Luteibacter rhizovicinus gen. nov., sp. nov., a yellow-pigmented gammaproteobacterium isolated from the rhizosphere of barley (*Hordeum vulgare* L.)

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Three strains of Gram-negative, aerobic, yellow-pigmented, chemo-organotrophic bacteria, motile by a polar flagellum, were isolated from the rhizosphere of spring barley (*Hordeum vulgare* L.) at a research field near Copenhagen, Denmark. The three strains, LJ79, LJ96^T and LJ99, formed visible colonies on one-tenth-strength tryptic soy broth supplemented with agar (1/10 TSBA) after incubation for 6 days at 15 °C. The strains hydrolysed starch, casein (skimmed milk), gelatin and various pentoses and hexoses and grew on MacConkey agar and full-strength TSBA. Growth on 1/10 TSBA occurred at 4–30 °C, pH 6–9 and 0–3 % (w/v) NaCl. The strains had identical 16S rRNA gene sequences and ERIC (enterobacterial repetitive intergenic consensus sequence) fingerprint profiles, but could be differentiated by their RAPD (random amplified polymorphic DNA) fingerprint patterns. Strain LJ96^T had a DNA G + C content of 64.3 mol% and the major fatty acids were 15:0 iso (23.4 %), 17:1 iso ω 9c (25.5 %) and 17:0 iso (18.1 %). Phylogenetic analysis of the 16S rRNA gene sequences of the three strains showed 96 % sequence similarity to *Rhodanobacter lindaniclasticus* LMG 18385^T, 95 % to *Frateuria aurantia* DSM 6220^T and 96 % to *Fulvimonas soli* LMG 19981^T. Using LJ96^T DNA as probe, DNA–DNA hybridizations documented the relationship of the three strains to a single species (87.4–98.7 % relatedness) and showed less than 30 % relatedness to *Frateuria aurantia* DSM 6220^T and *Fulvimonas soli* DSM 14263^T. *Rhodanobacter lindaniclasticus* LMG 18385^T is not extant and the strain not available from any public strain collections, thus DNA–DNA hybridization could not include this strain. On the basis of genotypic and phenotypic characteristics, the three yellow-pigmented strains could also be differentiated from *Frateuria aurantia*, *Fulvimonas soli* and *Rhodanobacter lindaniclasticus*. The name *Luteibacter rhizovicinus* gen. nov., sp. nov. is proposed, with the type strain LJ96^T (= DSM 16549^T = ATCC BAA-1015^T).

Soil adjacent to plant roots (the rhizosphere) usually supports a larger number and diversity of culturable bacteria than bulk soil (Lilley *et al.*, 1996; Marilley & Aragno, 1999; Johansen & Binnerup, 2002) probably due to release of exudates and sloughed off cells from the root tissue. The

culturable rhizosphere bacteria obtained on standard laboratory substrates are usually dominated by copiotrophic bacteria, such as *Bacillus* species (Germida *et al.*, 1998), *Cytophaga*-like bacteria (Johansen & Binnerup, 2002), *Enterobacter* species (Mahaffee & Kloepper, 1997), *Pseudomonas* species and *Xanthomonas* species (Lilley *et al.*, 1996).

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Abbreviations: ERIC, enterobacterial repetitive intergenic consensus sequence; RAPD, random amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the strains LJ79, LJ96^T and LJ99 are AJ580497–AJ580499, respectively.

Fatty acid profiles of LJ96^T and *Fulvimonas soli* DSM 14263^T and ERIC-PCR and RAPD fingerprints of LJ96^T and related species are available as supplementary material in IJSEM Online.

Members of the family *Xanthomonadaceae* (*Gammaproteobacteria*) are known to have diverse metabolic potential (Finkmann *et al.*, 2000; Mergaert *et al.*, 2002; Nalin *et al.*, 1999) and can be isolated from habitats like plants roots, contaminated soil and humans (Drancourt *et al.*, 1997; Hauben *et al.*, 1999; Swings *et al.*, 1980; Wells *et al.*, 1987). In recent years, several new genera have been included in the family *Xanthomonadaceae*. In the present paper, we suggest another genus of the family by providing a phylogenetic and phenotypic characterization of three

yellow-pigmented strains. The strains represent a novel species within a novel genus, *Luteibacter rhizovicinus* gen. nov., sp. nov.

The isolates originated from rhizosphere soil of a spring barley (*Hordeum vulgare* L.) plant grown on an organic field at Højbakkegaard (Taastrup, Denmark). See Johansen & Binnerup (2002) for further details concerning the isolation procedure. The strains appeared on 1/10 TSBA [3.0 g tryptic soy broth l⁻¹ solidified with 15.0 g agar l⁻¹ (Difco), pH 7.0] after 6 days of incubation at 15 °C in darkness. Single colonies were subcultured to ensure purity. The strains were preserved at -80 °C in a suspension of sterile MilliQ water and 15 % glycerol. *Rhodanobacter lindaniclasticus* LMG 18385^T originating from soil enriched with lindane (Nalin *et al.*, 1999), *Frateruia aurantia* DSM 6220^T isolated from the plant *Lilium auratum* Lindl (Swings *et al.*, 1980) and *Fulvimonas soli* DSM 14263^T originating from soil enriched with acetylated starch plastic (Mergaert *et al.*, 2002) were also included in the study. Since *Rhodanobacter lindaniclasticus* LMG 18385^T is no longer available from the BCCM/LMG, other public strain collections or from the authors (D. Janssens, Curator at BCCM/LMG Bacteria Collection, personal communication), only the genus and species description and the 16S rRNA gene sequence (GenBank accession no. AF039167) were used. Each of the three genera *Rhodanobacter*, *Frateruia* and *Fulvimonas* contains only a single species. *Frateruia aurantia* DSM 6220^T and *Fulvimonas soli* DSM 14263^T were cultured according to the recommendations of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

Transmission electron microscopy of LJ96^T was done using the whole mount preparation technique. Approximately 10 µl of a culture of LJ96^T, grown to exponential phase, was placed on a Formvar and carbon-coated grid and allowed to dry for about 30 min. Cells were killed by 2 % (v/v) osmium tetroxide solution vapour for 30 s. The grid was dried completely, washed in distilled water for 10 min and placed on a drop of 2 % (v/v) uranyl acetate for 5 min in darkness. Cells were examined in a JEOL electron microscope JEM-1010. Lysis with 3 % KOH was used to determine whether isolates were Gram-positive or -negative (Buck, 1982). Catalase activity was detected by the presence of bubbles after the addition of one drop of 3 % H₂O₂ to colonies growing on 1/10 TSBA plates. Cytochrome oxidase activity was considered positive when cells formed blue pigments after being streaked on filter paper wetted with 1 % N,N,N',N'-tetramethyl-*p*-phenyldiamine dihydrochloride (Merck). Change of colony colour (yellow to weak orange) was tested by adding 10 % (w/v) KOH to a 2-day-old colony grown on 1/10 TSBA. Hydrolytic enzyme activity of α-amylase, α-cellulase, β-mannanase, β-xylanase was tested by adding 0.5 g chromogenic azurine-cross-linked (AZCL) substrates l⁻¹ (MegaZyme) to 1/10 TSBA plates. Starch degradation was tested by adding 5.0 g starch l⁻¹ (Merck) to 1/10 TSBA plates. Clearing zones on starch-amended 1/10 TSBA plates were detected after dripping

Lugol solution (Fluka) on the surface of the plates. Protease activity was tested by addition of sterilized skimmed milk (Difco) (50.0 g l⁻¹) to 1/10 TSBA plates. The plates were incubated for up to 6 days at 20 °C and analysed daily for colour reactions (AZCL substrates) or clearing zones (skimmed milk and starch) around colonies. The ability to grow on 1/10 TSBA under anaerobic conditions was tested with the Oxoid Anaerobic system and agar plates were incubated for 3 days at 20 °C. The commercial systems API 20NE and API 50CHE (bioMérieux) were performed as recommended by the manufacturer using overnight cultures grown on 1/10 TSBA plates. All API tests were read after 3 days of incubation at 20 °C. Growth at different pH was studied on 1/10 TSBA plates by adjusting the pH with HCl or NaOH to pH 6, 7, 8 and 9. The salt tolerance was tested on 1/10 TSBA plates containing 0, 0.5, 1, 2.0, 3.0 and 5.0 % (w/v) NaCl. To test growth at different temperatures, 5 µl overnight cultures were placed on 1/10 TSBA plates and incubated at 5, 10, 15, 20, 30 and 37 °C for up to 25 days. The fatty acid methyl ester profile of strain LJ96^T was prepared by culturing at 20 °C for 3 days in 1/10 TSB and then identified by the DSMZ using standard methods (Sasser, 1990).

DNA was extracted by suspending a small amount of a colony in 200 µl Tris/EDTA buffer (1 M Tris/HCl, 0.5 M EDTA, pH 7.5) and boiling at 102 °C for 10 min. Cell debris was removed by centrifugation at 15 000 g for 5 min. The DNA-containing supernatant was placed in a new Eppendorf tube and stored at 4 °C. More than 95 % of the total 16S rRNA gene sequences of strains LJ96 (1474 bp), LJ79 (1471 bp) and LJ99 (1420 bp) were determined. PCR amplification (25 cycles at 94 °C for 30 s, 61 °C for 30 s and 72 °C for 2 min followed by 1 cycle at 72 °C for 6 min) of the 16S rRNA gene sequences was done in PCR tubes containing 1 µl of the DNA extract and 0.5 µM of each primer SDBact0008aS20 [5'-AGAGTTTGATC(AC)TGGCTCAG-3'] and S*UNIV518Aa18 (5'-GTATTACCGCGGCTGCTG-3'), 250 µM of each dNTP, 1 × PCR buffer, 0.5 mM MgCl₂ and 0.5 U *Taq* DNA polymerase. PCR products were purified using the QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions. MWG-BIOTECH (<http://www.mwg-biotech.com>) sequenced the PCR products on a NEN Global IR² DNA Sequencer Li-COR using the primer SDBact008aS20. Sequences were compared to sequences in nucleotide databases using the BLASTN 2.2.6 search program (<http://www.ncbi.nlm.nih.gov>) (Altschul *et al.*, 1997). Representatives of relatives were used to construct a phylogenetic tree where *Escherichia coli* K-12 (E05133) was used as an outgroup. Sequences were aligned using the algorithm CLUSTAL W (Thompson *et al.*, 1994) in the program BIOEDIT (Hall, 1999). All sequences were trimmed at the left and right ends and a total of 1338 bp were used for construction of the phylogenetic tree. Tree calculations were done in TREECON (version 1.3b) (Van de Peer & De Wachter, 1994) using the Jukes & Cantor (1969) substitution model followed by the neighbour-joining algorithm of Saitou & Nei (1987). Bootstrap values were collected for 1000

phylogenetic trees (Felsenstein, 1985). Genomic characterization by ERIC (enterobacterial repetitive intergenic consensus sequence) PCR on 1 µl DNA extracts was performed in a HYBAID pcrEXPRESS HBPX-220 thermocycler using a modified procedure of De Bruijn (1992). The primers used were R1CIRE (5'-CACTTAGGGGTCCTCGAATGTA-3') and ERIC2 (5'-AAGTAAGTGAAGGGGTGAGCG-3'). The program used was as follows: 1 cycle at 95 °C for 20 s followed by 35 cycles of 94 °C for 5 s, 54 °C for 1 min and 72 °C for 2 min, 1 cycle at 72 °C for 16 min and a final soak at 4 °C. Random amplified polymorphic DNA (RAPD) PCR was performed with three 10-base primers [OPA04 (5'-AATCGGGCTG-3'), OPA07 (5'-GAAACGGGTG-3') and OPA15 (5'-TTCCGAACCC-3'); Operon Technologies Inc.] according to Hansen & Winding (1998). DNA base composition (G+C content) was determined by the DSMZ by HPLC as described previously (Mesbah *et al.*, 1989; Tamaoka & Komagata, 1984; Visuvanathan *et al.*, 1989). DNA–DNA hybridization experiments were performed by the DSMZ using strains LJ79, LJ96^T, LJ99, *Frateruia aurantia* DSM 6220^T and *Fulvimonas soli* DSM 14263^T. DNA was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). The hybridization reactions were performed at 68 °C in 2 × SSC plus 10 % (v/v) DMSO (1 × SSC is 0.15 M NaCl plus 15 mM sodium citrate) as described by De Ley *et al.* (1970), with the modifications of Huß *et al.* (1983) and Escara & Hutton (1980), on a model 2600 spectrophotometer equipped with a model 2527-R thermoprogammer and plotter (Gilford Instrument Laboratories). Renaturation rates were computed with the TRANSFER.BAS program of Jahnke (1992).

Strains LJ79, LJ96^T and LJ99 were Gram-negative, motile rods (1.0 × 0.4 µm) with one polar flagellum 10–15 µm long (Fig. 1) and formed yellow colonies on 1/10 TSBA. They were catalase- and oxidase-positive and grew on 1/10 TSBA, full-strength TSBA and MacConkey agar. The colony colour changed from yellow to weak orange by addition of 10 % KOH. The colour change is a phenolate reaction that can be observed with several bacterial pigment types

Fig. 1. Scanning electron micrographs showing cells of LJ96^T. Bars, 2 µm.

(Reichenbach, 1992). Growth was observed on 1/10 TSBA between 5 and 30 °C but not at 37 °C, at pH 6–9 and at 0–3 % NaCl but not at 5 %. Other biochemical and physiological characteristics of the strains are presented in Table 1. The fatty acid composition of LJ96^T consisted mainly of branched 15:0 iso (23.4 %), 17:1 iso ω9c (25.5 %) and 17:0 iso (18.1 %) (detailed results are available in Supplementary Table S1 in IJSEM Online). Of the main branched fatty acids, 17:1 iso ω9c and 17:0 iso were found in larger proportions and 15:0 in a smaller proportion in LJ96^T than in *Fulvimonas soli* DSM 14263^T. Four fatty acids (15:0 iso, 15:0 anteiso, 16:0 and 16:1 ω9c) comprise 35–60 % of the total fatty acid profiles of xanthomonads (Norman *et al.*, 1997) and quantitative variation of these fatty acids has been used to differentiate among species of *Xanthomonas* (Chase *et al.*, 1992). LJ96^T had a fatty acid value of 30 % of the total profile (15:0 iso, 15:0 anteiso and 16:0) and this low value may be used as a differentiating character from other members of the family *Xanthomonadaceae*.

Analysis of the phylogenetic position of the strains demonstrated that they belonged to the family *Xanthomonadaceae* within the *Xanthomonadales* of the *Gammaproteobacteria* (Fig. 2). The highest similarity values were found for the genera *Rhodanobacter* (96 %), *Frateruia* (95 %) and *Fulvimonas* (96 %) (Fig. 2). Other members of the *Xanthomonadales* were distantly related (83–91 % similarity). DNA–DNA hybridization was performed with LJ96^T as a probe against LJ79, LJ99, *Frateruia aurantia* DSM 6220^T and *Fulvimonas soli* DSM 14263^T (*Rhodanobacter lindani-clasticus* LMG 18385^T was not available, as described above) to evaluate the closeness of their relationship. The results of the DNA–DNA hybridizations showed that LJ79, LJ96^T and LJ99 could be considered as one species (87.4 to 98.7 % relatedness to the probe) while *Frateruia aurantia* DSM 6220^T and *Fulvimonas soli* DSM 14263^T comprised two other species (i.e. below 31 % relatedness to the probe) (Stackebrandt & Goebel, 1994). The DNA G+C content of strains LJ96^T was 64.3 mol%, which is very close to the values reported for *Frateruia aurantia* DSM 6220^T (63.5 mol%) and *Rhodanobacter lindani-clasticus* LMG 18385^T (63.0 mol%) but considerably lower than the 71.7 mol% of *Fulvimonas soli* DSM 14263^T (Table 1). ERIC-PCR banding patterns of the three strains were identical but different from *Frateruia aurantia* DSM 6220^T and *Fulvimonas soli* DSM 14263^T (see Supplementary Fig. S1a in IJSEM Online). RAPD banding patterns, on the other hand, revealed differences between the three strains, and between the strains and *Frateruia aurantia* DSM 6220^T and *Fulvimonas soli* DSM 14263^T (Supplementary Fig. S1b), indicating a non-clonal origin of LJ79, LJ96^T and LJ99, a result also found by DNA–DNA hybridization (Table 1).

Three of the six closest relatives of LJ79, LJ96^T and LJ99 (Fig. 2) based on 16S rRNA gene sequence analysis were obtained by cloning of rRNA from different habitats. The sequences AJ318146 and AY081991 originated from soil

Table 1. Biochemical and physiological characteristics that differentiate strains LJ79, LJ96^T and LJ99 from their closest cultured relatives

Species/strains: 1, *Luteibacter rhizovincinus* gen. nov., sp. nov.; 2, *Rhodanobacter lindaniclasticus* LMG 18385^T (data from Nalin *et al.*, 1999); 3, *Frateruia aurantia* DSM 6220^T (Swings *et al.*, 1980; this study); 4, *Fulvimonas soli* DSM 14263^T (Mergaert *et al.*, 2002; this study); 5, *Xanthomonas campestris* LMG 568^T (Van den Mooter & Swings, 1990; Vauterin *et al.*, 1995). The genera *Rhodanobacter*, *Frateruia* and *Fulvimonas* consist of single species only. Characteristics are scored as: +, positive; −, negative; ±, weak; d, strain-dependent; ND, not determined. All *L. rhizovincinus* strains were positive for the following tests: catalase; utilization of *N*-acetylglucosamine, D-glucose, D-fructose, D-mannose, D-fucose, galactose, cellobiose, lactose, melibiose, sucrose and trehalose, growth at 5, 10, 15, 20 and 30 °C, pH 6, 7, 8 and 9 and in media containing 0, 0.5, 1, 2 and 3 % NaCl and change of colony colour (yellow to weak orange). All *L. rhizovincinus* strains were negative for the following tests: activity of the hydrolytic enzymes α-amylase, α-cellulase, β-xylanase and arginine dihydrolase, utilization of arginine, urea, maltose, mannitol, gluconate, caprate, adipate, malate, phenylacetate, glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β-xyloside, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, arbutin, salicin, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, D-turanose, D-tagatose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate, nitrate reduction, indole production, growth at 37 °C and in 5 % NaCl.

Characteristic	1		2	3	4	5
	LJ79, LJ99	LJ96 ^T				
Motility	+	+	−	+	+	+
Acid from D-glucose	−	−	−	+	+	+
Growth on:						
β-Gentiobiose	−/+	+	−	−	−	+
D-Lyxose	−/+	+	−	−	−	ND
Maltose	−	−	−	+	+	+
Citrate	−	−	+	−	−	ND
MacConkey agar	+	+	ND	−	−	ND
Enzyme activities:						
Oxidase	+	+	+	−	+	±
Protease (skimmed milk)	+	+	−	−	+	+
β-Galactosidase	+	+	−	−	+	ND
Hydrolysis of:						
Aesculin	+	+	+	−	+	+
Starch	+	+	ND	±	+	d*
Gelatin	+	+	ND	−	−	+
Growth at:						
5 °C	+	+	−	−	−	−
10 °C	+	+	ND	−	−	ND
37 °C	−	−	+	+	−	−
41 °C	−	−	+	−	−	−
Anaerobic growth on 1/10 TSBA	+	+	ND	+	+	−
DNA–DNA hybridization (%)	98.3/87.4	98.7	ND	29.7	30.3	ND
with LJ96 ^T as a probe†						
Mean DNA G + C (mol%)	ND	64.3	63.0	71.7	63.5	65.8–66.6

*75 % of 189 *X. campestris* strains tested positive (Van den Mooter & Swings, 1990).

†Determined at 68 °C in 2 × SSC plus 10 % (v/v) DMSO.

habitats contaminated with xenobiotics, whereas sequence AY218686 came from penguin dropping sediments from Ardley Island, Antarctica. The sequence AY162032 had 98.5 % similarity to the sequences of LJ79, LJ96^T and LJ99, and originated from a tropical forest soil isolate sampled

in Ghana. Sequence AY162032 was obtained by cultivation of samples in gel microdroplets (Zengler *et al.*, 2002). The three strains *Rhodanobacter lindaniclasticus* LMG 18385^T, *Frateruia aurantia* DSM 6220^T and *Fulvimonas soli* DSM 14263^T all originated from soil environments (as previously

Fig. 2. Neighbour-joining tree of 16S rRNA gene sequence similarity, showing the phylogenetic position of strains LJ79, LJ96^T, LJ99 and representatives of closely related isolates and other more distantly related genera of the family *Xanthomonadaceae*. Bootstrap percentages are indicated at branches and calculated from 1000 trees. *Escherichia coli* K-12 (E05133) was used as an outgroup. Sequence accession numbers are shown in parentheses.

described). Our stains were isolated from an agricultural field located in Denmark after growth on a standard soil bacterial substrate. Altogether, this suggests that members of the *Xanthomonadaceae* are adapted to soil habitats around the world and can be retrieved by a wide spectrum of cultivation and purification techniques.

In conclusion, we propose a novel genus within the *Xanthomonadaceae* with the name *Luteibacter* gen. nov., containing the species *Luteibacter rhizovicianus* gen. nov., sp. nov. (Fig. 2). This new genus can be separated from its nearest relatives by phenotypic characteristics (Table 1). *Luteibacter rhizovicianus* can be differentiated (96% 16S rRNA gene sequence similarity) from *Rhodanobacter lindanoclasticus* (of which the type strain is currently not available) by its mobility, protease activity and growth below 10 °C, and from *Frateuria aurantia* by being oxidase-positive, capable of growing on MacConkey agar, protease activity, hydrolysis of aesculin and gelatin, growth below 10 °C and G+C content. Further, *Fulvimonas soli* differs from our three strains by the absence of hydrolysis of gelatin and its failure to grow below 10 °C.

Description of *Luteibacter* gen. nov.

Luteibacter (Lu.te.i.bac'ter. L. adj. *luteus* yellow; N.L. masc. n. *bacter* rod; N.L. masc. n. *Luteibacter* yellow rod).

Cells are Gram-negative, single rod and motile by a polar flagellum; no spores or capsules are observed. Aerobic, chemo-organotroph, mesophilic (between 5–30 °C but not at 37 °C), pH range 6–9 and tolerant to 0–3% (w/v) NaCl. Aesculin, starch and gelatin are hydrolysed. Colony colour is yellow after growth on 1/10-strength and full-strength TSBA. Colony colour change from yellow to weak orange by

addition of 10% KOH. Catalase- and oxidase-positive. Fatty acids are mainly branched (15:0 iso, 17:1 iso ω9c, 17:0 iso). The genus *Luteibacter* belongs to the family *Xanthomonadaceae* in the class *Gammaproteobacteria*. The type species is *Luteibacter rhizovicianus*.

Description of *Luteibacter rhizovicianus* sp. nov.

Luteibacter rhizovicianus (rhi.zo.vi.ci'nus. Gr. n. *rhiza* root; L. masc. adj. *vicinus* neighbouring; N.L. masc. adj. *rhizovicianus* neighbouring a root, referring to the rhizosphere, soil closely related to plant roots, from where the type strain was isolated).

Displays the following properties in addition to those given in the genus description. Yellow, low-convex colonies, 3–5 mm in diameter and with an entire edge after 48 h at 20 °C when grown on 1/10 TSBA. Characteristics of the species are presented in Table 1. The fatty acid composition consists mainly of branched 15:0 iso (23.4%), 17:1 iso ω9c (25.5%) and 17:0 iso (18.1%) (detailed results are available in Supplementary Table S1). The DNA G+C content of the type strain is 64.3 mol%.

The type strain is LJ96^T (=DSM 16549^T=ATCC BAA-1015^T), isolated from the rhizosphere of a spring barley plant (*Hordeum vulgare* L.) at Højbakkegaard (Taastrup, Denmark).

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