NOTE

Pseudomonas sp. strain KC represents a new genomovar within *Pseudomonas stutzeri*

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***Pseudomonas* sp. strain KC (= ATCC 55595 = DSM 7136) is a denitrifying aquifer isolate that produces and secretes pyridine-2,6-bis(thiocarboxylate) (PDTC), a chelating agent that fortuitously transforms carbon tetrachloride without producing chloroform. Although KC has been used successfully for full-scale bioremediation of carbon tetrachloride, its taxonomy has proven difficult to resolve, as it retains properties of both *Pseudomonas stutzeri* and *Pseudomonas putida*. In the present work, a polyphasic approach was used to conclude that strain KC represents a new genomovar (genomovar 9) within the species *P. stutzeri*.**

Keywords: *Pseudomonas* strain KC, phylogeny and taxonomy, genomovar, carbon tetrachloride biodegradation, pyridine-2,6-bis(thiocarboxylate)

Bacterial strain KC (= ATCC 55595 = DSM 7136) is a denitrifying bacterium originally isolated from an aquifer in Seal Beach, CA, USA (Criddle *et al*., 1990). Under iron-limiting conditions, strain KC induces genes for the production and secretion of pyridine-2,6-bis(thiocarboxylate) (PDTC), a molecule that can rapidly dechlorinate carbon tetrachloride (CCl_4), yielding CO_2 and non-volatile compounds, under anoxic conditions (Criddle *et al*., 1990; Dybas *et al*., 1995; Lee *et al*., 1999; Lewis & Crawford, 1993; Sepúlveda-Torres *et al*., 1999). This activity is important for bioremediation applications in aquifer sediments because it is rapid, with half-lives of only a few minutes (Tatara *et al*., 1995), and occurs without the accumulation of chloroform. Cells of strain KC attach to aquifer sediments, but can also exist in a free-swimming, highly motile form that is chemotactic towards nitrate, and cells can sustain dechlorination activity during migration (Witt *et al*., 1999a, b). Recent developments underscore the unique environmental significance of this strain. Lewis *et al*. (2000) reported that a laboratory culture of strain KC spontaneously lost a 170 kb fragment containing genes necessary for PDTC biosynthesis on a 25 kb fragment of the lost DNA. This fragment was not detected in three *Pseudomonas stutzeri* strains. Strain KC also has significance for biotechnology, because of its use in one of the first full-scale aquifer bioaugmentation applications (Hyndman *et al*., 2001). Large volumes of strain KC were grown on-site and injected into a CCl_4-contaminated aquifer in Schoolcraft, MI, USA. The added cells colonized the aquifer sediment, creating a biocurtain that has efficiently removed CCl_4 from groundwater passing through it for over 3 years.

Strain KC was originally classified as a *P. stutzeri*-like organism because of its ability to reduce nitrate and to use maltose, citrate, malonate and glycerol as carbon sources, and a preliminary fatty acid profile (Criddle *et al*., 1990), and some previously published studies have referred to it as a strain of *P. stutzeri*. Nevertheless, no exhaustive studies have been performed to elucidate its taxonomy. The present investigation was performed to establish conclusively the systematic classification of strain KC based on physiological and genotypic studies. The results obtained from DNA–DNA hybridization, DNA fingerprinting, analysis of 16S rRNA gene and 16S–23S internal transcribed spacer

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Abbreviations: ITS1, 16S–23S internal transcribed spacer region; PDTC, pyridine-2,6-bis(thiocarboxylate).

The GenBank accession numbers for the 16S rRNA gene and ITS1 sequences of strain KC are AF067960 and AF063219 (16S rRNA) and AF356514 (ITS1).

Table 1. Bacterial strains used in this study

Strain	Other designation(s)	Origin of isolation	Reference(s)
Pseudomonas sp. strain KC	ATCC 55595, DSM 7136	Aquifer isolate, CCl_4-degrader	Criddle *et al.* (1990)
P. stutzeri CCUG 11256^T gv. 1	ATCC 17588^T, Stanier strain 221^T	Clinical isolate	Stanier *et al.* (1966)
P. stutzeri ATCC 17591 gv. 2	Stanier strain 224	Clinical isolate	Stanier *et al.* (1966)
P. stutzeri DSM 50227 gv. 3	ATCC 11607	Clinical isolate	Van Niel & Allen (1952)
P. stutzeri 19SMN4 gv. 4	DSM 6084	Marine isolate, naphthalene-degrader	Rosselló *et al.* (1991)
P. stutzeri DNSP21 gv. 5	DSM 6082	Wastewater isolate	Rosselló *et al.* (1991)
Pseudomonas balearica DSM 6083^T gv. 6	–	Wastewater isolate, naphthalene-degrader	Bennasar *et al.* (1996); Rosselló *et al.* (1991)
P. stutzeri DSM 50238 gv. 7	ATCC 17832, Stanier strain 419	Soil isolate	Stanier *et al.* (1966)
P. stutzeri JM300 gv. 8	DSM 10701	Soil isolate	Carlson & Ingraham (1983)
Pseudomonas putida ATCC 12633^T	DSM 50202^T	Lactate enrichment	Skerman *et al.* (1980); Stanier *et al.* (1966)
P. putida DSM 3601	–	Tomato plant isolate, produces PDTC	Ockels *et al.* (1978)

gv., Genomovar. The strains listed are the reference strains of the indicated genomovars.

region (ITS1) sequences and *gyrB* PCR studies were combined with substrate oxidation, antibiotic resistance and fatty acid methyl ester analyses to establish that strain KC should be classified as a reference strain for a novel *P. stutzeri* genomovar.

Bacterial strains and phenotypic studies. The strains used in this study, their source of isolation and relevant references are provided in Table 1. Carbon source oxidation capabilities were tested by inoculating BIOLOG GN2 plates, in triplicate, with fresh bacterial cultures normalized to an OD_{600} of 0·195–0·205 and grown at 30 °C for 48 h. Forty of the 95 carbon sources tested were used differently by the bacterial strains, as shown in Table 2. This result is consistent with previous reports that indicate a high degree of physiological heterogeneity amongst *P. stutzeri* strains (Palleroni *et al.*, 1970; Rosselló *et al.*, 1994; Stanier *et al.*, 1966). Strain KC is similar to these *P. stutzeri* strains in its ability to oxidize dextrin, glycogen, maltose, itaconic acid, α-ketobutyric acid and L-leucine and its inability to oxidize L-arabinose, D-sorbitol, phenyl ethylamine and 2,3-butanediol, which are carbon sources not oxidized by any of the *P. stutzeri* strains tested. Strain KC was the only organism, of the 11 tested, that was capable of oxidizing *m*-inositol.

Even though significant differences were observed in carbon-source utilization, the behaviour of strain KC and *P. stutzeri* strains in antibiotic-resistance tests and fatty acid methyl ester analysis was more homogeneous. The antibiotic-susceptibility tests were performed in tryptic soy agar plates containing 0·7 cm diameter filter discs with one of 12 antibiotics. The results obtained for strain KC coincided with the consensus for the majority of *P. stutzeri* strains and diverged from the *Pseudomonas putida* pattern for all of the 12 antibiotics tested except trimethoprim/sulfamethoxazole, an antibiotic combination that showed variability among all strains tested. The identity and abundance of cellular fatty acids were determined by Microbial ID using a procedure described previously (Sasser, 1990). The fatty acid profiles for strain KC were very similar in composition and abundance to those of *P. stutzeri* and *Pseudomonas balearica* strains, diverging from the patterns observed in the two *P. putida* strains tested. This result is congruent with previous observations that have indicated that *P. stutzeri* strains have similar fatty acid patterns, making this technique unsuitable for strain differentiation (Rosselló *et al.*, 1994; Stead, 1992; Veys *et al.*, 1989).

DNA-based analyses. When strain KC was compared to *P. stutzeri* strains by DNA reassociation studies, using a modification of the hydroxyapatite method (Marmur, 1961; Ziemke *et al.*, 1998), the similarity indices were below the 70% threshold. Genomovar 5 was the closest relative, with a similarity index of 66·3%. DNA–DNA similarity values are usually higher than 70% for members of the same genomovar, between 40 and 60% for members of different genomovars and under 20% when a *P. stutzeri* strain is compared with other *Pseudomonas* species (Rosselló *et al.*, 1991; Rosselló-Mora *et al.*, 1993, 1996). The results obtained for strain KC are consistent with the aforementioned values. Low similarity profiles were also observed when strain KC and the *P. stutzeri* strains were studied by DNA fingerprinting with REP, BOX and ERIC PCR (Schneider & de Bruijn, 1996; Versalovic *et al.*, 1991). The low similarity indices observed in these assays may be explained by the large chromosomal plasticity detected in *P. stutzeri* (Ginard *et al.*, 1997); any chromosomal rearrangements would interfere with experiments that depend on DNA sequence homogeneity.

When commonly used regions of DNA with phylogenetic relevance such as 16S rDNA, ITS1 and *gyrB* were analysed to deduce phylogenetic relationships, strain KC clustered distinctively within the *P. stutzeri* phylogenetic branch. The 16S rRNA gene was amplified by PCR with modified

Table 2. Substrate utilization by strain KC and various *P. stutzeri, P. balearica* and *P. putida* strains

P. stutzeri strains tested are indicated as: 1, CCUG 1126^T; 2, ATCC 17591; 3, DSM 50227; 4, 19SMN4; 5, DNSP21; 6, DSM 50238; 7, JM300. The remaining strains are indicated as: 8, *P. balearica* DSM 6083^T; 9, *P. putida* ATCC 12633^T; 10, *P. putida* DSM 3601. Utilization is scored as: +, positive; −, negative; w, weakly positive. The following carbon sources were oxidized by all strains tested: Tweens 40 and 80, α-D-glucose, methyl pyruvate, *cis*-aconitic acid, D-gluconic acid, β-hydroxybutyric acid, α-ketoglutaric acid, DL-lactic acid, malonic acid, L-alanine, L-asparagine, L-glutamic acid, L-proline, glycerol, mono-methylsuccinate, acetic acid, citric acid, D-galacturonic acid, D-glucuronic acid, quinic acid, succinic acid, bromosuccinic acid, D-alanine and L-aspartic acid. The following carbon sources were not oxidized by any of the strains tested: α-cyclodextrin, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, cellobiose, *i*-erythritol, L-fucose, D-galactose, gentiobiose, α-D-lactose, lactulose, D-melibiose, methyl β-D-glucoside, D-psicose, D-raffinose, L-rhamnose, sucrose, turanose, xylitol, D-galactonic acid lactone, D-glucosaminic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, urocanic acid, uridine, thymidine, α-DL-glycerol phosphate, glucose 1-phosphate, glucose 6-phosphate and L-arabinose.

BIOLOG carbon source	Strain KC	1	2	3	4	5	6	7	8	9	10
Dextrin	+	+	+	+	+	+	+	+	+	−	−
Glycogen	+	+	+	+	+	+	+	+	+	−	−
L-Arabinose	−	−	−	−	−	−	−	−	−	−	+
D-Arabitol	+	−	−	−	−	−	−	+	−	−	−
D-Fructose	+	−	+	−	−	−	−	+	−	+	−
m-Inositol	+	−	−	−	−	−	−	−	−	−	−
Maltose	+	+	+	+	+	+	+	+	+	−	−
D-Mannitol	+	−	+	w	−	+	−	+	−	−	−
D-Mannose	−	−	−	−	−	−	−	+	−	−	+
D-Sorbitol	−	−	−	−	−	−	−	−	−	+	−
Trehalose	+	−	w	−	−	−	−	w	−	−	−
Formic acid	w	−	w	w	−	−	w	+	w	+	+
α-Hydroxybutyric acid	+	+	w	w	+	−	+	+	w	+	−
γ-Hydroxybutyric acid	−	−	w	w	−	−	w	−	−	−	−
p-Hydroxyphenylacetic acid	−	+	−	−	−	w	w	w	w	+	w
Itaconic acid	+	+	+	+	+	+	+	+	+	+	−
α-Ketobutyric acid	+	+	w	w	+	w	+	+	w	−	−
α-Ketovaleric acid	w	−	−	−	+	−	−	+	−	+	−
Propionic acid	+	+	+	+	+	−	+	+	+	+	+
D-Saccharic acid	+	+	w	+	−	w	+	w	w	+	+
Sabacic acid	−	+	−	+	+	−	−	+	−	−	−
Succinamic acid	+	−	−	−	−	−	−	+	−	−	−
Glucuronamide	+	+	+	w	−	−	−	+	−	+	+
Alaninamide	+	+	+	+	+	−	w	+	+	+	+
L-Alanyl glycine	w	+	−	w	+	−	w	w	−	−	−
L-Histidine	−	+	−	w	w	−	+	w	w	+	+
Hydroxy-L-proline	−	+	w	w	w	w	+	w	w	+	+
L-Leucine	+	+	+	+	+	+	+	+	+	+	−
L-Ornithine	−	−	−	−	−	−	−	w	−	+	−
L-Phenylalanine	−	−	−	−	−	−	−	+	−	−	−
L-Pyroglutamic acid	+	+	+	+	+	−	−	−	w	−	−
D-Serine	−	+	−	−	−	−	−	−	w	+	−
L-Serine	−	+	−	−	−	−	+	+	w	+	+
L-Threonine	+	+	w	−	−	−	−	+	−	+	−
DL-Carnitine	−	−	−	w	−	w	+	w	w	+	+
γ-Aminobutyric acid	+	+	−	+	+	+	+	w	w	+	+
Phenylethylamine	−	−	−	−	−	−	−	−	−	+	−
Putrescine	w	+	w	w	−	w	+	w	w	+	+
2-Aminoethanol	+	−	+	−	+	−	+	+	−	+	−
2,3-Butanediol	−	−	−	−	−	−	−	−	w	+	−

universal eubacterial primers fD1 and rP1 (Zhou *et al.*, 1995), ligated into a PCR cloning vector (Invitrogen) and sequenced by *Taq* cycle sequencing using fluorescent dye-labelled dideoxynucleotides. The sequence from strain KC exhibited 99·4% similarity to the 16S rDNA sequences of members of genomovar 3 and 99·2% similarity to sequences

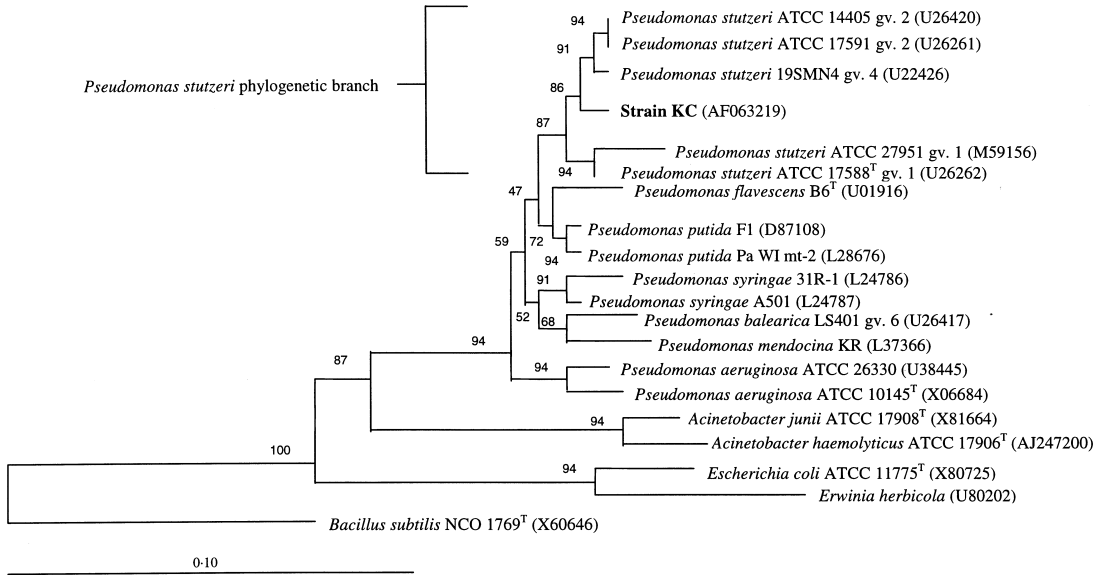

Fig. 1. Bootstrap parsimony tree obtained when the 16S rRNA gene of strain KC was compared to some sequences available in the Ribosomal Database Project. Numbers on the branches indicate bootstrap confidence estimates obtained with 100 replicates. Multiple sequence alignment was done with the PILEUP program in the Genetics Computer Group software package (Devereux *et al.*, 1984). The alignment was edited for the appropriate analysis by using the SUBALIGN and GDE programs from the Ribosomal Database Project (Maidak *et al.*, 1999). The phylogenetic analyses were performed in the DNA distance program ARB using neighbour-joining with Felsenstein's correction (O. Strunk, Technische Universität München, Germany). Scale bar, 0·1 substitutions per nucleotide position.

of members of genomovar 4 (Fig. 1). 16S rRNA gene sequence comparisons support the relationship among the genomovars and have further sustained the genomovar concept, because similarities between 16S rRNA genes range from 99·8 to 100 % for members of the same genomovar and 98·0 to 99·7 % for reference strains of different genomovars (Bennasar *et al.*, 1996). Strain KC shows more than 98 % 16S rDNA sequence similarity to the reference strains of *P. stutzeri* genomovars but slightly lower similarities of 96 % to *P. putida*, *Pseudomonas aeruginosa* and *P. balearica*. These results are consistent with previous observations that have reported 16S rDNA similarities of less than 97 % for strains of different species (Stackebrandt & Goebel, 1994). Furthermore, when the 16S rRNA gene of strain KC was amplified with the *P. stutzeri*-specific primers fps150 and rps1271 (Bennasar *et al.*, 1998), it showed the *Bam*HI restriction pattern that is observed for *P. stutzeri* strains but is absent from other related species like *P. balearica* or *P. putida*. PCR amplification of *gyrB* also excluded the possibility that strain KC belongs to the species *P. putida*, since the *gyrB* gene of strain KC was not amplified by PCR with the *P. putida*-specific primers UP-1 and UP-2r (Yamamoto & Harayama, 1995).

The intragenic, 16S–23S ITS1 region has also been used as a tool to confirm genomovar assignments (Guasp *et al.*, 2000). The sequence of ITS1 is assumed to be less susceptible to selective pressures, due to its non-coding function, and should have accumulated a higher percentage of mutations than the rRNA genes (Tyrrell *et al.*, 1997). Comparison of ITS1 sequences indicates that the considerable variation in length and sequence makes these regions good candidates for discriminating closely related taxa (Gürtler & Stanisich, 1996). ITS1 sequences are identical within all the strains of a *P. stutzeri* genomovar and deletions or insertions in this portion of DNA can be used as a taxonomic tool to differentiate strains at the genomovar level (Guasp *et al.*, 2000). ITS1 was amplified, according to Guasp *et al.* (2000), by PCR with oligonucleotide primers 16F945 and 23R458. The primers used for sequencing were rrn16S and rrn23S (Jensen *et al.*, 1993), designed to anneal to conserved positions in the 3′ and 5′ regions of the bacterial 16S rRNA and 23S rRNA genes, respectively.

Strain KC is closely linked to the genomovar 3–genomovar 4 cluster (Fig. 2), since base differences were only observed towards the end of the sequence. The ITS1 region of strain KC has 11 bp and 2 bp insertions separated by four bases, and nine mismatches were also observed in the vicinity of these insertions. These differences indicate that strain KC could be a new genomovar of *P. stutzeri*, since it shows more than 80 % identity at the sequence level to *P. stutzeri* strains and less than 70 % sequence identity to other closely related species such as *P. putida*, *P. aeruginosa* and *Pseudomonas mendocina*.

Our results demonstrate that strain KC is a member of the species *P. stutzeri* and that strain KC does not belong to any described genomovar within the species. The phenotype of strain KC fits the description of the overall phenotype of the species, except for the ability to oxidize *m*-inositol and the capability to degrade CCl_4. Recent nitrite reductase (*nirS*) gene sequencing results published by Grüntzig *et al.* (2001) provide further evidence that strain KC does not belong to genomovar 5 or 4, the genomovars that are most similar to strain KC on the basis of DNA–DNA hybridizations and the sequencing of 16S rDNA and ITS1. *nirS* is an important gene for the definition of the species *P. stutzeri*. The *nirS* gene of strain KC is more similar to the '*P. stutzeri*-type', while the *nirS* sequences of members of genomovars 4 and 5 are more similar to the '*P. aeruginosa*-type'. We therefore

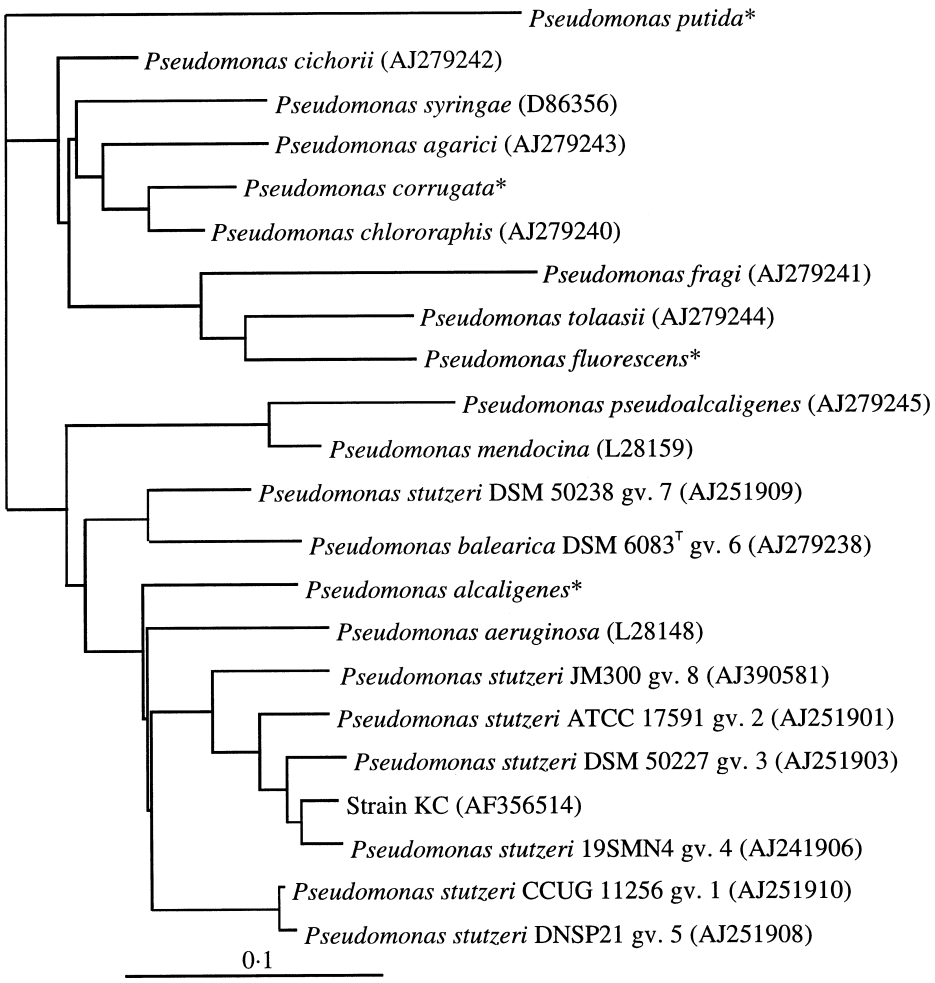

Fig. 2. Dendrogram depicting phylogenetic relationships between strain KC, several *P. stutzeri* strains and type strains of some other *Pseudomonas* species, as estimated by comparing ITS1 sequences. ITS1 sequences were aligned using the computer program CLUSTAL W (Thompson *et al.*, 1994) with a final manual adjustment (Rabaut, 1996). Evolutionary distances were calculated from pairwise sequence similarities (Jukes & Cantor, 1969) and estimations of relationships were generated using the FITCH program within PHYLIP (Felsenstein, 1989). Scale bar, 0·1 substitutions per nucleotide position. *, Accession numbers not listed by Guasp *et al.* (2000).

propose that strain KC be classified as the sole representative of a new genomovar, genomovar 9, following the enumeration of Rosselló *et al.* (1991) and Rosselló-Mora *et al.* (1996). The isolation and characterization of new strains belonging to the same genomovar as strain KC may help to clarify whether the unique phenotypic characteristics of strain KC are sufficient to propose its reclassification as a new species within the genus *Pseudomonas*, or if these attributes simply reflect unusual physiological traits within the diverse species *P. stutzeri*.

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