Actinospica robiniae gen. nov., sp. nov. and Actinospica acidiphila sp. nov.: proposal for Actinospicaceae fam. nov. and Catenulisporinae subord. nov. in the order Actinomycetales

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Two novel Gram-positive, acidophilic bacterial strains were isolated from forest soil. According to their 16S rRNA gene sequences, these strains are related closely to each other and form a distinct cluster within the order Actinomycetales. They show the typical features of filamentous actinomycetes, with branched vegetative hyphae and production of aerial hyphae. The distinct phylogenetic positions and the combination of chemotaxonomic characteristics of these strains justify the proposal of Actinospica gen. nov. Both strains display 3-hydroxydiaminopimelic acid plus traces of meso-diaminopimelic acid, the phospholipids diphosphatidylglycerol, phosphatidylethanolamine, methylphosphatidylethanolamine and phosphatidylglycerol, the predominant cellular fatty acids i-C15:0, i-C16:0 and ai-C15:0 and the whole-cell sugars mannose and rhamnose. They differ in the fatty acid profiles, in the quantitative ratios of the major menaquinones MK-9(H4), MK-9(H6) and MK-9(H8) and in the occurrence of additional whole-cell sugars (arabinose and xylose in strain GE134769T and galactose in strain GE134766T). Differences in the phenotypic characteristics and in the 16S rRNA gene sequences suggest the description of two species, Actinospica robiniae gen. nov., sp. nov. (the type species) and Actinospica acidiphila sp. nov., with the type strains GE134769T (=DSM 44927T =NRRL B-24432T) and GE134766T (=DSM 44926T =NRRL B-24431T), respectively. The DNA G+C contents of strains GE134769T and GE134766T are 70.8 and 69.2 mol%, respectively. Due to the large phylogenetic distance from known actinomycete genera, it is proposed to accommodate Actinospica gen. nov. in Actinospicaceae fam. nov. In addition, Catenulisporinae subord. nov. is proposed to harbour Actinospicaceae fam. nov. and the newly proposed family Catenulisporaceae, described in the accompanying paper.

INTRODUCTION

There is now substantial evidence indicating that currently known bacteria represent a small fraction of the existing diversity (Hugenholtz, 2002). Earlier data based on phylogenetic evidence from cloned DNA have contributed

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Abbreviation: FESEM, field emission scanning electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains GE134766T and GE134769T are AJ865861 and AJ865863, respectively.

Supplementary figures showing FESEM images and a phylogenetic tree of strains GE134766T and GE134769T are available in IJSEM Online.

to a renewed interest in strain isolation, leading to the recent cultivation of strains belonging to several novel lineages within the domain Bacteria (e.g. Janssen et al., 2002; Hahn et al., 2004; Stevenson et al., 2004). It is worth emphasizing that novel taxa are often recovered from common soil sources by small but effective modifications of known isolation methods. Strains belonging to presumably novel lineages have also been isolated within the order Actinomycetales (Sait et al., 2002; Joseph et al., 2003), an order that has been subjected to intensive screening because of its biotechnological potential.

In the accompanying paper, we describe Catenulispora acidiphila and propose the novel family Catenulisporaceae.
(Busti et al., 2006). Here, we report the characterization and classification of the strains GE134766T and GE134769T, proposing their affiliation to Actinospicia gen. nov. as two distinct species, Actinospicia robindae gen. nov., sp. nov. and Actinospicia acidiphila sp. nov., respectively. We also propose the description of Actinospiciaae fam. nov., in which to accommodate the novel genus Actinospicia, and of Catenulisporinae subord. nov., in which to accommodate the novel families Actinospicaceae and Catenulisporaceae.

**METHODS**

Isolation and cultivation of strains GE134766T and GE134769T. Strains GE134766T and GE134769T were isolated from a soil sample collected in a wooded area in Gerenzano, Italy, as described in the accompanying paper (Busti et al., 2006). Strains GE134766T and GE134769T were maintained on ISP2 agar (Shirling & Gottlieb, 1966) acidified to pH 5 with HCL. Inocula for cultural, physiological and chemotaxonomic analyses were obtained by growing strains GE134766T and GE134769T for 4–8 days (until the end of the vegetative growth phase) at 28 °C on a rotary shaker at 200 r.p.m., using ATSB medium (Busti et al., 2006).

**Morphological and physiological characterization.** For morphological, cultural and physiological characterization, we used the media (acidified to pH 4.8–5.5) and procedures described previously (Busti et al., 2006). For carbon-source utilization, ISP4 (without starch and acidified to pH 5.0–5.5) and ISP9 were used as basal media, both supplemented with the CMM vitamin solution (Busti et al., 2006). Field emission scanning electron microscopy (FESEM) was performed as described previously (Busti et al., 2006).

**Chemotaxonomic characterization.** Menaquinones and polar lipids were analysed as described by Groth et al. (1997). Analysis of the amino acid composition of the peptidoglycan followed the method described by Schleifer & Kandler (1972), as modified by Willems et al. (1997). Whole-cell sugars were determined according to Staneck & Roberts (1974). Cellular fatty acid methyl esters were obtained by the method of Miller (1982). Identification and quantification of the fatty acid methyl esters were performed by using standard MIS Library Generation software (Microbial ID).

**DNA base composition.** The DNA G+C content was determined by reversed-phase HPLC of nucleosides according to Mesbah et al. (1989).

**DNA sequencing and phylogenetic analysis.** Genomic DNA was purified with a GenElute Bacterial Genomic DNA kit (Sigma). Amplification of the nearly complete 16S rRNA gene, DNA sequencing, similarity searches and phylogenetic analyses were performed as described previously (Monciardini et al., 2003).

**RESULTS AND DISCUSSION**

**Strain morphology**

Strains GE134766T and GE134769T appeared on isolation plates only after prolonged incubations at 28 °C. As observed by light microscopy on HSA5 plates after a 3 week incubation at 28 °C, strain GE134766T forms branched vegetative and aerial mycelia. Aerial mycelium appears as single filaments or as tufts of relatively long, straight to flexuous hyphae. FESEM images show aerial hyphae proceeding towards different maturation stages, as revealed by the different level of hyphal septation (Fig. 1a), to produce chains of arthrospores. Spores are cylindrical (Fig. 1b), except those borne on the apical part of the sporogenous hyphae, which have a rounded tip (arrows) and are 0.6–0.8 µm long with a mean diameter of 0.5 µm. Spore chains contain up to 30 spores (or occasionally more). Spore surface is slightly rugose; spores do not show motility.

The aerial mycelium of strain GE134769T appears as tufts of short, straight to slightly flexuous hyphae starting to septate into chains of squat to cylindrical (0.6–0.7 to 1.0–1.2 µm long by 0.6–0.7 µm wide) arthrospores (Fig. 2a). Up to 30 spores were counted in a single chain. Spores showed a slightly rugose surface (Fig. 2b). No motile elements were observed. Further images of the strains are available as Supplementary Figs S1 and S2 in IJSEM Online.

**Cultural and physiological characteristics**

Table 1 reports the appearance of strains GE134766T and GE134769T on various agar media. Strain GE134766T usually grows better than GE134769T. On ISP5 and ISP7 plates, GE134766T also shows a higher degree of differentiation than GE134769T, which does not produce any aerial mass or pigmentation under these conditions.

Both strains grow in the temperature range 17–33 °C, are incapable of growing at 14 or 37 °C, are positive for H2S

![Fig. 1. Scanning electron microscopy of strain GE134766T grown on HSA5 agar for 3 weeks at 28 °C. (a) A young, unseptated hypha runs together with a partially septated one and with a matured one, divided into a spore chain; (b) cylindrical spores with rugose surface. See text for description. Bars, 2 µm.](image-url)
production and negative for gelatin liquefaction, tyrosine reaction and nitrate reduction. In addition, they are sensitive to 100 mg lysozyme ml\(^{-1}\). Whilst both strains are obligate acidophiles, they exhibit different pH ranges (4.2–6.0 and 4.8–6.2 for GE134766\(^T\) and GE134769\(^T\), respectively) and optima (5.0 and 5.5, respectively) for growth. In addition, 1% NaCl disturbs the growth of GE134769\(^T\) significantly, whereas it is well tolerated by GE134766\(^T\). We could observe starch hydrolysis only with GE134766\(^T\). Traces of casein hydrolysis could be observed for strains GE134769\(^T\) and GE134766\(^T\) only after 5 and 6 weeks at 28°C, respectively, even if good growth was observed after 3 weeks. No significantly enhanced growth was observed when the basal media were supplemented with any carbon source. Thus, we cannot say whether GE134766\(^T\) or GE134769\(^T\) differ in carbon-source utilization.

**Chemotaxonomic characteristics**

Strains GE134766\(^T\) and GE134769\(^T\) contain 3-hydroxydiaminopimelic acid as diagnostic diamino acid of the peptidoglycan in addition to glycine, glutamic acid and alanine. Only small traces of meso-diaminopimelic acid could be detected. The occurrence of 3-hydroxydiaminopimelic acid, alone or in combination with meso-diaminopimelic acid, has already been reported for members of the family *Micromonosporaceae* (Koch et al., 1996; Lee & Hah, 2002) within the order *Actinomycetales*.

The major menaquinones of strains GE134766\(^T\) and GE134769\(^T\) are MK-9(H\(_4\)), MK-9(H\(_6\)) and MK-9(H\(_8\)), with different ratios in the two strains (see Table 2). Among members of the suborder *Frankineae*, the closest phylogenetic cluster (see below), the major menaquinone patterns differentiate the isolates from *Nakamurella multipartita* and *Quadrisphaera granulorum*, showing MK-8(H\(_4\)) and MK-8(H\(_2\)), respectively, whilst other members of that suborder also contain partially hydrogenated menaquinones with nine isoprenoid units (Tamura et al., 1998; Tao et al., 2004; Maszenan et al., 2005). The isolates show cellular fatty acid profiles with the predominant components i-C\(_{15:0}\), i-C\(_{16:0}\) and ai-C\(_{15:0}\). Detailed profiles are reported in Table 3. The cellular fatty acid profiles of the two isolates differ in amounts and in the content of the C\(_{17:1}\) isomers and do not match any entry of the TSBA40 identification database.

Both strains display the phospholipids diphosphatidylglycerol, phosphatidylethanolamine, methylphosphatidylethanolamine and phosphatidylinositol and contain the whole-cell sugars mannose and rhamnose. Whereas strain GE134769\(^T\) shows the additional whole-cell-wall sugar galactose, strain GE134766\(^T\) additionally contains arabinose and xylose.

**Phylogenetic analysis**

The almost-complete 16S rRNA gene sequences for strains GE134769\(^T\) and GE134766\(^T\) [1421 and 1446 nt, corresponding to 92.0% and 93.6%, respectively, of the *Escherichia coli* sequence (Brosius et al., 1978)] were determined. The two sequences share 97.5% identity with each other. The highest pairwise identity levels with cultured bacteria (ranging from 97.0% to 99.3%) were found with the recently released sequences of several undescribed strains, identified as ‘acidophilic actinobacteria’ (e.g. GenBank accession numbers AB180758, AB180762 and AB180777). Apart from these, the closest relative (93.1% sequence identity) was *Catenulispora acidiphila* ID139908\(^T\) (Busti et al., 2006). Among species with validly published names, the highest pairwise identity (92.3%) was found between the sequences of GE134766\(^T\) and of *Cryptosporangium aurantiacum* IMSNU 22120 (GenBank accession no. AJ293746), but...
representatives of several actinomycete families have sequence identities to GE134766\textsuperscript{T} and GE134769\textsuperscript{T} of between 90 and 92\%. Although strains GE134766\textsuperscript{T} and GE134769\textsuperscript{T} clearly belong to the order \textit{Actinomycetales}, they – together with the above-mentioned acidophilic strains – do not seem to belong to any of the described families within the order.

The 16S rRNA gene sequences of strains GE134766\textsuperscript{T} and GE134769\textsuperscript{T}, together with those of some of the closest relatives found in GenBank, were aligned with those of representatives of the major actinomycete lineages. For the suborder \textit{Frankineae}, we included several type species in order to have a better representation of the diversity within this suborder containing the closest described relatives. The resulting phylogenetic tree is shown in Fig. 3. Strains GE134766\textsuperscript{T} and GE134769\textsuperscript{T} represent, together with strains Aac-2, Aac-35, Aac-50 and Aac-85, a novel group within the \textit{Actinomycetales} line of descent. They are associated consistently with another coherent clade, represented by the family \textit{Catenulisporaceae} (Busti \textit{et al.}, 2006). Bootstrap analysis suggests a monophyletic origin for the two clades. In order to better understand the degree of diversity within the novel group, we aligned the 16S rRNA gene sequences of strains GE134766\textsuperscript{T} and GE134769\textsuperscript{T} with those of similar strains isolated in our laboratory and of strains reported as GenBank entries only (see Supplementary Fig. S3, available in IJSEM Online). The lineage presents two major clades, represented respectively by strains GE134766\textsuperscript{T} and GE134769\textsuperscript{T}, but phylogenetic diversity of each of the two groups appears quite large.

The 16S rRNA gene sequences of all the 28 sequences included in Supplementary Fig. S3 (available in IJSEM Online) were scanned for the signature nucleotide patterns of the order \textit{Actinomycetales} and its suborders (Stackebrandt \textit{et al.}, 1997). All signature nucleotides of the order \textit{Actinomycetales} were found, with two exceptions. The first is at position 449, where a C is found instead of an A, as also observed for other members of the \textit{Actinomycetales}. The second exception is at positions 450 : 483, where C–G is found instead of G–C, as observed for the order \textit{Bifidobacteriales} (Stackebrandt \textit{et al.}, 1997). However, sequences of strains GE134766\textsuperscript{T} and GE134769\textsuperscript{T} do not present any of the other signatures typical of the order \textit{Bifidobacteriales} and clearly cluster within the \textit{Actinomycetales} in phylogenetic analyses. In addition, they do not contain the signature pattern of any of the described suborders. Phylogenetic data therefore suggest that these strains belong to a novel family within the \textit{Actinomycetales}. Relatively high bootstrap values and the presence of a common pattern of signature nucleotides (see below) support the placement of this family in the same suborder as the newly proposed family \textit{Catenulisporaceae} (Busti \textit{et al.}, 2006).

The combination of morphological, chemotaxonomic and phylogenetic data reported here for strains GE134766\textsuperscript{T} and GE134769\textsuperscript{T} support the proposal for a novel genus to include them both. Due to the morphology of the aerial structures, the name \textit{Actinospica} gen. nov. is proposed.
fam. nov. to harbour propose the description of the branch depth observed in phylogenetic trees, we also harbour the families

Although the level of DNA relatedness between the two strains was not analysed, we consider the differences observed between the two strains in terms of morphology, physiology (pH growth range and NaCl tolerance), chemotaxonomy (menaquinones and fatty acid patterns), G + C content of their DNAs and level of 16S rRNA sequence identity to be sufficient to support the description of two different species.

Stackebrandt et al. (1997) proposed that affiliation to higher hierarchical taxa in the class Actinobacteria should be based on phylogenetic criteria. Accordingly, strains GE134766T and GE134769T are related phylogenetically to, but are clearly distinct from, representatives of the genus Catenulispora (Busti et al., 2006). We thus propose Actinospicaceae fam. nov. to harbour Actinospica gen. nov. and, on the basis of the branch depth observed in phylogenetic trees, we also propose the description of Catenulisporineae subord. nov. to harbour the families Actinospicaceae and Catenulisporaceae.

Table 2. Chemotaxonomic characteristics of strains GE134766T and GE134769T

<table>
<thead>
<tr>
<th>Chemotaxonomic marker</th>
<th>GE134766T</th>
<th>GE134769T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptidoglycan diamino acids</td>
<td>hydroxy-Dpm, meso-Dpm (traces)</td>
<td>hydroxy-Dpm, meso-Dpm (traces)</td>
</tr>
<tr>
<td>Whole-cell sugar pattern</td>
<td>Man, Rha, Ara, Xyl</td>
<td>Man, Rha, Gal</td>
</tr>
<tr>
<td>Major menaquinones (%)</td>
<td>MK-9(H₄) 23</td>
<td>MK-9(H₄) 2</td>
</tr>
<tr>
<td></td>
<td>MK-9(H₆) 40</td>
<td>MK-9(H₆) 25</td>
</tr>
<tr>
<td></td>
<td>MK-9(H₈) 20</td>
<td>MK-9(H₈) 59</td>
</tr>
<tr>
<td>Polar lipids</td>
<td>PI, DPG, PE, methyl-PE</td>
<td>PI, DPG, PE, methyl-PE</td>
</tr>
<tr>
<td>G + C content of DNA (mol%)</td>
<td>69.2</td>
<td>70.8</td>
</tr>
</tbody>
</table>

Although the level of DNA relatedness between the two strains was not analysed, we consider the differences observed between the two strains in terms of morphology, physiology (pH growth range and NaCl tolerance), chemotaxonomy (menaquinones and fatty acid patterns), G + C content of their DNAs and level of 16S rRNA sequence identity to be sufficient to support the description of two different species.

Stackebrandt et al. (1997) proposed that affiliation to higher hierarchical taxa in the class Actinobacteria should be based on phylogenetic criteria. Accordingly, strains GE134766T and GE134769T are related phylogenetically to, but are clearly distinct from, representatives of the genus Catenulispora (Busti et al., 2006). We thus propose Actinospicaceae fam. nov. to harbour Actinospica gen. nov. and, on the basis of the branch depth observed in phylogenetic trees, we also propose the description of Catenulisporineae subord. nov. to harbour the families Actinospicaceae and Catenulisporaceae.

Table 3. Cellular fatty acid composition (%) of strains GE134766T and GE134769T

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>GE134766T</th>
<th>GE134769T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Branched</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i-C₁₄:0</td>
<td>3.88</td>
<td>5.26</td>
</tr>
<tr>
<td>i-C₁₅:0</td>
<td>30.17</td>
<td>14.88</td>
</tr>
<tr>
<td>i-C₁₆:0</td>
<td>27.40</td>
<td>37.65</td>
</tr>
<tr>
<td>i-C₁₇:0</td>
<td>4.60</td>
<td>1.82</td>
</tr>
<tr>
<td>ai-C₁₅:0</td>
<td>8.56</td>
<td>15.41</td>
</tr>
<tr>
<td>ai-C₁₇:0</td>
<td>6.19</td>
<td>10.52</td>
</tr>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₁₅:0</td>
<td>1.48</td>
<td>2.72</td>
</tr>
<tr>
<td>C₁₆:0</td>
<td>2.86</td>
<td>1.10</td>
</tr>
<tr>
<td>Unsaturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₁₇:09c</td>
<td>–</td>
<td>2.94</td>
</tr>
<tr>
<td>C₁₇:10c</td>
<td>7.18</td>
<td>–</td>
</tr>
<tr>
<td>i-C₁₇:09c</td>
<td>–</td>
<td>2.72</td>
</tr>
<tr>
<td>ai-C₁₇:09c</td>
<td>1.06</td>
<td>1.19</td>
</tr>
<tr>
<td>Other</td>
<td>6.62</td>
<td>3.78</td>
</tr>
</tbody>
</table>

Description of Catenulisporineae subord. nov.
(Ca.te.nu.li.spo.ri’ne.ae. N.L. fem. n. Catenulispora type genus of the suborder; -ineae ending to denote a suborder; N.L. fem. pl. n. Catenulisporineae the Catenulispora suborder).

The suborder contains the families Catenulisporaceae and Actinospicaceae. The pattern of 16S rRNA gene signatures consists of nt 127:234 (G–C), 138:225 (U–A), 139:224 (C–G), 140:223 (C–G), 141:222 (A–U), 157:164 (G–C), 449 (C), 589:650 (C–G), 602:636 (R–U), 603:635 (A–U), 694 (G) and 1251 (G). The phylogenetic analysis is presented in this study. The type genus of the suborder is Catenulispora.

Description of Actinospicaceae fam. nov.
(Ac.ti.no.spi.ca’ce.ae. N.L. fem. n. Actinospica type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. Actinospicaceae the Actinospica family).

The family contains the type genus Actinospica. The pattern of 16S rRNA gene signatures consists of nt 127:234 (G–C), 129:232 (C–G), 344 (G), 449 (C), 450:483 (C–G), 560 (U), 576 (G), 590:649 (C–G), 591:648 (U–R), 859 (G), 952:1229 (C–G), 1122:1151 (G–C), 1123:1150 (U–G), 1124:1149 (A–U), and of seven to nine extra nucleotides between positions 1134 and 1140. The phylogenetic analysis is presented in this study.

Description of Actinospica gen. nov.
(Ac.ti.no.spi.ca. Gr. n. actinos a ray; L. fem. n. spica tuft; N.L. fem. n. Actinospica an actinomycete with tufts of aerial hyphae).

Aerobic, Gram-positive micro-organisms that form non-framenting vegetative mycelia. The aerial mycelium appears as tufts of straight to slightly flexuous hyphae, carrying at maturity chains of cylindrical spores. Tufts originate from very short sporophorous branching in few sporogenous hyphae. Motile elements are not produced. Obligately acidophilic, growing at pH values not higher than 6.2. Mesophilic; best growth occurs at 22–28 °C. Grow
better on acidic yeast extract–malt extract agar and acidic oatmeal agar. Cell wall contains 3-hydroxydiaminopimelic acid. The polar lipids consist of diphosphatidylglycerol, phosphatidylethanolamine, methylphosphatidylethanolamine and phosphatidylinositol. The predominant cellular fatty acids are i-C15:0, i-C16:0 and ai-C15:0. Contain the major menaquinones MK-9(H4), MK-9(H6) and MK-9(H8) and the cell-wall sugars mannose and rhamnose. The G+C content of the DNA ranges between 69 and 71 mol%. The type species of the genus is \textit{Actinospica robiniae}.

\textbf{Description of \textit{Actinospica robiniae} sp. nov.}

\textit{Actinospica robiniae} (ro.bi'ni.ae. N.L. fem. n. \textit{Robinia} scientific name of a genus of tree; N.L. fem. gen. n. \textit{robiniae} of \textit{Robinia}, isolated from a wood of \textit{Robinia pseudoacacia}).

The chemotaxonomic and general characteristics are the same as given above for the genus. The type strain contains galactose as an additional whole-cell sugar. Aerial mycelium produces long chains of up to 30 cylindrical spores (0.5–0.8 x 0.5 mm) and occasionally more. Spore surface is rugose. Growth occurs between pH 4.0 and 6.0, with optimal rates at pH 5.0. Greenish pigments are produced in some media. H2S is produced. Nitrates are not reduced. Starch is hydrolysed. Gelatin is not liquefied. The G+C content of the DNA is 69.2 mol%. The type strain, GE134766T (\(=\)DSM 44927T = NRRL B-24432T), was isolated from temperate forest soil.

\textbf{Description of \textit{Actinospica acidiphila} sp. nov.}

\textit{Actinospica acidiphila} (a.ci.di'phi.\textit{la}. N.L. neut. n. \textit{acidum} acid; Gr. adj. \textit{philos} loving; N.L. fem. n. \textit{acidiphila} acid-loving).

The chemotaxonomic and general characteristics are the same as given above for the genus. The type strain contains arabinose and xylose as additional whole-cell sugars. Aerial mycelium produces long chains of up to 30 cylindrical spores (0.6–0.8 x 0.5 mm) and occasionally more. Spore surface is rugose. Growth occurs between pH 4.2 and 6.2, with optimal rates at pH 5.0. Greenish pigments are produced in some media. H2S is produced. Nitrates are not reduced. Starch is hydrolysed. Gelatin is not liquefied. Catalase-positive. Growth occurs between 17 and 33 °C and is optimal at 28 °C; no growth occurs at 14 or 37 °C. Up to 1 % (w/v) NaCl is tolerated, whereas 100 µg lysozyme ml\(^{-1}\) is not. The G+C content of the DNA is 69.2 mol%.

The type strain, GE134766T (\(=\)DSM 44927T = NRRL B-24432T), was isolated from temperate forest soil.

\textbf{ACKNOWLEDGEMENTS}

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