DNA Cleavage Patterns as Indicators of Genotypic Heterogeneity among Strains of *Acholeplasma* and *Mycoplasma* Species

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Electrophoretic patterns of digestion products of *Acholeplasma* and *Mycoplasma* DNA by restriction endonucleases were compared. The patterns of *Acholeplasma axanthum* strains isolated from a variety of hosts and habitats differed markedly from each other, indicating considerable genotypic heterogeneity among strains included in this species. Heterogeneity was less marked among the *Acholeplasma oculi* strains tested, and was minimal among strains of the avian pathogen *Mycoplasma gallisepticum*. Strains of *Mycoplasma genitalium* isolated from the urethra of patients with non-gonococcal urethritis and from the urethra of an experimentally infected chimpanzee yielded identical cleavage patterns, indicating a high degree of genetic homogeneity of these strains. The data support the notion that mycoplasma species of strict host and tissue specificity exhibit marked genetic homogeneity. The advantages and deficiencies of the use of DNA cleavage patterns for classification purposes are discussed.

INTRODUCTION

The current concept of species in prokaryotes is ill-defined. Ideally, a prokaryotic species should consist of a cluster of strains of identical or nearly identical genetic composition. However, it is apparent that in the process of evolution, mutagenesis and selective pressures have resulted in the appearance of strains differing genotypically to varying degrees from the original strain, a factor which constitutes the major obstacle in bacterial classification. In practice, most prokaryotic species have been established on the basis of a few, readily-determined phenotypic characteristics, in the absence of supportive genetic data. In most cases this somewhat arbitrary speciation proved to be effective, serving the purpose of distinguishing bacteria of special medical, industrial and agricultural importance. Nevertheless, it is clear that in order to put bacterial classification on a more scientific basis, speciation should be determined according to genetic relatedness. Direct analysis of the base composition and nucleotide sequence of the bacterial chromosome may provide the necessary data. A variety of DNA–DNA hybridization techniques have been employed in assessment of genetic relatedness among prokaryotic strains, as these provide a measure of the degree of homology of nucleotide sequences between the chromosomes of the tested organisms. DNA base composition analysis and hybridization tests have indeed become useful tools in classification of prokaryotes, including mycoplasmas (Somerson *et al.*, 1967; Aulakh *et al.*, 1979, 1983; Stephens *et al.*, 1983).

We have recently promoted the use of cleavage patterns of mycoplasmal DNA digested by restriction endonucleases as ‘finger-prints’ indicating genetic relatedness among mycoplasma strains (Razin *et al.*, 1983; Chandler *et al.*, 1982). These enzymes cleave double-stranded DNA at specific recognition sites consisting of four or six nucleotides arranged in a specific order. The

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electrophoretic patterns of the resulting oligonucleotide fragments exhibit highly reproducible patterns specific for strains and species. By using this method nine serotypes of *Ureaplasma urealyticum* were shown to fall in two distinct clusters representing apparently two genotypes, whereas the DNA from five representative strains of *Mycoplasma pneumoniae* produced very similar cleavage patterns indicating the marked genetic homogeneity of this species (Chandler *et al.*, 1982; Razin *et al.*, 1983). Species specific cleavage patterns were also reported for the DNA of *Spiroplasma citri* strains cleaved by *EcoRI* (Bove & Saillard 1979; Bove *et al.*, 1982) and for *Mycoplasma hyorhinis* DNA digested by a variety of restriction endonucleases (Darai *et al.*, 1981, 1982).

Recent DNA-DNA hybridization tests (Aulakh *et al.*, 1983) revealed low nucleotide sequence homology (2 to 21%) among the eight established species of *Acholeplasma*, providing genetic support for their distinction. However, considerable variation in nucleic acid homology (ranging between 48 to 100%) was found among strains within each of the species *Acholeplasma laidlawii* and *Acholeplasma axanthum* (Stephens *et al.*, 1983). It seemed worthwhile to test whether or not this intraspecies genotypic heterogeneity can be observed by the restriction endonuclease method. Strains of *A. axanthum*, for which hybridization data were available, were selected for our study. Strains of *Acholeplasma oculi*, the avian pathogen *Mycoplasma gallisepticum* and the newly discovered human genital mycoplasma *Mycoplasma genitalium* (Tully *et al.*, 1981, 1983) were also included. The data presented here show that DNA cleavage patterns can be used as a convenient measure of intraspecies genotypic homogeneity or heterogeneity and support the notion that mycoplasma species of strict host and tissue specificity exhibit marked genetic homogeneity.

METHODS

Organisms and growth conditions. The designation of the strains tested and their origin are listed in Table 1. All the strains were cloned by the filtration procedure (Subcommittee on the Taxonomy of Mollicutes, 1979). The acholeplasmas and the *Mycoplasma gallisepticum* strains were grown in 200–400 ml quantities of 1% (w/v) bovine serum fraction broth medium (Tully & Razin, 1969) for 24–48 h at 37 °C, and harvested by centrifugation at 27000 g for 30 min at 4 °C. The cell pellets of the acholeplasmas were washed once in 0.25 M-NaCl containing 0.1 M-Na₂EDTA while those of *M. gallisepticum* were washed in 0.25 M-NaCl alone. The washed pellets were kept at –70 °C until used for DNA extraction. The *Mycoplasma genitalium* strains were grown in plastic tissue culture flasks (T-175) containing 200 ml SP4 medium (Tully *et al.*, 1977). The bottles were incubated horizontally at 37 °C for 88 h. The medium was decanted and the surface attached growth was washed twice with 10 ml 0.25 M-NaCl and then scraped off into another 10 ml of the salt solution. The organisms were collected by centrifugation at 18000 g for 10 min, and the cell pellets were used immediately for DNA extraction.

DNA extraction and purification. Portions of the washed cell pellets (containing about 20–50 mg cell protein) were resuspended in 0.1 ml TE buffer (50 mM-Tris, pH 8.0 and 10 mM-EDTA) and the organisms were lysed by adding 1 ml 1% (w/v) SDS in TE buffer. To minimize self-digestion of DNA by the endogenous DNAases of acholeplasmas (Razin *et al.*, 1964; Pollack *et al.*, 1965) the concentration of EDTA in the TE buffer was raised to 20 mM when acholeplasma pellets were lysed. The lysate was treated for 30 min at 37 °C with preheated (boiled for 2 min) RNAase A (Worthington Biochemical Corporation) at a final concentration of 50 µg ml⁻¹, and then for 60 min at 37 °C with preheated proteinase K (Boehringer) at 100 µg ml⁻¹. The SDS in the lysate was then precipitated by adding 100 µl 5 M-potassium acetate and incubated in ice for 30 min. The precipitate was sedimented by centrifugation at 15000 r.p.m. for 10 min in an Eppendorf microcentrifuge. The supernatant fluid was transferred to another microcentrifuge tube and mixed with an equal volume of redistilled phenol/chloroform (1:1, v/v) mixture. The aqueous phase was separated by centrifugation at 15000 r.p.m. for 15 min in the microcentrifuge and the DNA was precipitated by adding two volumes of cold ethanol. The DNA precipitate was collected by centrifugation for 5 min in the microcentrifuge. The resulting pellet was dried under vacuum and dissolved in 25–100 µl 10 mM-Tris, pH 7.5 containing 1 mM-Na₂EDTA. A sample (5 µl) of the DNA solution was diluted with 0.8 ml phosphate-buffered saline, and the absorbance was measured at 260 and 280 nm. The values of A_{260}/A_{280} were about 1.7 to 2.0, indicating the purity of the DNA preparations. The DNA content of the original solution was calculated according to the A_{260} value of the diluted solution, assuming that an A_{260} of 1.0 corresponds to 50 µg DNA ml⁻¹ (Archer *et al.*, 1981).

DNA digestion. Restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.). The reaction mixtures (total volume 20 µl) contained about 5 µg mycoplasmal DNA, 20–40 units of the tested endonuclease, 10 mM-Tris/HCl, pH 7.5, 10 mM-MgSO₄ and 50 mM-NaCl for digestion by *Bam*HI, *Pst*RI and

Table 1. Designation and origin of strains tested

Species and strain designation	Isolated from:	Isolated by:	Received from:
<i>Acholeplasma oculi</i>			
19L	Goat, eye	J. M. Al-Aubaidi	J. M. Al-Aubaidi
3557 TC	Tissue culture	R. A. DelGiudice	R. A. DelGiudice
Goat 5	Goat, eye	O. H. V. Stalheim	O. H. V. Stalheim
70-538 TC	Tissue culture	R. A. DelGiudice	R. A. DelGiudice
X5045	Swine, nasal	R. F. Ross	R. F. Ross
75151	Guinea pig, genital tract	E. V. Langford	E. V. Langford
<i>Acholeplasma axanthum</i>			
S-743	Tissue culture	C. Friend	J. G. Tully
1190	Lagoon	R. F. Ross	R. F. Ross
D-1	Swine, joint	D. Schimmel	L. Stipkovits
H86N	Bovine, nasal	N. O. Olson	J. G. Tully
1025C	Kale	N. L. Somerson	N. L. Somerson
0501	Broccoli	N. L. Somerson	N. L. Somerson
J544	Coconut palm	S. J. Eden-Green	S. J. Eden-Green
J248	Coconut palm	S. J. Eden-Green	S. J. Eden-Green
<i>Acholeplasma laidlawii</i>	Sewage	P. P. Laidlaw & W. J. Elford	D. G. ff. Edward
<i>Mycoplasma gallisepticum</i>			
PG31 (x95)	Chicken, trachea	F. S. Markham	Reference reagent NIH
S6	Turkey, brain	D. V. Zander	J. Noel
F(R)	Chicken, trachea	R. Yamamoto & H. E. Adler	J. Fabricant
293-1	Chicken, trachea	J. Taylor	J. Fabricant
<i>Mycoplasma genitalium</i>			
G-37*	Man, genital tract	J. G. Tully & D. Taylor-Robinson	J. G. Tully
G-37 (chimp)†	Chimpanzee, genital tract (experimentally infected)	J. G. Tully & M. F. Barile	J. G. Tully
M-30‡	Man, genital tract	J. G. Tully & D. Taylor-Robinson	J. G. Tully

* Isolated from the urethra of a non-gonococcal urethritis patient in SP4 broth at 37 °C (Tully *et al.*, 1981). Two cloned cultures were tested, one at passage level 8 and the other at 17.

† Isolated on SP4 medium (passage 1) from the urethra of a chimpanzee 8 weeks after experimental intra-urethral infection with the G-37 cloned strain at passage 8.

‡ Isolated from the urethra of another non-gonococcal urethritis patient on SP4 broth at 30 °C, cloned and tested at the fourth passage.

*Hind*III, and 50 mM-Tris/HCl, pH 7.5, 10 mM-MgSO₄ and 100 mM-NaCl for *Xho*I. For the enzymes *Hpa*I and *Kpn*I, the salt solution was composed of 10 mM-Tris/HCl, pH 7.5, and 10 mM MgSO₄, and for *Sma*I, the solution contained in addition 20 mM-KCl. A reaction mixture containing the tested endonuclease, the appropriate salt solution and 1 µg of phage lambda DNA (Bethesda Research Laboratories, BRL, Gaithersburg, Md.) served as a control of specific enzyme activity. Digestions were carried out at 37 °C for 60 min. The reaction was stopped by adding 2 µl of tracking dye solution consisting of 0.07% (w/v) bromophenol blue, 7% (w/v) SDS, and 33% (v/v) glycerol in water.

Electrophoresis of digestion products. The total volume of the reaction mixture was subjected to electrophoresis in slab gels made of 1% (w/v) agarose (Type 1, Low EEO; Sigma) prepared in Tris/borate buffer (89 mM-Tris, pH 8.2, 2.5 mM-EDTA, and 89 mM-boric acid) used also as electrophoresis buffer. A vertical electrophoresis apparatus (BRL, model V16) was used at 100 volts for 3 h until the dye marker reached the bottom of the gel. The gel was stained for 15 min with ethidium bromide (0.4 µg ml⁻¹) and photographed under UV light (Chromato-Vue transilluminator model C-61, Ultra-Violet Products, San Gabriel, Calif.) using Polaroid type 55 P/N film and a Polaroid MP-4 land camera with an orange filter. The negatives obtained were used to prepare prints in which the polynucleotide bands appear dark on a light background. A control preparation of undigested DNA (DNA preparations without the addition of restriction endonucleases) from each mycoplasma was also subjected to electrophoresis to detect extrachromosomal DNA. Only one chromosomal band was seen with no evidence of extrachromosomal DNA by the electrophoretic procedure.

Fig. 1. Cleavage patterns of the DNA of *A. axanthum* strains (lanes 2–9) and of phage lambda DNA (lane 1) digested by *Kpn*I. The strains tested were S-743 (lane 2); 0501 (lane 3); D-1 (lane 4); 1025C (lane 5); H86N (lane 6); J544 (lane 7); 1190 (lane 8) and J248 (lane 9). Of the eight strains tested, the patterns of only strains 0501 and 1025C (both isolated from vegetables) appear similar. The 'smears' seen in the gels indicate that some degradation of DNA occurred during DNA extraction.

RESULTS

The DNA cleavage patterns of *Acholeplasma axanthum* strains, particularly those obtained with restriction endonucleases with recognition sites rich in G + C, support the intraspecies genetic heterogeneity of this species indicated by the DNA hybridization data (Stephens *et al.*, 1983). Thus, of the eight *A. axanthum* strains tested only the DNA of strains 0501 and 1025C (both isolated from vegetables, Table 1) produced identical cleavage patterns with *Kpn*I (recognition site GGTAC/C) and with *Bam*HI (recognition site G/GATCC), whereas the DNAs of the other six strains each yielded a different pattern (Figs 1 and 2). On the other hand, the cleavage patterns of the DNAs of the five *A. oculi* strains by *Kpn*I and *Bam*HI, though not being identical, resembled each other (Fig. 3). In addition, Fig. 3 shows that the *A. oculi* DNAs can be distinguished from those of *A. axanthum* in being cleaved at many more points when digested with *Kpn*I, and the cleavage pattern of the *A. laidlawii* PG8 DNA by this enzyme differs from the patterns of the *A. axanthum* and *A. oculi* strains. *Pst*I, despite sharing with *Bam*HI and *Kpn*I a recognition site rich in G + C (CTGCA/G) cleaved the acholeplasmal DNAs at many points, producing highly similar patterns with the *A. oculi* strains, but different patterns with the *A. axanthum* and *A. laidlawii* strains (Fig. 4).

The restriction endonucleases *Hpa*I and *Hind*III which have recognition sites poor in G + C (GTT/AAC and A/AGCTT, respectively) cut the G + C poor acholeplasmal DNA at many sites, producing cleavage patterns with numerous bands, making comparison of patterns more difficult. Again, the patterns of the *A. axanthum* strains obtained with these enzymes differed from each other, as against the similarity of patterns exhibited by the selected *A. oculi* strains tested (data not shown). On the other hand, digestion of the acholeplasmal DNAs by *Xho*I (recognition site C/TCGAG) produced patterns with no or very few visible cleavage bands, rendering this enzyme not useful for our purpose. *Sma*I, the enzyme with a recognition site CCC/GGG, did not produce any visible cleavage fragments when applied to the DNAs of the five *A. oculi* strains, and three of the *A. axanthum* strains (D-1, 1190, and S743) but produced four visible cleavage bands with *A. laidlawii* PG8 DNA (data not shown).

Fig. 2. Cleavage patterns of the DNA of *A. axanthum* strains (lanes 2-9) and of phage lambda DNA (lane 1) digested by *Bam*HI. The order of the strains in the lanes is as depicted in Fig. 1. Only strains 0501 and 1025C produced similar or identical patterns.

Fig. 3. Cleavage patterns of the DNA of *A. oculi* strains by *Bam*HI (lanes 1-5) and by *Kpn*I (lanes 6-10). The cleavage pattern of *A. laidlawii* PG8 DNA by *Kpn*I is included for comparison in lane 11. The *A. oculi* strains tested were: 19L (lane 1); 3557TC (lanes 2 and 6); goat 5 (lanes 3 and 7); 70-538TC (lanes 4 and 8); X5054 (lanes 5 and 9) and 75151 (lane 10).

Fig. 4. Cleavage patterns of phage lambda DNA (lane 1) and the DNAs of *A. laidlawii* PG8 (lane 2), *A. axanthum* D-1 (lane 3) and *A. oculi* strains (lanes 4-8) digested by *Pst*I. The *A. oculi* strains tested were: X5045 (lane 4); 70-538TC (lane 5); goat 5 (lane 6); 3557TC (lane 7) and 19L (lane 8). The marked similarity of the *A. oculi* patterns to each other, and their dissimilarity to the *A. axanthum* and *A. laidlawii* patterns can be seen.

Fig. 5. Cleavage patterns of the DNA of *M. gallisepticum* strains by *Bam*HI (lanes 1-4) and by *Kpn*I (lanes 5-8). The strains tested were: PG-31 (lanes 1 and 5); S6 (lanes 2 and 6); F(R) (lanes 3 and 7) and strain 293-1 (lanes 4 and 8). The marked similarity of the patterns is particularly noticeable with *Bam*HI. The patterns of strains PG-31 and 293-1 are practically identical with both enzymes.

Fig. 6. Cleavage patterns of lambda phage DNA and DNAs of *M. gallisepticum* strains by *Hpa*I (lanes 1–5) and by *Xho*I (lanes 5–10). The strains tested: PG-31 (lanes 2 and 7); S6 (lanes 3 and 8), F(R) (lanes 4 and 9) and 293-1 (lanes 5 and 10). The essential identity of the patterns of strains PG-31 and 293-1 can be seen.

Fig. 7. Cleavage patterns of the DNA of *M. genitalium* strains and of phage lambda DNA by *Kpn*I (lanes 1–5) and by *Hpa*I (lanes 6–10). The strains tested: G-37, passage 17 (lanes 1 and 6); G-37, passage 8 (lanes 2 and 7); G-37 isolated from the urethra of an experimentally infected chimpanzee (lanes 3 and 8); M-30, passage 4; lambda phage DNA (lanes 5 and 10). The identity of the cleavage patterns of all strains can be seen with both enzymes.

Based on the known close serologic relatedness, similarities in electrophoretic patterns of cell proteins (Razin & Rottem, 1967; Rhoades *et al.*, 1974) and narrow host specificity of *M. gallisepticum* strains (Jordan, 1979) we had predicted that the cleavage patterns of the DNAs of the various *M. gallisepticum* strains would be similar. This, indeed, proved to be the case, as the cleavage patterns of the DNAs of the four *M. gallisepticum* strains by *Bam*HI (Fig. 5), *Hpa*I (Fig. 6) and *Pst*I (data not shown) were almost identical. Nevertheless, the cleavage patterns obtained with *Xho*I and *Kpn*I, enzymes which cut the *M. gallisepticum* DNA at fewer points, revealed some differences among the strains, although PG-31 and 293-1 appeared identical in all cases (Figs 5 and 6). As with the *A. axanthum* and *A. oculi* DNAs, *Sma*I failed to produce visible cleavage bands with the *M. gallisepticum* DNA, apart from one band with the S6 DNA (data not shown). Genetic homogeneity was even more striking with the human genital *M. genitalium* strains. The cleavage patterns by *Kpn*I and *Hpa*I of the DNA of strains G-37 and M-30 isolated from the urethra of different patients (Tully *et al.*, 1981) were essentially identical (Fig. 7). Moreover, the organisms recovered from a chimpanzee 8 weeks after it had been inoculated with *M. genitalium* strain G-37, exhibited DNA cleavage patterns identical with those of the original strain (Fig. 7). The same results were obtained with *Bam*HI and *Pst*I (results not shown).

DISCUSSION

The working hypothesis that genetic heterogeneity among strains of the same species is associated with their recovery from diverse habitats has been put forward by Stephens *et al.* (1983) on the basis of DNA hybridization data. Accordingly, the hypothesis would suggest that adaptation of an organism to a new host had pressured it to genotypically change in order to survive or that colonization occurred through a selective process. The DNA cleavage data reported here support the above notion by demonstrating the marked genotypic heterogeneity of the *A. axanthum* strains isolated from a variety of hosts (Table 1). The *A. oculi* strains, though appearing closer in DNA structure to each other than the *A. axanthum* strains, still exhibit significant variations in their cleavage patterns, a fact which may be related to the variety of hosts from which the *A. oculi* strains were isolated (Table 1). Unfortunately, in the case of *A. oculi* DNA hybridization data are unavailable for comparison with the DNA cleavage data.

In contrast to the marked heterogeneity of the two *Acholeplasma* species, the DNA cleavage patterns of *M. gallisepticum* strains indicated marked genetic homogeneity, despite the fact that the strains were isolated in different laboratories, and were subcultured numerous times. It can be argued in this case that *M. gallisepticum* has a much more restricted ecological niche than the *acholeplasmas*, as it only inhabits the respiratory tract of chickens and turkeys (Jordan, 1979). In genetic homogeneity, *M. gallisepticum* resembles another avian pathogen *M. meleagridis* (Elmahi *et al.*, 1982) and the human respiratory pathogen *M. pneumoniae* (Chandler *et al.*, 1982; Razin *et al.*, 1983). In the case of *M. pneumoniae* the ecological niche is even more restricted, as this mycoplasma is found in the respiratory tract of man only. *Mycoplasma pneumoniae* has not been isolated under natural conditions from any other animal, including primates (Barile, 1973). Data on the occurrence of *M. genitalium* are still very scarce. Nevertheless, the DNA cleavage patterns suggest that this mycoplasma, considered as a potential pathogen in the urogenital tract (Tully *et al.*, 1981; Taylor-Robinson *et al.*, 1981) constitutes another genotypically homogeneous species.

DNA cleavage data are available for another human mycoplasma, *Ureaplasma urealyticum* (Razin *et al.*, 1983). In this case, the cleavage patterns, as well as DNA hybridization data and electrophoretic patterns of cell proteins, indicate the presence of two genetically distinct clusters of strains. However, as against the marked diversity exhibited by the *A. axanthum* strains, the strains in each of the two *U. urealyticum* clusters show remarkable homogeneity.

The experience gained so far from the use of restriction endonucleases for classification purposes enables the drawing of some conclusions as to the advantages and deficiencies of this approach. The advantages include: (1) Need for a small quantity of organisms, as only a few µg of DNA suffice for each test. (2) No interference by culture medium components particularly proteins, a problem complicating the electrophoretic analysis of cell proteins of poorly-growing

mycoplasmas in serum-rich media (see Razin *et al.*, 1983). (3) Easy reading of results based on visual comparison of patterns. Densitometer tracings of the patterns can also be prepared (Bove *et al.*, 1982). (4) The DNA cleavage patterns are excellent tools to test for genetic homogeneity. Identical patterns obtained by several restriction enzymes strongly indicate genetic homogeneity of subcultures. The case of the *M. genitalium* strains illustrates this point. Also, the susceptibility of the DNA of a tested strain to cleavage by restriction endonucleases with G + C rich recognition sites, provides an indication for assessment of the G + C content of this particular DNA. Thus, the relatively G + C rich *M. pneumoniae* DNA (G + C about 40 mol%) is cleaved by *Sma*I (recognition site CCC/GGG) at about 35 sites, whereas the DNAs of other *Mycoplasma* and *Acholeplasma* species, which are poorer in G + C, usually resist digestion by this enzyme (present results and Razin *et al.*, 1983).

Possible deficiencies of the DNA cleavage approach can be summarized as follows: (1) Undegraded DNA of high purity is required. This is not easy to obtain with the *Acholeplasma* species, as they are particularly rich in endogenous DNAases which are activated on cell lysis (Razin *et al.*, 1964; Pollack *et al.*, 1965). We found that by increasing the EDTA concentration in the lysis solution, and by processing relatively small pellets of cells, the self-digestion of the mycoplasmal DNA can be minimized. We have not encountered this problem with *Mycoplasma* species. (2) The presence of extrachromosomal DNA, such as that of plasmids or viruses, complicates the interpretation of cleavage patterns (Bove *et al.*, 1982). It can be argued that the cleavage patterns of *A. axanthum* strains are so different from each other due to the presence of different plasmids and/or viruses in these strains. Although this possibility cannot be totally ruled out, electrophoresis of undigested DNA of these strains failed to show any bands additional to that of the chromosomal DNA. (3) Comparison of cleavage patterns is done visually by direct comparison of bands on photographs or on densitometer tracings of the photographs. This suffices for determining complete identity, close-similarity, or total non-identity of DNA structure. The problem lies in cases of partial similarity, as the cleavage data are not expressed by numbers, such as percent homology provided by DNA hybridization tests.

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