Clarithromycin emerged as one of the antibiotics of choice because of its low MIC, which is relatively unaffected by lowering the pH, as well as its high concentration in gastric mucosa. In binding experiments the tightest interaction for a macrolide-ribosome complex observed to date was found for the binding of clarithromycin to *H. pylori* ribosomes (10, 11). However, clarithromycin used as a single antibiotic cannot eradicate more than 70% of strains, and resistant strains have been isolated from patients who were not cured.

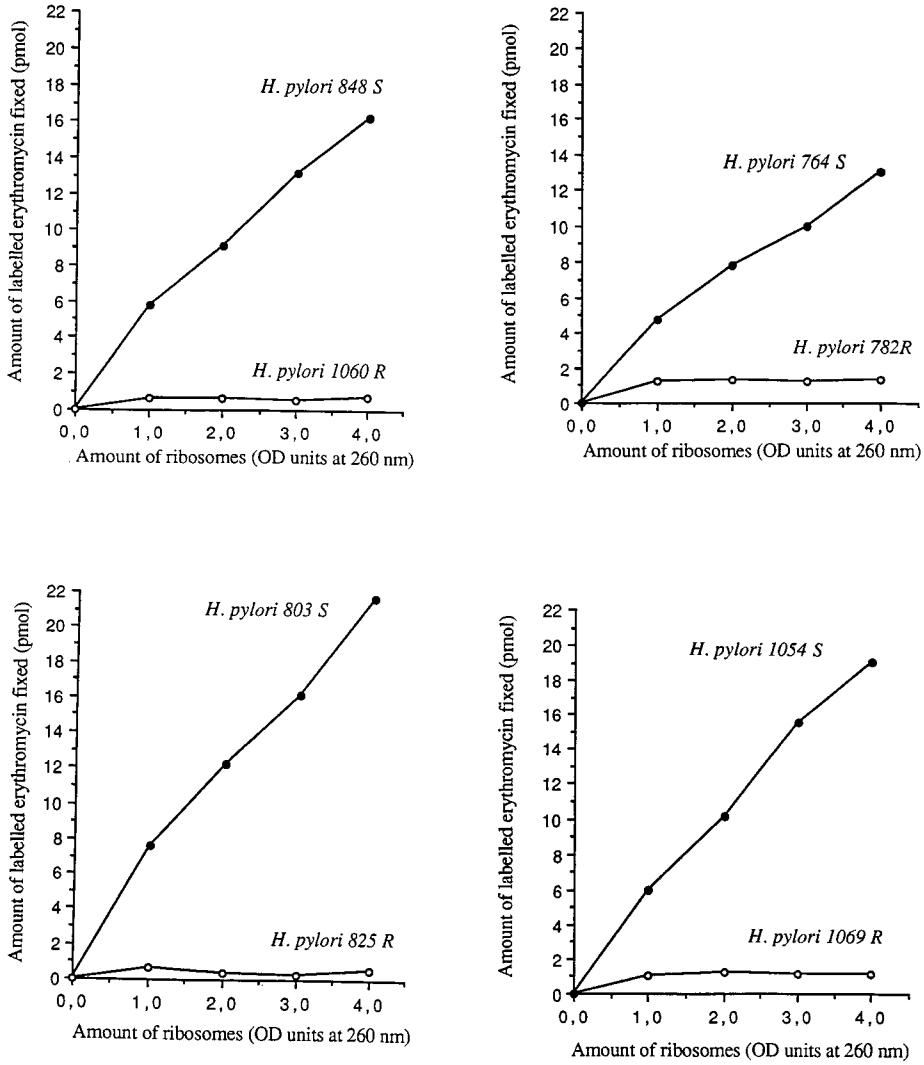

FIG. 3. Binding of [*N-methyl*-^{14}C]erythromycin (200 nM) to increasing concentrations of 70 S ribosomes (optical densities [OD], 1, 2, 3, 4) purified from four pairs of clarithromycin-susceptible and -resistant *H. pylori* isolates.

When a second antibiotic was added, the success rate increased to around 90% but resistant strains were still isolated from case failures (17). Furthermore, the actual level of resistance observed (between 1 and 10%, depending on the country) jeopardizes the success of therapy and seems to be related to the extent of consumption of macrolides. It is hypothesized, but not proven, that the use of macrolides to treat respiratory infection leads to resistance in *H. pylori*. Data from France where the consumption of macrolides for treating respiratory tract infection has been at a high level since 1982 have shown a stable resistance frequency in the range of 10% since 1985 (18).

This important problem of clarithromycin resistance led Versalovic et al. (33) to explore the genetic basis of the phenomenon in *H. pylori*. It had been reported that a mutation in the 23S rRNA peptidyltransferase domain was associated with macrolide resistance in *E. coli* (27, 34), *M. avium* (22), *M. intracellulare* (20), and *Mycoplasma pneumoniae* (15). Versalovic et al. studied 12 strains isolated from seven patients in the U.S. and also found that the peptidyltransferase loop of 23S rRNA contained a mutation. They described point mutations at positions 2143 (three cases) and 2144 (four cases). In a recent study performed in The Netherlands (5) five cases of each of these two mutations were described. In our study we confirmed that these mutations are also found in France, a country with a relatively high prevalence of clarithromycin resistance (10%). Furthermore, recent research on rRNA methylases using a conserved-primer PCR assay (2) did not reveal any evidence of *erm*-like genes in *H. pylori* strains (5).

We could also show by sequencing the entire domain of the 23S rRNA that no mutation was present in another site of the domain V loop, for example at positions 2032, 2057, and opposite site 2611, sites described for *E. coli* (7, 32). Furthermore we detected a different mutation (24) at position 2143 (A→C instead of A→G), which was also reported recently (29). The MICs of other macrolides and related compounds determined for these strains confirmed that resistance was of concern for all macrolides but that the *H. pylori* strains remained susceptible to pristinamycin, except in the one case. The identity of the strains for a given patient was indicated by similar RAPD profiles before and after treatment including clarithromycin. This result shows that resistance was the result of the selection of variants of the infecting strain rather than infection with a different resistant strain.

In this study, testing of macrolide binding to free *H. pylori* ribosomes from resistant strains was also performed for the first time. Because of the cross-resistance observed between the different macrolides, labelled erythromycin was used in these experiments. The dose response observed, showing the binding of labelled erythromycin to the susceptible parent strains and the absence of binding to the resistant strains, strongly suggests that the lack of binding is the mechanism involved in the occurrence of resistance. We can therefore postulate that this lack of binding is due to a modification in the ribosomal structure following the mutation.

In summary a strong association between point mutations at positions 2143 and 2144 in the 23S rRNA genes, conformational changes in the ribosome at the macrolide binding site, and cross-resistance to macrolides was found. These results suggest that there is a causal relationship. The number of copies of the rRNA operons is relevant to the resistance mechanism. If one copy is present, the resistance is always expressed. Bukanov and Berg (3) and Tomb et al. (31) reported that *H. pylori* had two copies of the rRNA operon, but only two strains were studied. Our results and those of others nevertheless indicate the presence of only one copy because, when sequencing was performed, only the allelic mutant was found. Nevertheless, Versalovic et al. described a heterozygote resistant strain in one instance. The fraction of resistant ribosomes alone may be sufficient to confer a selectable resistance in *Streptomyces ambofaciens* as well (25).

We think that the possibility of a variable number of copies of the 23S rRNA depending on the strain exists, especially in the light of the important genome variability of *H. pylori.*

Sequencing is obviously the best approach to detect mutations, but it is rather fastidious and time consuming even when automated sequencing is used; therefore, we took advantage of the occurrence of new restriction sites on the amplified fragment to detect the mutation by PCR-RFLP. Using enzymes *Bsa*I and *Bbs*I we were able to discriminate between the mutation A→G at positions 2144 and 2143, respectively. This approach could be useful when exploring the epidemiology of macrolide resistance in the future. However, mutation A→C at position 2143 did not produce a restriction site for common enzymes.

The factors that most influence the emergence of drug-resistant strains include inappropriate treatment regimens and lack of compliance of patients. However because of the difficulties in obtaining cultures of *H. pylori* and the delay in obtaining the results, susceptibility testing is rarely carried out before a first treatment or when a second treatment is administered following a treatment failure.

The characterization of resistance mechanisms in *H. pylori* and their easy detection will facilitate the choice of appropriate treatment regimens and ultimately the control of infection.

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