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Survey of Plasmids and Resistance Factors in *Campylobacter jejuni* and *Campylobacter coli*

FRED C. TENOVER,[1,2]* SUE WILLIAMS,[1] KATHRYN P. GORDON,[1] CHARLES NOLAN,[3,4] AND JAMES J. PLORDE[1,2,4]

Seattle Veterans Administration Medical Center, Seattle, Washington 98108[1]; Seattle King County Department of Health, Seattle, Washington 98104[3]; and Departments of Laboratory Medicine[2] and Medicine,[4] University of Washington, Seattle, Washington 98105

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A total of 688 isolates of *Campylobacter jejuni* and *Campylobacter coli* were screened for the presence of plasmid DNA by agarose gel electrophoresis and were tested for susceptibility to ampicillin, chloramphenicol, erythromycin, streptomycin, and tetracycline. Of the isolates examined, 32% were noted to harbor plasmid DNA, ranging in size from 2.0 to 162 kilobases. Only tetracycline resistance was noted to correlate with the presence of plasmids. Plasmids capable of transferring tetracycline resistance via conjugation ranged in size from 42 to 100 kilobases. The *BglII* and *BclI* restriction endonuclease profiles of 31 plasmids examined showed marked diversity in their banding patterns. Although a high degree of DNA-DNA homology was noted among the *Campylobacter* spp. plasmids, no homology was noted between these plasmids and tetracycline R factors commonly found in the family *Enterobacteriaceae*.

Campylobacter jejuni and *Campylobacter coli* now have been established as agents of acute diarrheal disease in both animals and humans worldwide (3, 5, 19). Although there have been many reports describing the epidemiology and clinical aspects of campylobacter infections (for reviews, see references 3 and 4), little has been reported about the extrachromosomal gene pool of the organisms.

The first report of plasmids in the genus *Campylobacter* and their possible association with antibiotic resistance was published by Austen and Trust (1). Of the 29 strains of "related vibrios" they screened for extrachromosomal DNA, 6 harbored plasmids. No attempt was made to determine whether specific antibiotic resistance genes were plasmid encoded.

The first plasmid shown to mediate antibiotic resistance in *C. jejuni* was described by Taylor and associates (21). This 38-megadalton R factor was capable of transferring tetracycline resistance via conjugation to other *Campylobacter* strains including *C. jejuni* and *Campylobacter fetus* but not to *Escherichia coli*. A similar replicon was noted by Tenover and associates in isolates of *C. jejuni* recovered from simians (23).

More recent studies by Taylor and co-workers (22) have demonstrated a high degree of homology among six *Campylobacter* R factors obtained from strains isolated in Canada, Belgium, and the United States, despite differences in their restriction endonuclease profiles. These same R factors shared no DNA homology with tetracycline R plasmids common to the family *Enterobacteriaceae*.

The goals of this study were three: first, to establish the prevalence of plasmids within isolates of *C. jejuni* and *C. coli* in King County, Wash.; second, to determine whether these plasmids carried antibiotic resistance genes; and third, to characterize the R factors that were identified.

(This study was presented in part at the 23rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Las Vegas, Nev., 24 to 26 October 1983. [F. C. Tenover, S. Williams, K. Gordon, M. Coyle, C. Nolan, and J. J. Plorde, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 1983, abstr. no. 886, p. 246].)

MATERIALS AND METHODS

Bacterial isolates. A total of 688 isolates of *C. jejuni* and *C. coli* were obtained from the Seattle-King County Department of Public Health between February 1982 and March 1984 as part of an ongoing study examining the flow of these organisms from food sources to humans. The majority of nonhuman isolates were collected from chickens processed at a local poultry processing plant. Eleven isolates were obtained from domestic pets. One control organism, PS793, was obtained from a cynomologous monkey as previously described (23). Another, *C. jejuni* PS1559, containing plasmid pMAK 175 was kindly provided by Diane Taylor, University of Alberta, Edmonton, Alberta, Canada (21). Organisms were identified by standard techniques (11) and transported to the Seattle Veterans Administration Medical Center in the semisolid medium described by Wang et al. (26). *C. jejuni* and *C. coli* were differentiated by the hippurate hydrolysis test as described by Harvey (9). Isolates were maintained on Columbia Blood Agar plates (Prepared Media Laboratories, Renton, Wash.) in a GasPak jar containing a Campy Pak (BBL Microbiology Systems, Cockeysville, Md.). Strains of *E. coli* containing the class-specific tetracycline-resistant determinants described by Mendez and co-workers (15) were obtained from Esther Lederberg at the Plasmid Reference Center, Stanford, Calif. The plasmids included Class A, RP1; Class A1, R386; Class B, R100; Class B2, R27; Class C, pSC101; and Class D, RA1.

Characterization of plasmids. Organisms were screened for plasmid DNA by a previously described procedure (23) modified only in the adjustment of the lysis buffer to pH 12.0. The DNA was purified by cesium chloride-ethidium bromide density centrifugation (23). Restriction endonucleases were purchased from New England Biolabs, Beverly, Mass., or Bethesda Research Laboratories, Gaithersburg, Md., and used according to the directions of the manufacturers. Restriction endonuclease fragments prepared from campylobacter plasmid DNA were transferred from agarose

* Corresponding author.

gels to nitrocellulose filters (BA 85; Schleicher Schuell, Inc., Keene, N.H.) by the method of Southern (20). Probe DNA was labeled with ^{32}P by nick translation, using a commercial kit (New England Nuclear Corp., Boston, Mass.), and hybridized to the filters at high stringency in 50% formamide by the method of Wahl et al. (25). The filters were exposed to Kodak X-Omat AR X-ray film with intensifying screens for 48 h at −70°C.

Conjugation studies. Overnight matings between tetracycline-resistant, plasmid-containing isolates of *C. jejuni* and a streptomycin-resistant, tetracycline-susceptible, plasmidless strain of *C. coli* were performed on Columbia Blood Agar plates at 42°C by a previously described method (23). Transconjugants were selected on brucella agar containing 15 μg of tetracycline per ml and 100 μg of streptomycin per ml. Suspected transconjugants were recloned three times on antibiotic-containing media, tested for the ability to hydrolyze hippurate, and screened for plasmids. Antibiotic-resistant, plasmid-containing isolates not capable of hydrolyzing hippurate were scored as transconjugants. All matings were repeated three times. *C. coli* isolates were not tested for plasmid transfer.

Antimicrobial susceptibility testing. Isolates were screened for resistance to ampicillin, chloramphenicol, erythromycin, streptomycin, and tetracycline by a disk diffusion method. Organisms were suspended in saline to the density of a 2 McFarland standard, diluted 1:20, and streaked by the method of Bauer et al. (2) on Mueller-Hinton agar. Plates were incubated for 24 h at 37°C in a GasPak jar containing a Campy Pak. Zone sizes were interpreted by National Committee for Clinical Laboratory Standards (17), as recommended by Vanhoof et al. (24). Determination of the MICs of tetracycline for selected isolates was done by a modification of the agar dilution method of Mendez and co-workers (15). Briefly, isolates of *C. jejuni* were grown overnight both on Columbia Blood Agar and on brucella agar plates (Difco Laboratories, Detroit, Mich.) containing 1 μg of tetracycline per ml. The growth was suspended in 0.1 M phosphate-buffered saline (pH 7.2) to the density of the 0.5 McFarland standard (ca. 5×10^8 CFU/ml). The suspension was diluted 1:20 in phosphate-buffered saline and inoculated onto both Mueller-Hinton agar and brucella agar containing doubling concentrations of tetracycline, ranging from 8 to 256 μg/ml. All MIC determinations were performed in duplicate at both 37 and 42°C in GasPak jars containing Campy Paks.

Colony hybridization with class-specific DNA probes. Approximately 0.5 μg of plasmid DNA from each of 15 test organisms was spotted on nitrocellulose filters and air dried. The DNA was denatured by placing the filters on Whatman 3MM paper soaked in 0.5 N NaOH containing 1 M NaCl for 5 min and then on paper soaked in 1 M Tris (pH 7.5) containing 1.5 M NaCl for 5 min. After air drying, the filters were baked in a vacuum oven for 2 h at 80°C and sent to B. Marshall and S. Levy, Tufts University, Boston, Mass., for hybridization studies with the class-specific DNA probes (14). These were performed in duplicate.

RESULTS

Presence of plasmid DNA. The results of plasmid screening are summarized in Table 1. Approximately 31% of *C. jejuni* isolates and 40% of *C. coli* isolates harbored plasmids, regardless of their source of isolation. Eighty-four percent contained only a single plasmid, but some contained as many as five replicons. The plasmids ranged in size from 2.0 to 162 kilobases (kb). An agarose gel displaying several of the more commonly seen plasmid patterns is shown in Fig. 1.

TABLE 1. Summary of plasmid-containing isolates of *C. jejuni* and *C. coli*

Isolate	No. of plasmids per isolate[a]		
	None	One	Multiple
C. jejuni			
Human	184 (65)	88 (31)	10 (4)
Animal	247 (72)	84 (24)	15 (4)
C. coli			
Human	20 (71)	5 (18)	3 (11)
Animal	16 (50)	8 (25)	8 (25)

[a] The numbers within parentheses are percentages of total isolates in each category. The total percentages of plasmids per isolate are: none, 68%; one, 27%; and multiple, 5%.

Susceptibility to antimicrobial agents. None of the 688 isolates were resistant to chloramphenicol (MIC, ≤12.5 μg/ml), although several were resistant to the other antibiotics tested (ampicillin [MIC, ≥32 μg/ml], 8%; streptomycin [MIC, ≥20 μg/ml], 10%; erythromycin [MIC, ≥8 μg/ml], 4%). Resistance to tetracycline (MIC, ≥12 μg/ml) was noted most frequently, occurring in 24% of strains. Tetracycline-susceptible isolates routinely demonstrated MICs of ≤2 μg/ml to this antibiotic. (The details of the susceptibility patterns of these organisms will be presented elsewhere [M. Coyle, manuscript in preparation]). The MICs of tetracycline for 12 isolates of *C. jejuni* were determined with and without preexposure to 1 μg of tetracycline per ml. No significant difference was noted between the two procedures when tested on either brucella agar or Mueller-Hinton agar. The MICs of these organisms are shown in Table 2.

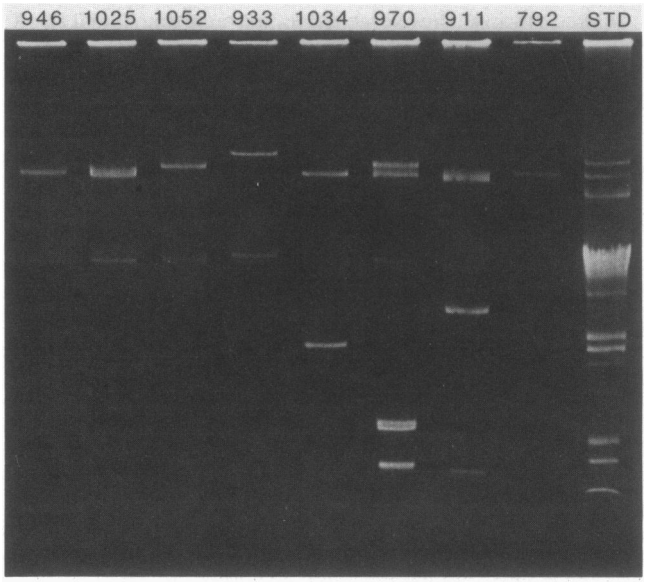

FIG. 1. Agarose gel electrophoresis of plasmid DNA from seven isolates of *C. jejuni* and one isolate of *C. coli* (911). The isolates were obtained from the following sources: 946, chicken (48 kb); 1025, human (53 kb); 1052, human (65 kb); 933, chicken (89 kb); 1034, chicken (51 and 5.4 kb); 970, chicken (58, 43, 3.2, 3.0, and 2.7 kb); 911, human (51, 7.4, and 2.6 kb); 792, monkey (57 kb). STD, Molecular weight standards (104, 66, 54, 34, 7.2, 5.6, 5.1, 3.9, 3.0, 2.7, and 2.1 kb). The molecular sizes reported are the averages of three determinations calculated from agarose gel electrophoresis experiments.

TABLE 2. Size, tetracycline MIC, frequency of transfer, and source of plasmid-containing isolates of *C. jejuni*

Strain no.	Plasmid size (kb)	Tetracyline MIC[a]	Frequency of transfer[b]	Source
PS793	57	32	2.3×10^{-5}	Monkey
PS946	48	64	4.3×10^{-4}	Chicken
PS972	44	128	1.8×10^{-7}	Chicken
PS1025	53	64	1.3×10^{-7}	Human
PS1052	65	128	1.8×10^{-5}	Human
PS1135	45	32	4.5×10^{-7}	Chicken
PS1137	42	32	4.6×10^{-7}	Chicken
PS1156	45	64	2.1×10^{-8}	Human
PS1240	133	64	$<1 \times 10^{-9}$	Chicken
PS1318	109	64	4.6×10^{-7}	Chicken
PS1559[c]	57	32	4.2×10^{-6}	Human
PS1147	42	1	ND	Human

[a] The MIC was determined by the agar dilution method.
[b] Frequencies are expressed as the number of transconjugants per input donor cells. ND, Not determined.
[c] Control strain obtained from D. Taylor, University of Edmonton, Alberta, Canada.

Relationship of tetracycline resistance to plasmid carriage. The relationship of tetracycline resistance to plasmid carriage is summarized in Fig. 2. All 160 tetracycline-resistant isolates harbored plasmid DNA. Of the plasmid-containing *C. jejuni* isolates, 77 (78%) of human origin and 74 (75%) of those from animals were resistant to tetracycline. All 10 of the human *C. jejuni* strains and 12 of the 15 animal strains containing multiple plasmids were resistant to tetracycline. Of the *C. coli* isolates from humans (5 of 8), 63%, and 50% of those from animals (8 of 16), also were resistant to this agent. The majority of the plasmids associated with tetracycline-resistant strains were between 40 and 60 kb in size.

Conjugal transfer of tetracycline resistance. Thirty-one tetracycline-resistant isolates of *C. jejuni* harboring plasmids ranging in size from 42 to 133 kb were tested for their ability to transfer tetracycline resistance by conjugation. Only a single isolate, containing a 133-kb plasmid, failed to demonstrate plasmid transfer. Frequencies of transfer ranged from 5×10^{-3} to 5×10^{-9} transconjugants per input donor cell. All transconjugates contained plasmids identical in size to that of the donor strain. Although several of the donors demonstrated resistance to other antimicrobials, transconjugants were resistant only to tetracycline. Since our recipient *C. coli* strain appears to harbor a chromosomal beta-lactamase gene, the transfer of ampicillin resistance could not be assessed. Three strains, cured of their plasmids by successive transfer on brucella agar plates, simultaneously became susceptible to tetracycline but not to other antibiotics. Twelve of the plasmids, their sources, and their frequencies of conjugation are shown in Table 2.

Restriction endonuclease analysis of plasmid DNA. Thirty-one plasmids ranging in size from 40 to 58 kb were cleaved with the restriction endonucleases *Bcl*I, *Bgl*II, and *Hin*cII. The *Hin*cII digests for the plasmids were quite similar, each demonstrating two large fragments and two small fragments (data not shown). However, 18 distinct banding patterns were observed for each of the *Bcl*I and *Bgl*II enzymes. The *Bcl*I and *Bgl*II fragment patterns for six of the plasmids are shown in Fig. 3. The *Bgl*II restriction profile of pFKT1000 from the simian isolate PS793 is included for comparison. Despite the diversity, all fragments demonstrated homology with a whole plasmid DNA probe prepared from the R factor of *C. jejuni* PS1025(pFKT1025), a tetracycline-resistant clinical isolate (Fig. 3, bottom).

Relationship of *Campylobacter* R factors to the tetracycline R factors of the family *Enterobacteriaceae*. This same plasmid (pFKT1025) was hybridized to a Southern filter containing plasmid DNA from each of the four classes and two subclasses of tetracycline determinants commonly found in the family *Enterobacteriaceae*, as described by Mendez and co-workers (15). No homology was noted between the *C. jejuni* R factor and any of these R factors (data not shown). To confirm these findings, purified plasmid DNA from 12 tetracycline-resistant, plasmid-containing isolates of *C. jejuni* and a tetracycline-susceptible control organism (PS1147) (Table 2) was spotted on nitrocellulose filters and hybridized with class-specific DNA probes described by Marshall et al. (14). No homology was noted between the *Campylobacter* R factors and these probes (B. Marshall and S. Levy, personal communication).

DISCUSSION

Approximately one-third of the *Campylobacter* isolates in this study harbored plasmids, ranging in size from 2.0 to 162 kb; 16% of these harbored multiple plasmids. In both *C. jejuni* and *C. coli* the plasmids demonstrated considerable

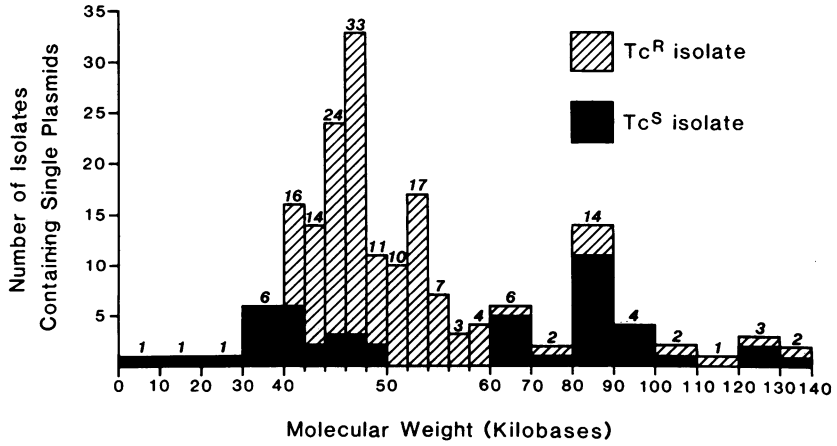

FIG. 2. *Campylobacter* plasmid distribution by molecular weight: total of 182 isolates. Tc^R, Tetracycline-resistant isolate; Tc^S, tetracycline-susceptible isolate. Note the change in scale between 40 and 60 kb.

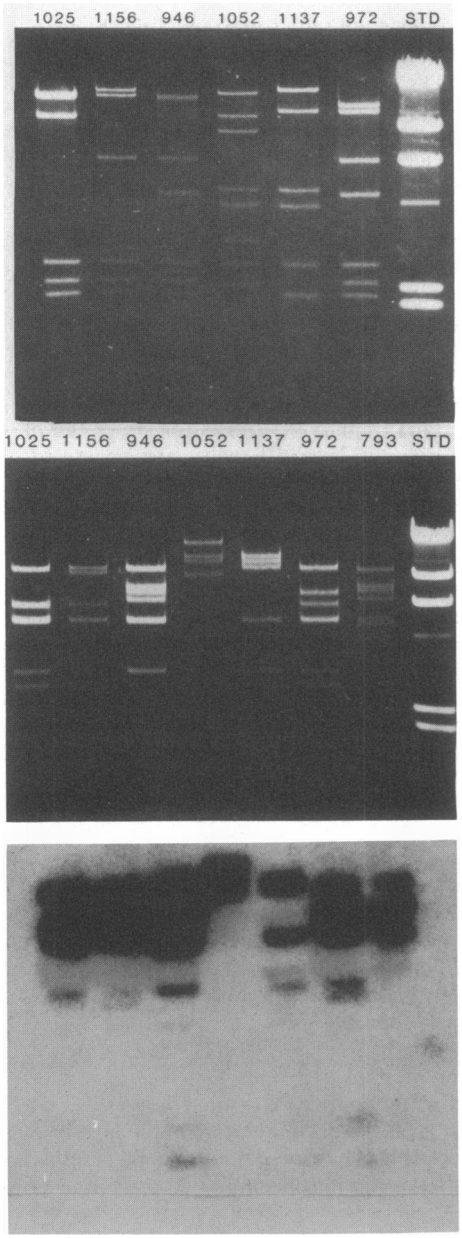

FIG. 3. Restriction endonuclease digests of *Campylobacter* plasmids separated on 0.8% agarose gels, and autoradiogram of *Bgl*II digests probed with ^{32}P-labeled *Campylobacter* R-factor pFKT1025 DNA. Top: *Bcl*I digests. Middle: *Bgl*II digests. Bottom: autoradiogram. Source of isolates: 793, cynomologous monkey (21); 972, chicken; 1137, chicken; 1052, human; 946, chicken; 1156, human; 1025, human. STD, Molecular weight standards-*Hin*dIII digest of phage lambda (23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb).

size variation. There clearly was no plasmid type common to all campylobacters such as the 3.9-kb cryptic plasmid typically seen in *Neisseria gonorrhoeae* (18).

There do not appear to be any significant differences in the size or distribution of plasmids between *C. jejuni* and *C. coli*. Although DNA-DNA homology studies carried out by several investigators have indicated that these two organisms are indeed separate species (8, 10), they appear to share a common pool of extrachromosomal elements.

There was a strong correlation between tetracycline resistance and plasmid carriage. Although not all plasmid-containing isolates were resistant to this antibiotic, all tetracycline-resistant strains harbored plasmids. Three strains of *C. jejuni* became susceptible to tetracycline after loss of their plasmids during repeated subculture, suggesting the tetracycline resistance was plasmid mediated. Thirty plasmid-containing isolates of *C. jejuni* were able to transfer the tetracycline resistance phenotype to a *C. coli* recipient. The lack of a suitable antibiotic-resistant strain of *C. jejuni* prevented our analysis of *C. coli* plasmid transfer. No other antibiotic resistance markers were noted to transfer during conjugation. There were no differences in the MICs of tetracycline for the isolates tested with and without preexposure to the antibiotic, leading us to conclude that tetracycline resistance is not inducible in *Campylobacter* species.

Our initial work with R factors obtained from cynomologous monkeys (23) and the early studies with human isolates of Taylor and associates (21) described a conjugal 57-kb (38-megadalton) plasmid, suggesting that R factors mediating tetracycline resistance in *C. jejuni* and *C. coli* would be of a single type and size.

Within the first 3 months of this present study, however, it became apparent that plasmids both larger and smaller than 57 kb were capable of transferring resistance to tetracycline within *Campylobacter* species. Although the *Bgl*II and *Bcl*I endonuclease patterns of these plasmids were quite diverse, careful analysis revealed that two *Bgl*II and one *Bcl*I fragments were conserved in all but two plasmids. Preliminary mapping studies showed these *Bgl*II and *Bcl*I fragments to be in the same area of the plasmids. Thus, it was not surprising that these R factors showed a high degree of nucleotide homology with one another when examined by the Southern hybridization technique. These data confirm and extend the data recently published by Taylor and associates (22), who also noted a high degree of nucleotide homology among *Campylobacter* R factors.

Two explanations can be put forth to account for the diversity noted among the plasmids. First, the differences in the restriction endonuclease profiles may be the result of intramolecular rearrangements due to insertion sequences or similar elements that promote in vivo rearrangements (6, 7, 12, 16). Alternatively, if these plasmids are capable of functioning as high-frequency recombinants, the differences could be due to imprecise integration into, and excision from, the host chromosome. If the process of excision were inefficient, as in the F′ plasmids (13), then chromosomal DNA of various lengths would be incorporated into the plasmid DNA as it exits from the host chromosome. Preliminary studies have demonstrated that the R-factor pFKT1025 does show homology to purified *C jejuni* DNA from five tetracycline-susceptible, plasmidless isolates, when tested by Southern hybridization analysis. Further studies to delineate possible sites of integration are in progress.

The lack of DNA-DNA homology between the campylobacter and enteric plasmids noted in the Southern gel and colony hybridization studies suggests that the campylobacter R factors are not an extension of those commonly found in the family *Enterobacteriaceae*. Both previous studies (23) and those of Taylor and co-workers (21) demonstrated that the campylobacter R factors could not be introduced into *E. coli* by conjugation or transformation, suggesting that these plasmids are incapable of replicating in *E. coli*. Whether these plasmids could be transferred and maintained in other members of the bowel flora, such as in *Bacteroides* species, remains to be tested.

In summary, it appears that *Campylobacter* spp. harbor a unique and very dynamic set of extrachromosomal elements

which undergo a considerable degree of intramolecular rearrangements. These rearrangements may be due to insertion-like sequences or may be generated during integration and excision events related to high-frequency-recombinant formation. Experiments are currently in progress to evaluate both of these possibilities.

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