Desulfovibrio africanus subsp. *uniflagellum* subsp. nov., a sulfate-reducing bacterium from a uranium-contaminated subsurface aquifer

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The bacterial strain SR-1^T was isolated from subsurface sediments of a uranium-contaminated site in Shiprock, New Mexico, USA. Cells are vibrioid and motile by means of a single polar flagellum. Strain SR-1^T grows on sulfate, oxidizing formate, lactate and H₂, but not malate, and ferments pyruvate. The DNA sequences of the 16S rRNA gene and the 16S–23S internal transcribed spacer of strain SR-1^T showed 99.9 and 99.4 % similarity, respectively, to those of the type strain *Desulfovibrio africanus* DSM 2603^T. The DNA sequence of the ITS region is 300 bases in length and contains two tRNA genes (tRNA^{Ile}, tRNA^{Ala}). The partial DNA sequence of the *dsrAB* gene showed 94.6 % amino acid sequence similarity to that of *D. africanus*. The DNA G+C content of strain SR-1^T was 62.4 mol% and it showed 72 % DNA–DNA similarity to *D. africanus*. DNA typing methods that target gene clusters and whole genomes revealed characteristic genomic fingerprints for strain SR-1^T. A small plasmid was detected by gel electrophoresis. On the basis of distinct phenotypic and genotypic characteristics, strain SR-1^T represents a novel subspecies of *D. africanus*, for which the name *Desulfovibrio africanus* subsp. *uniflagellum* subsp. nov. is proposed. The type strain is SR-1^T (=JCM 15510^T =LS KCTC 5649^T).

Sulfate-reducing bacteria (SRB) are primarily isolated from environmental sources, such as soil, water and sediments (Postgate & Campbell, 1966). A number of SRB have been isolated from a variety of subsurface habitats where sulfate reduction has been shown to be important. Examples include granitic aquifers (Motamedi & Pedersen, 1998), deep sandstones (Krumholz *et al.*, 1999; Sass & Cypionka, 2004), a petroleum-contaminated aquifer (Allen *et al.*, 2008) and oil reservoirs (Davidova *et al.*, 2006). Species of the genus *Desulfovibrio* are members of the class *Deltaproteobacteria*, with more than 47 proposed species. Species of the genus *Desulfovibrio* isolated from freshwater and marine settings include *Desulfovibrio africanus*, which has typical lophotrichous flagella and has been reported to oxidize malate but not to ferment pyruvate (Campbell *et al.*, 1966). Based on phylogenetic analysis, *Desulfovibrio burkinensis* and *Desulfovibrio sulfodismutans* are the closest relatives of *D. africanus* (Thabet *et al.*, 2007). In this study, we report the isolation and characterization of strain SR-1^T

and compare it to the closely related species *D. africanus*. Phenotypic and genotypic differences include morphology, phylogenetic analysis (*dsrAB* gene), DNA G+C mol%, DNA–DNA hybridization, genomic fingerprints using PFGE, 16S rRNA gene sequence, BOX-PCR and the presence of a small plasmid. Based on these differences, we suggest that this strain represents a novel subspecies of *D. africanus*.

Strain SR-1^T was isolated from a uranium-contaminated, shallow, subsurface aquifer in Shiprock-New Mexico, where the sediment contained at least 4.27×10^4 SRB g⁻¹ (Castañeda Carrión, 2001). The geochemistry of the aquifer has been previously described (Chang *et al.*, 2001; Elias *et al.*, 2003; Schryver *et al.*, 2006) and the aquifer has been shown to have abundant sulfate (up to 140 mM) and highly variable salinity, with sodium concentrations reaching as high as 411 mM (Nevin *et al.*, 2003) in some wells. Sulfate reduction has been shown to be an important respiratory processes in this sulfate-rich aquifer (Elias *et al.*, 2003). The isolation of strain SR-1^T was carried out at 30 °C by liquid enrichment of a 10⁻⁶ dilution of subsurface sediments in basal mineral medium containing minerals, trace metal solution, vitamins, 0.05 % yeast extract, 10 mM Na₂SO₄, 25 mM sodium lactate, 0.5 mM Fe(NH₄)₂(SO₄)₂ and 20 mM TES, pH 7.2, prepared under 80 % N₂/20 % CO₂ (Widdel & Bak, 1992). Sterile PdCl₂ and NaHCO₃

Abbreviations: ITS, internal transcribed spacer; SRB, sulfate-reducing bacteria.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA and *dsrAB* gene sequences of strain SR-1^T are EU659693 and EU716165, respectively.

Differential PFGE fingerprint patterns between strain SR-1^T and *D. africanus* are available with the online version of this paper.

were added to final concentrations of 0.005 % and 0.07 %, respectively (Castañeda Carrión, 2001). The headspace contained 10 ml H_2 gas. After three transfers, the enrichment culture was serially diluted in the same medium up to 10^{-8} , and then anaerobically plated onto solid basal medium containing 1.5 % agar. The inoculum was added to a mineral medium supplemented with 0.8 % agar, overlaid onto the solid agar and incubated under 4 % H_2/N_2 atmosphere in an anaerobic glovebag. After 1 week of incubation, black colonies appeared on plates that had been inoculated from the 10^{-5} dilution. Colonies were picked and streaked onto the solid medium described above. After a further 2 weeks of incubation, black colonies were transferred to liquid mineral medium. Pure cultures were maintained on basal liquid medium lacking $Fe(NH_4)_2(SO_4)_2$ and reduced with 1 mM $Na_2S_9H_2O$ instead of $PdCl_2$ and H_2 (for ease of manipulation) and all subsequent incubations were at 37 °C. Desulfovibrin was tested as described by Postgate (1984). Motility was observed by phase-contrast light microscopy, and the morphological properties were analysed by transmission electron microscopy according to standard protocols for the negative stain with uranyl acetate (Bozzola & Russell, 1999).

The range of electron donors and electron acceptors was determined in basal medium containing 0.05 % yeast extract and 1 mM $Na_2S_9H_2O$ [lacking $Fe(NH_4)_2(SO_4)_2$]. Electron donors were tested in medium supplemented with sterile stock solutions (2–20 mM) of donor and with 10 mM sulfate as the terminal electron acceptor. Hydrogen gas, when tested, was added into the gas phase of tubes. The use of electron acceptors was determined with 10 mM lactate as the electron donor. Controls lacked donor for donor tests or acceptor for acceptor tests. After inoculation (1 %, v/v), growth rates were determined by monitoring an increase in OD_{600} , and were regarded as positive if this exceeded 0.1 within 14 days. Fermentative growth on pyruvate was tested in sulfate-free basal medium, and acetate accumulation in the culture media was determined by gas chromatography (Mouttaki *et al.*, 2007). Growth at a range of temperatures, pH and NaCl concentrations was determined in a modified lactate/sulfate medium (Castañeda Carrión, 2001; Rapp & Wall, 1987). The pH range tested was from 3 to 8.5. The buffer systems were 25 mM sodium citrate (pH 3–6.2), 25 mM MOPS (pH 6.5–7), 25 mM HEPES (pH 7–8) and 25 mM sodium carbonate (pH 8.5). The NaCl concentrations tested were from 0.5 to 8 %. Growth temperature was tested at 15, 20, 25, 30, 37, 40 and 45 °C. For analysis of small-subunit rRNA sequence, internal transcribed spacer (ITS) and DSR sequences, the cultures were grown in lactate/sulfate medium and genomic DNA was isolated from 10 ml cultures using the Easy-DNA kit (Invitrogen). DNA templates were amplified in a total reaction of 50 µl containing 500 ng of chromosomal DNA, 400 µM dNTPs, 400 nM primers, 2.5 mM $MgCl_2$ and 2.5 U of Platinum Taq DNA polymerase (Invitrogen). Thermal cycling

conditions were adjusted according to the T_m of primers and the length of the fragment to be amplified. PCR products were cloned into the TOPO-TA cloning vector according to the manufacturer's instructions (Invitrogen). For each analysis, twenty clones were picked randomly, screened by restriction digestion, and two clones were sequenced at the Oklahoma Medical Research Foundation (Oklahoma City, OK, USA). DNA sequences were subjected to a BLAST search against the GenBank database using the BLASTN algorithm. The 16S rRNA gene was amplified by using primers 8F and 1496R (Kane *et al.*, 1993). The 16S–23S rRNA ITS was amplified with the custom-designed primer F-16S (5'-TG TAGGAGTGAAA-TCCGTAG-3') and the consensus primer R-23S (García-Martínez *et al.*, 1999). Complete 16S rRNA gene sequences from other species of the genus *Desulfovibrio* were aligned via CLUSTAL_X (Thompson *et al.*, 1997), and distance trees were constructed with the PAUP 4.0* software package using the neighbour-joining algorithm and Jukes–Cantor correction. The ITS structure was analysed with IWoCS software (D'Auria *et al.*, 2006) and tRNA genes were detected with the program tRNAscan-SE v.1.21 (Lowe & Eddy, 1997). The *dsrAB* gene (1983 bases) was amplified using the primer pair DSR1F and DSR4R (Wagner *et al.*, 2005). Sequencing was completed with primers DSR1F-2 (5'-TTCCGACTTCTCGGTCATCG-3') and DSR4R-2 (5'-TCCAGGATCTCGTGGTACAG-3). *DsrAB* amino acid sequences deduced from *Desulfovibrio dsrAB* gene sequences greater than 1941 nucleotides were considered for comparative analysis. The 327 amino acid positions of the α -subunit and the 216 amino acid positions of the β -subunit were used for phylogeny inference, and a distance matrix was generated using PHYLIP and the Jones-Taylor-Thorton model (Wagner *et al.*, 2005). A phylogenetic tree based on the FITCH distance method was generated using ARB software (Ludwig *et al.*, 2004).

The spectroscopic DNA–DNA hybridization between strain SR-1^T and *D. africanus* DSM 2603^T, and the DNA G+C content of strain SR-1^T were determined by the Deutsche Sammlung von Mikroorganismen und Zellkulturen. PFGE from 3 ml exponential phase cultures was performed according to standard protocols on a CHEF-DF III system (Bio-Rad Laboratories). Briefly, the agarose embedded chromosomal DNA was digested with 20 U of *PmeI* in a 200 µl restriction mixture at 37 °C for 16 h. Running conditions were 14 °C, 6.0 V cm^{-1} for 24 h, and a switching time linearly ramped from 60–120 s. The 16S rRNA gene fingerprints were detected by Southern blot hybridization. Genomic DNA from each strain was digested with *HindIII*, *PstI*, *SphI*, *BmgBI* and *XmaI*. Approximately 5 µg of digested genomic DNA was separated by agarose gel electrophoresis on 0.8 % agarose gels in 1 × TAE buffer (pH 7.9) at 90 V for 4 h. Gels were blotted by downward capillary transfer on positively charged nylon membranes. The 227 bp labelled probe was amplified with a PCR DIG Probe synthesis kit (Roche) by using the custom designed primers F-16S-Hyb (5'-

GGTGTAGGAGTGAAATCCGTAG-3') and R-16S-Hyb (5'-AGTTTCAGCCTTGCGACCGTAC-3'). The reproducibility of the 16S rRNA fingerprints was assessed similarly using new genomic DNA samples. Repetitive PCR genomic fingerprints were obtained with the BOX A1R primer (Versalovic *et al.*, 1994). The 50 µl reaction mixtures contained 4 mM MgCl₂, 200 µM dNTPs, 1 µM primer, 2.5 U of Platinum Taq DNA polymerase, and 400 ng of genomic DNA. Thermal cycling conditions were: (i) 95 °C for 5 min; (ii) 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 8 min; followed by (iii) a final extension of 8 min at 72 °C. The reproducibility and stability of BOX-PCR fingerprint patterns were verified in two independent reactions. Cultures from exponential and stationary phase were assayed for the presence of plasmids using the Qiagen plasmid mini prep protocol, except that the bacterial lysate was directly precipitated with 2-propanol and 70 % ethanol.

Here, we report the isolation and characterization of an abundant sulfate-reducing bacterium from uranium(VI)- and sulfate-containing sediments (Elias *et al.*, 2003). Strain SR-1^T was isolated as a black colony that grew well with H₂, lactate and sulfate in the medium used for enrichment and isolation. Substrates tested which supported growth when sulfate (10 mM) was the electron acceptor included lactate (10 mM), formate (20 mM), pyruvate (10 mM), ethanol (10 mM) and H₂ (10 ml). Growth with H₂ was weak. Pyruvate (10 mM) supported fermentative growth, although at low levels. Substrates which did not support growth in the presence of sulfate included acetate (10 mM), butyrate (10 mM), malate (15 mM) propionate (10 mM), methanol (10 mM), choline (10 mM), glucose (2 mM) and fructose (2 mM). Both thiosulfate and sulfite (10 mM each) were used as electron acceptors and reduction of elemental sulfur occurred. Growth was not detected when nitrate (10 mM) or polysulfide (6 mM) was used as the electron acceptor. The differential phenotypic and genotypic characteristics are shown in Table 1.

Table 1. Differential characteristics between strain SR-1^T and closely related species of the genus *Desulfovibrio*

Strains: 1, Strain SR-1^T; 2, *Desulfovibrio africanus* DSM 2603^T [data from this study, Campbell *et al.* (1966) and Skyring & Jones (1972)]. Optimum values are shown in parentheses.

Characteristic	1	2
Cell shape	Vibrio	Sigmoid
Flagellation	Single polar	Lophotrichous
Cell size (µm)	0.5–3.5	0.5 × 5.0–10
pH range	6.25–7.65 (7)	6.6–7.65 (7)
Temperature range (°C)	20–40 (37)	20–40 (37)
NaCl maximum (%)	4.25	4
DNA G + C content (mol%)	62.4	63.1
Plasmid (8568 bp)	Yes	No

Electron microscopy analysis of negatively stained cells showed a single polar flagellum on strain SR-1^T and lophotrichous flagella on *D. africanus* (Fig. 1). Lophotrichous polar flagella have been described as a morphological characteristic of *D. africanus*, which was independently isolated from two different sites in Africa; both strains were identical in morphology, physiology and DNA base composition regardless of isolation source (Campbell *et al.*, 1966). Considering that the number of flagella is a relevant morphological characteristic in species of the genus *Desulfovibrio*, strain SR-1^T and *D. africanus* may be considered as different strains.

The growth of strain SR-1^T in sulfate-free pyruvate medium coupled with acetate production (6 mM) confirmed the fermentative metabolism of pyruvate. Growth with pyruvate as a carbon source is considered taxonomically significant for species affiliation to the genus *Desulfovibrio* (Postgate & Campbell, 1966). No difference in pyruvate fermentation has been observed between *Desulfovibrio* subspecies (Loubinoux *et al.*, 2002; Walker *et al.*, 2006). Additionally, it has been reported that pyruvate does not support growth of *D. africanus* in the absence of sulfate (Campbell *et al.*, 1966). However, our

Fig. 1. Electron micrographs (negative stain with uranyl acetate) of strain SR-1^T, showing the vibrioid shape and a single polar flagellum (a, b), and *Desulfovibrio africanus* DSM 2603^T, showing the lophotrichous flagella (c). Bars: 0.5 µm (a), 0.2 µm (b, c).

experiments showed that low levels of growth occurred for both the type strain and strain SR-1^T on pyruvate.

For phylogenetic comparison, we sequenced 1880 nucleotides from the ribosomal operon of strain SR-1^T, which includes the full-length 16S rRNA gene (1542 nucleotides), ITS region (300 nucleotides), and the 5' 23S rRNA gene. Comparative analysis of 16S rRNA gene sequences revealed that strain SR-1^T is closely related to *D. africanus*, with 99.9% sequence similarity, which is above the 97% threshold value generally used to delineate prokaryotic species (Wayne *et al.*, 1987). However, the discriminatory power of 16S rRNA sequences is limited when closely related species, and even different species within the same genus, are compared (Stackebrandt & Goebel, 1994). In the genus *Desulfovibrio*, *D. alaskensis* and *D. vietnamensis* (Feio *et al.*, 2004), and *D. magneticus* and *D. burkinensis* (Sakaguchi *et al.*, 2002), are species with high 16S rRNA sequence similarities (>98.7%). The DNA sequences of the 16S–23S ITS regions of strain SR-1^T and *D. africanus* were 300 and 299 bases in length, respectively, with each containing two tRNA genes (tRNA^{Ile}, tRNA^{Ala}). Other characterized species of the genus *Desulfovibrio* have similar 16S–23S ITS regions containing either tRNA^{Ile} or tRNA^{Ile} and tRNA^{Ala} genes (Loubinoux *et al.*, 2002). Whole-genome sequences of species of the genus *Desulfovibrio* (NC_002937 and NC_008751) have also revealed that the ITS regions are conserved in size and type of tRNA genes. Comparative analysis revealed that the ITS sequence of strain SR-1^T is 99% similar to that of *D. africanus*. Thus, the ITS region is not a useful marker to differentiate these closely related *Desulfovibrio* strains. Based on phylogenetic analysis of DsrAB amino acid sequences (Fig. 2), strain SR-1^T is related to *D. africanus* with a 94.66% sequence similarity. A similar phylogenetic affiliation has been reported for *Desulfovibrio aerotolerans* and *D. burkinensis*, with a 96.8% DsrAB amino acid sequence similarity (Mogensen *et al.*, 2005). There is no threshold value of *dsrAB* gene sequence divergence for species affiliation, but complete genome sequences of closely related strains of *Desulfovibrio vulgaris* have confirmed that they have identical *dsrAB* sequences. Our results indicate that the *dsrAB* gene may offer an advantage over the 16S rRNA gene and 16S–23S ITS region in

defining sequence divergence between closely related *Desulfovibrio* strains.

Considering that comparisons between some species of the genus *Desulfovibrio* with high 16S rRNA gene sequence similarities (Feio *et al.*, 2004; Sakaguchi *et al.*, 2002) did not meet the 70% DNA–DNA hybridization criterion for inclusion in the same species (Wayne *et al.*, 1987), the DNA–DNA hybridization between strain SR-1^T and *D. africanus* was assayed and shown to be 72%. It is important to note that a 90% DNA–DNA hybridization value was reported when closely related *Desulfovibrio vulgaris* strains were inspected (Brandis & Thauer, 1981). In the genus *Pseudomonas*, phenotypic differences have been considered to reclassify *Pseudomonas aureofaciens* DSM 6698^T as a subspecies of *Pseudomonas chlororaphis*, despite a 73–81% DNA–DNA similarity with other subspecies and a 16S rRNA gene sequence similarity of greater than 99.4% (Peix *et al.*, 2007). The DNA G+C content of 62.4 mol% obtained for strain SR-1^T differs from the value of 63.1 mol% reported for the type strain *D. africanus* (Skyring & Jones, 1972). Since determination of the genomic G+C content lacks resolution for determining relationships between strains (Rossello-Mora & Amann, 2001), the DNA mol% G+C determination for strain SR-1^T constitutes complementary information for its description. PFGE revealed the presence of three *PmeI*-fragments in strain SR-1^T and six *PmeI*-fragments in *D. africanus* (Supplementary Fig. S1, available in IJSEM Online). The separation of macrorestriction fragments by PFGE generates DNA banding patterns that can be used to evaluate interspecies and intraspecies genetic variation. Here we have considered that bacterial isolates differing by one to three bands are closely related strains (Tenover *et al.*, 1995). Considering this difference, strain SR-1^T and *D. africanus* may be considered closely related strains. 16S rRNA gene fingerprint patterns (Fig. 3) indicate that strain SR-1^T can be distinguished from *D. africanus* based on variation of restriction sites within (*SphI*, *XmaI*) and around (*BmgBI*, *HindIII*, *PstI*) the 16S rRNA gene. The number of 16S rRNA gene hybridization bands depends on the restriction enzyme and the location of the restriction sites in the genome (Pukall, 2006). Despite the same number of hybridization bands, the different 16S ribo-

Fig. 2. Phylogenetic tree of *Desulfovibrio* DsrAB amino acid sequences showing the affiliation of strain SR-1^T. Sequences were retrieved from the GenBank database; accession numbers are given in parentheses. Bootstrap values (%) are based on 1000 replicates. The sequence of *Desulfomonile tiedjei* was included as the outgroup. Bar, 0.01 substitutions per site.

Fig. 3. Differential 16S rRNA gene sequence (a) and BOX-PCR (b) fingerprint patterns of strain SR-1^T as compared with *D. africanus*. (a) Southern blot hybridization of genomic DNA digested with *Hind*III, *Pst*I, *Sph*I, *Bmg*BI and *Xma*I. (b) Agarose gel of 10 µl aliquots of PCR products amplified with the BOX A1R primer. SR-1, Strain SR-1^T; DSM, *D. africanus* DSM 2603^T; M, DNA ladder.

somal fingerprint patterns generated suggested that the genomes of strain SR-1^T and *D. africanus* have a different organization. Identical BOX-PCR fingerprint patterns were observed on replicate gels, but significant differences in the fingerprint patterns between strains were observed (Fig. 3). Rep-PCR techniques, which include BOX-PCR, have been shown to be valuable tools for classifying and typing a variety of Gram-negative bacteria (Versalovic *et al.*, 1994). ERIC- and REP-PCR fingerprints have been used in an attempt to differentiate soil from intestinal strains of *Desulfovibrio desulfuricans* (Dzierzewicz *et al.*, 2003). The results presented here suggest that strain SR-1^T has a different genomic organization from *D. africanus*.

A small plasmid was purified only from either exponential or stationary phase cultures of strain SR-1^T. The absence of plasmids in *D. africanus* has been previously reported (Postgate, 1984) and was confirmed in the present study.

The ad hoc committee on reconciliation of approaches to bacterial systematics has proposed that subspecies designations can be used for genetically close organisms that can be differentiated by some phenotypic characteristics (Wayne *et al.*, 1987). Salient phenotypic characteristics of strain SR-1^T (single polar flagellum), and differential DNA fingerprints (*Pme*I-PFGE, 16S rRNA gene sequence and BOX-PCR), indicate that strain SR-1^T constitutes a novel subspecies of *D. africanus*, for which the name *Desulfovibrio africanus* subsp. *uniflagellum* subsp. nov. is proposed.

Description of *Desulfovibrio africanus* subsp. *africanus* Campbell *et al.* 1966, subsp. nov.

Desulfovibrio africanus subsp. *africanus* (a.fri.ca'nus. L. adj. *africanus* pertaining to Africa).

Motile, vibrio-shaped cells with multiple polar flagella (lophotrichous) and 0.5 × 5–10 µm in size. Grow at pH 6.6 to 7.65 and at 20–40 °C. No growth is observed at 15 or 45 °C. Does not require NaCl for growth, but can

grow with up to 4.0 % NaCl. Lactate, pyruvate, ethanol and formate are oxidized with sulfate reduction. Substrates that do not support growth with sulfate include acetate, propionate, malate, butyrate, choline, glucose and fructose. Sulfite and thiosulfate are used as electron acceptors and elemental sulfur is weakly reduced (10 mM each). Nitrate is not used as an electron acceptor. The DNA G+C content of the type strain is 63.1 mol%. DNA–DNA hybridization between the type strain and the type strain of *D. africanus* subsp. *uniflagellum* is 72 %.

The type strain is ATCC 19996^T (=DSM 2603^T =NCIB (now NCIMB) 8401^T =VKM B-1757^T).

Description of *Desulfovibrio africanus* subsp. *uniflagellum* subsp. nov.

Desulfovibrio africanus subsp. *uniflagellum* [uni fla.gel'lum. L. adj. *unus* only one, one; L. n. *flagellum* whip; N.L. neut. n. (nominative in apposition) *uniflagellum* the only one whip].

Motile, vibrio-shaped cells with a single polar flagellum and 0.5–3.5 µm in size. Grow at pH 6.25 to 7.65, with optimum growth at pH 7.0, and at 20–40 °C, with optimum growth at 37 °C. No growth is observed at 15 or 45 °C. Does not require NaCl for growth, but can grow with up to 4.25 % NaCl. Lactate, formate, pyruvate, ethanol and hydrogen are oxidized with sulfate reduction. Substrates that do not support growth with sulfate include acetate, propionate, malate, butyrate, choline, glucose and fructose. Pyruvate supports fermentative growth. Nitrate is not used as an electron acceptor. Habitat: shallow subsurface aquifer. The DNA G+C content of the type strain is 62.4 mol%. DNA–DNA hybridization between the type strain and the type strain of *D. africanus* subsp. *africanus* is 72 %.

The type strain is SR-1^T (=JCM15510^T =KCTC 5649^T), which was isolated from subsurface sediments of a

uranium-contaminated site in Shiprock, New Mexico, USA.

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