A strictly anaerobic, Gram-positive, non-spore-forming bacterium was isolated from sewage sludge which grew on creatinine as sole source of carbon and energy. This new isolate, designated strain KRE 4T, totally degraded creatinine via creatine, sarcosine and glycine to the products acetate, monomethylamine, ammonia and carbon dioxide. Growth on creatinine or creatine was selenium-dependent and stimulated by formate, indicating the involvement of a creatine reductase, sarcosine reductase and/or glycine reductase. This was substantiated by the fact that creatine, sarcosine and glycine were reduced by cell-free extracts. Growth on creatinine or creatine was also possible in the absence of formate, but with an increase in doubling time. The new bacterium occurred as rod-shaped cells, which exhibited an angular form (2-6 µm long and 0.7-1.1 µm wide) and showed motility by means of peritrichous flagella. The G+C content of the DNA was 30 mol%. Comparative 16S rRNA sequence analysis demonstrated that strain KRE 4T represents a new subline within the genus Tissierella. Due to its very restricted substrate spectrum and the inability of whole cells to utilize sarcosine and glycine as intermediates of creatine breakdown, this organism can be readily separated from currently described species of Tissierella. Therefore, based on the phenotypic and phylogenetic distinctiveness of the new isolate, it is proposed that the bacterium be classified as a new species of the genus Tissierella, Tissierella creatinophila sp. nov. The type strain is KRE 4 (= DSM 69117).

**Keywords:** Tissierella creatinophila, creatinine, creatine
Pseudomonas putida
Pseudomonas ovalis
C. Harms and others

Methods

Isolation and cultivation of creatinine-degrading strains. Five strains were isolated as pure cultures from enrichment cultures that had been inoculated anaerobically with sediment from sewage sludge by employing standard enrichment techniques (Zindel et al., 1988) and creatinine plus formate as the selective carbon-, nitrogen- and energy sources. Enrichments and pure cultures were grown at 30°C in a defined anaerobic, bicarbonate-buffered, sulfide-reduced medium at pH 8.0, which was prepared by the Hungate technique under a nitrogen gas atmosphere. The medium contained: 50 mM creatinine, 40 mM sodium formate, 7 mM NaCl, 13 mM KCl, 2 mM MgSO₄, 1 mM CaCl₂, 47 mM NH₄Cl and 1.5 mM KH₂PO₄. During autoclaving, 75% of the creatine may become converted to creatinine (Möller et al., 1986). One millilitre trace element solution SL-A, 1 ml resazurin solution (1 g l⁻¹) and 5 ml vitamin stock solution were added per litre of medium as described previously (Hormann & Andreessen, 1989). The following sterilized components were added separately to the autoclaved medium to give the indicated final concentrations: 50 mM NaHCO₃ and 2.7 mM Na₂S. Cultivation in peptone/yeast glucose extract medium (PYG) and chopped meat medium was performed as described previously (Holdemann et al., 1977). Two of the five isolated strains, KRE 4 and KRE 2, were deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) with the accession numbers DSM 6911 and DSM 6912, respectively.

Determination of cell density. Growth was followed in anaerobic Hungate tubes by measuring the turbidity (optical density) at 578 nm with a Spectronic 88 spectrophotometer (Bausch & Lomb).

Phenotypic characterization. Strains KRE 4 and KRE 2 were initially characterized by using a variety of standard physiological tests directed to the Eubacterium species-complex (Andreessen, 1992; Zindel et al., 1988) and were found to be similar to T. creatinini, strain BN 11° described by Gauglitz (1988). Key enzymes, such as glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, glutamate- and malate dehydrogenases, from strain KRE 4° were determined according to the methods described by Bergmeyer (1983).

Genotypic characterization. Isolation of DNA was performed according to a modification of the method of Marmur & Doty (1962). For estimation of the G+C content of the DNA, the method of Tomoka & Komagata (1984) was used. Escherichia coli K-12 strain DSM 485 (G+C content 51.7 mol%) was used as control. Phylogenetic analysis was performed by comparative 16S rRNA gene sequence analysis. A large fragment of the 16S rRNA gene (corresponding to positions 30–1521 of the E. coli 16S rRNA gene) was amplified by PCR using conserved primers closed to the 3' and 5' ends of the gene. The PCR products were purified using a Prep-A-Gene kit (Bio-Rad) according to the manufacturer's instructions and directly sequenced using a Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A, Applied Biosystems). The closest known relatives of the new isolates were determined by performing a database search using the program FASTA of the Genetics Computer Group package (Devereux et al., 1984). These sequences, and those of other known related strains, were retrieved from the GenBank or Ribosomal Database Project (RDP) libraries and aligned with the newly determined sequences using the program PILEUP (Devereux et al., 1984). The resulting multiple sequence alignment was corrected manually and approximately 100 bases at the 5' end of the rRNA were omitted from further analyses because of alignment ambiguities. A continuous stretch of 1320 bases was used for distance matrix analysis. A distance matrix was calculated...
using the programs PRETTY (Devereux et al., 1984) and DNADIST (using the Kimura-2 correction parameter) (Felsenstein, 1989). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR (Felsenstein, 1989). The stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

**Chemical analysis of substrates and products.** Analyses of substrates and products were performed with the supernatant of anaerobically harvested cells. Creatinine, creatine, N-methylhydroxypyridine and N-carbamoylsarcosine were determined by HPLC. The HPLC system comprised an 879 pump module (Du Pont), a UVICON 729 LC spectrophotometer (Kontron Analytik), a Chromatopac C-R1A integrator (Shimadzu) and an RP-18 column, no. NAK 32136 (Kontron). Column temperature was maintained at 24 °C and the mobile phase consisted of 25 mM ammonium acetate with 1% methanol and 1% acetonitrile, pH 6.0. Before use the mobile phase was filtered through a 0.5-μm pore-size filter membrane under reduced pressure and then delivered at a flow rate of 1 ml min⁻¹.

Formate was determined using the method of Lang & Lang (1972). The simultaneous quantification of urea and ammonia was performed according to a test system supplied by Boehringer Mannheim containing glutamate dehydrogenase and urease. For the determination of acetate the method of Dorn et al. (1978) was used. N-Methylguanidine was determined by the method of Nakao et al. (1983). Estimation of monomethylamine was carried out using an amino acid analyser (Typ 4151 Alpha Plus, Pharmacia).

Fermentation products (acetate, ethanol, butanol) were determined by gas chromatography using a Perkin Elmer (Shimadzu). A Porapack QS column was used with N-methylhydantoin and incubated for at least 5 min at 37 °C. The reaction was stopped by adding 100 μl test solution with N-methylhydantoin hydrolase. ATP (1 mM) was added to check for ATP-dependence of the reaction. After incubation, the enzyme activity of N-methyl-THF reductase was tested anaerobically in a final volume of 3 ml: 90 mM glycine buffer, pH 8.9, 3 mM dithioerythritol, 20 mM N-methylhydroxypyridine, pH 8.9 and 0.1 ml cell-free extract (10 mg protein ml⁻¹). ATP (1 mM) was added to check for ATP-dependence of the reaction (Gauglitz, 1988). The reaction was started with N-methylhydroxypyridine and incubated for at least 5 min at 37 °C. The reaction was stopped by adding 100 μl test solution to 900 μl mobile phase (100 °C). The concentration of the substrate was determined by HPLC as described above. On completion, the enzyme activity of N-methylhydroxypyridine hydrolase was also determined by incubating 100 μl crude extract with 100 μl N-methylhydroxypyridine (100 mM in 50 mM potassium phosphate buffer, pH 7.5) for 30 min at 37 °C. After incubation, the test system was diluted 1:20 and a 20 μl aliquot was used for the determination of the N-carbamoylsarcosine formed according to the method of Siedel et al. (1988). N-Carbamoylsarcosine amidohydrolase activity was determined in the same manner.

**Harvesting of cells and preparation of cell-free extracts.** The cells were anaerobically mass-cultured in 201 carboys at 30 °C and anaerobically harvested in the late-exponential growth phase by centrifugation under a nitrogen atmosphere. One gram of cells was resuspended in 1 ml 50 mM potassium phosphate, pH 7.8, then lysozyme (1 mg ml⁻¹), DNase I (0.1 mg (g cells)⁻¹) and PMSF (0.2 M in 2-propanol; 1 μl ml⁻¹) were added. The cell suspension was incubated at 37 °C for 30 min. The suspensions were usually passed twice through a precooled French pressure cell at 147 MPa, then the extract was frozen at −20 °C and further lysozyme, DNase I and PMSF were added. After this treatment, the cells were passed up to ten times through the French pressure cell. The cell debris was removed by centrifugation for 30 min at 10000 g at 4 °C. The resulting supernatant (crude extract) was used for enzyme assays.

**Enzyme assays.** If the assays were performed anaerobically, cuvettes (1 ml assay mixture, 0.5 ml N₂, pathlength 1 cm) sealed with soft rubber stoppers were employed. One unit is defined as the amount of enzyme which converts 1 μmol substrate min⁻¹ at 37 °C.

Creatine reductase, sarcosine reductase and glycine reductase were determined by the formation of [¹⁴C]acetate from [¹⁴C]creatine, [²⁻¹⁴C]sarcosine or [¹⁴C]glycine as described in general by Stadtman (1970) using the modifications of Hormann & Andreesen (1989). In addition, a non-radioactive enzyme assay was used by measuring the formation of acetyl phosphate (Meyer et al., 1995).

For the determination of creatine amidohydrolase activity, the method of Tsuru et al. (1976) was used. This enzyme activity was also assayed by incubating 100 μl crude extract with 100 μl creatine (85 mM in 50 mM Tris, pH 7.5). The sarcosine formed was measured in a coupled enzyme assay with sarcosine oxidase and peroxidase as described above. Quantification of creatinine deiminase activity was performed by the determination of the N-methylhydantoin formed as described by Siedel et al. (1988). The test system, composed of 100 μl crude extract and 100 μl creatine (85 mM, pH 7.4), was incubated for 30 min at 34 °C.

Determination of N-methylhydroxypyridine hydrolase was performed using in a final volume of 3 ml: 90 mM glycine buffer, pH 8.9, 3 mM dithioerythritol, 20 mM N-methylhydroxypyridine, pH 8.9 and 0.1 ml cell-free extract (10 mg protein ml⁻¹). ATP (1 mM) was added to check for ATP-dependence of the reaction (Gauglitz, 1988). The reaction was started with N-methylhydroxypyridine and incubated for at least 5 min at 37 °C. The reaction was stopped by adding 100 μl test solution to 900 μl mobile phase (100 °C). The concentration of the substrate was determined by HPLC as described above. On completion, the enzyme activity of N-methylhydroxypyridine hydrolase was also determined by incubating 100 μl crude extract with 100 μl N-methylhydroxypyridine (100 mM in 50 mM potassium phosphate buffer, pH 7.5) for 30 min at 34 °C. After incubation, the test system was diluted 1:20 and a 20 μl aliquot was used for the determination of the N-carbamoylsarcosine formed according to the method of Siedel et al. (1988). N-Carbamoylsarcosine amidohydrolase activity was determined in the same manner.

5,10-Methylene-THF reductase was tested anaerobically in the direction of methyl-THF oxidation to methane-THF with dichlorophenol/indophenol (50 mM) (Spormann & Thauer, 1988). The assay mixture contained: 200 mM TES (0.5 mM, pH 7.5), 1 mM dichlorophenol/indophenol and 290 μg cell-free extract protein. The reaction was started by adding 0.13 mM S-5-THF and the reduction of dichlorophenol/indophenol was followed at 522 nm (ε₅₂₂ = 8.6 mM⁻¹ cm⁻¹).
Methylene-THF dehydrogenase was measured according to the method of Uyeda & Rabinowitz (1967). Two microlitres of crude extract (30 mg protein ml⁻¹) was added to a mixture containing 400 mM potassium malate buffer, pH 7.0, 3.3 mM NADP, 300 mM mercaptoethanol and 0.32 mM methylene-THF solution in a final volume of 1 ml to start the enzyme reaction. The reaction was stopped by diluting 50 μl test aliquots with 950 μl 0.36 M HCl. The concentration of the methenyl-THF formed was measured by determining the increase in absorbance at 350 nm using a molar extinction coefficient of 24.9 mM⁻¹ cm⁻¹.

Formyl-THF synthetase was assayed anaerobically in the direction of formyl-THF formation from formate, THF and ATP, and was quantified at 350 nm (Spormann, 1988).

Formate dehydrogenase was measured under anaerobic conditions at 37 °C in the direction of formate oxidation according to the method of Meyer et al. (1995). Formate dehydrogenase activity was routinely assayed using methyl viologen as electron acceptor. In some experiments benzyl viologen replaced methyl viologen.

CO dehydrogenase (carbon monoxide:methyl viologen oxidoreductase) was measured anaerobically by following the reduction of methyl viologen at 600 nm (ε₉₀₀ = 13 mM⁻¹ cm⁻¹) (Spormann & Thauer, 1988).

**Chemicals.** [2-¹⁴C]Sarcosine was custom made and [2-¹⁴C]glycine was purchased from Amersham-Buchler. [1-¹⁴C]Creatine was from ICN Biomedicals. Enzymes and coenzymes were obtained from Boehringer Mannheim. PMSF, vitamins, THF, 5-methyl-THF and dithioerythritol were purchased from Sigma. All other chemicals were of the highest purity available and were obtained from Merck.

**RESULTS**

**Isolation of a creatinine-degrading anaerobe, strain KRE 4ᵀ, and its classification**

Five strains were isolated by anaerobic enrichment cultures from sewage sludge using a liquid medium at pH 8.0 which contained creatinine and formate as sole sources of carbon, nitrogen and energy. Pure cultures were obtained by plating in agar flat bottles and finally by using the agar shake technique. One of these physiologically quite similar isolates, strain KRE 4ᵀ, was studied in more detail. The rod-shaped cells of strain KRE 4ᵀ exhibited an angular form, being 2–6 μm long and 0.7–1.1 μm wide. Cells were single or in chains in the exponential growth phase. Cells stained Gram-negative, but showed a Gram-positive cell wall structure as revealed by electron micrographs. Using electron microscopy, a high similarity between cells of *T. creatinini* and cells of strain KRE 4ᵀ was observed. In contrast to *T. creatinini*, peritrichous flagella were observed in strain KRE 4ᵀ by which these bacteria were very motile. No sporulation was detected, neither in very old cultures on blood agar plates nor in enrichment medium to which soil extract had been added. No survival was observed after pasteurization (10 min, 80 °C). According to these results strain KRE 4ᵀ is a non-spore-forming organism. Colonies on PYG agar were small, circular, flat with a rough surface, greyish and milky. No haemolysis occurred in blood-containing media; no respiratory menaquinones were detected. Nitrate, sulfate and sulphur were not reduced.

Strain KRE 4ᵀ grew extremely slowly when cultivated on PYG. The following end products of metabolism were detected after 2 weeks incubation: 9.3 mM acetate, 5.2 mM ethanol and 0.13 mM butyrate (Table 1). The formation of spheroplasts was observed on PYG. During growth on chopped meat medium, 18.1 mM acetate, 4.5 mM ethanol, 0.65 mM butanol and 0.16 mM butyrate was estimated in the supernatant. However, in this medium chains and clusters of bacteria were formed and motility was lost.

The G+C content of the DNA of strain KRE 4ᵀ was 30 mol%. Comparative 16S rRNA gene sequencing demonstrated that strain KRE 4ᵀ was a member of the *Clostridium* subphylum of Gram-positive bacteria (data not shown). Highest 16S rRNA gene sequence similarities were shown with species of the genus *Tissierella* and *Clostridium hastiforme* (approximately 95%). A tree depicting the phylogenetic position of

**Table 1.** Comparison of *T. praecuta*, *T. creatinini* and *T. creatinophila* strain KRE 4ᵀ

<table>
<thead>
<tr>
<th>Organism</th>
<th>Major end products from PYG*</th>
<th>Metabolism†</th>
<th>MDH‡</th>
<th>GDH‡</th>
<th>G6PDH‡</th>
<th>6PGDH‡</th>
<th>Major long-chain fatty acids</th>
<th>Menaquinones</th>
<th>G+C content (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. praecuta</em>§</td>
<td>B, A, iV</td>
<td>NF</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>iso-C₁₅:₀</td>
<td>–</td>
<td>28</td>
</tr>
<tr>
<td>*T. creatinini</td>
<td>ND</td>
<td>NF</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>C₁₈:₁, C₁₃:₀</td>
<td>–</td>
<td>32</td>
</tr>
<tr>
<td>Strain KRE 4ᵀ</td>
<td>A, E, B</td>
<td>NF</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>30</td>
</tr>
</tbody>
</table>

* A, acetate; B, butyrate; E, ethanol; iV, isovaleric acid. *T. creatinini* and strain KRE 4ᵀ showed only very weak growth in PYG.
† NF, Non- or weakly fermentative.
‡ MDH, malate dehydrogenase; GDH, glutamate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; –, not produced; ND, not determined.
§ Collins & Shah (1986).
|| Farrow et al. (1995).
**Tissierella creatinophila sp. nov.**

- **Tissierella creatinophila**
- **Clostridium hastiforme**
- **Tissierella creatinophila** [sp. nov.]

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**Fig. 1.** Unrooted tree based on 16S rRNA gene sequences showing the phylogenetic position of *Tissierella creatinophila* sp. nov. (strain KRE 4T) within the *Clostridium* subphylum of Gram-positive bacteria. The tree was constructed using the neighbour-joining method. Significant bootstrap values (calculated from 500 trees) are indicated at the branching points. Clusters refer to Collins et al. (1994).

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**Fig. 2.** Possible metabolic reactions during creatinine degradation in strain KRE 4T discussed. Enzymes are shown in italics.

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Strain KRE 4T is shown in Fig. 1 and confirmed the close affinity of the unknown isolate with the genus *Tissierella*. The biochemical characterization of strain KRE 4T revealed differences with the two described species of *Tissierella*, *T. praeacuta* and *T. creatinini* (Table 1). In contrast to *T. praeacuta* (Farrow et al.,...
strains, strain KRE 4^T formed malate dehydrogenase and glutamate dehydrogenase whereas 6-phosphogluconate dehydrogenase activity was absent. There were also differences in the major end products from PYG (Table 1). In all three species, menaquinones could not be detected.

**Nutritional characterization**

Maximal growth of strain KRE 4^T was possible on creatinine or creatine plus formate as substrates. Similar compounds or metabolic products of creatinine, such as N-methylhydantoin (25, 50 mM), N-carbamoylsarcosine (50 mM), sarcosine (25 mM), glycine (10, 25 mM), hydantoin (50 mM) or hydantoic acid (50 mM), did not allow growth of strain KRE 4^T in contrast to *T. creatinini*, which was able to utilize all the above-mentioned compounds for growth (Farrow *et al.*, 1995). In addition, no growth was obtained on betaine (25 mM), arginine (10 mM), ornithine (10 mM), trimethylamine (25 mM), dimethylamine (25 mM) or choline (20 mM). Strain KRE 4^T was unable to utilize the following as substrates in the presence or absence of formate: carbohydrates (glucose, galactose, fructose, lactose, ribose; each 10 mM), compounds of central metabolism (pyruvate, malate, succinate, fumarate, aspartate, acetate, serine; each 10 mM), pyrimidines and purines (adenine, cytosine, thymine, uric acid, guanidine, xanthine; each 10 mM) and several possible component pairs of Stickland reactions (isoleucine + glycine, alanine + glycine, leucine + sarcosine, valine + sarcosine, phenylalanine + sarcosine, leucine + ornithine; each 10 mM). Many substrates were also examined in combination with creatinine as an additional starting, energy-yielding substrate. Thus, serine, arginine, threonine, glycine and sarcosine, and combinations of these compounds, were tested in the presence of creatinine and formate, which have been found to stimulate the growth of *T. creatinini* (Farrow *et al.*, 1995). However, in the case of strain KRE 4^T, an observed increase in optical density was due only to the amount of creatinine added.

Growth on creatinine was also possible without the addition of formate but with a twofold increase in doubling time. The effect of formate on growth with creatinine was determined in liquid medium containing 18 or 38 mM creatinine with or without 10–50 mM formate. A doubling time of 14.5–19.9 h was found in the absence of formate. The doubling time was considerably shorter in the presence of formate: 10.2 h with 10 mM formate and 8.7 h with 20–50 mM formate. Growth on creatinine/formate was dependent on the concentration of selenite. No growth was obtained without selenite addition. The observed doubling times were 52 h at 10^{-8} M Na_{2}SeO_{3}, 20 h at 10^{-6} M, 9 h at 10^{-7} M, 8.5 h at 10^{-8} M, 9.6 h at 10^{-9} M and 10 h at 10^{-4} M Na_{2}SeO_{3}. Thus, optimal growth of strain KRE 4^T was observed in the presence of 10^{-4} M selenite in the liquid medium, indicating an involve-ment of a creatine reductase, sarcosine reductase or glycine reductase in the creatinine degradation pathway.

Methanol and hydrogen were tested as alternative electron donors to formate. Hydrogen could replace formate. Strain KRE 4^T did not oxidize methanol, although the enzymes of general C_1 metabolism were present. Autotrophic growth was not observed with a hydrogen/carbon dioxide gas phase or with formate in the absence of creatinine.

**Growth and product formation on creatinine**

Growth on creatinine in the presence of formate as a cosubstrate led to the formation of acetate, monomethylamine and ammonia as final products (Fig. 2). Formate and creatinine were metabolized with the same stoichiometry (Fig. 3). In summary, 29.8 mM formate and 27.3 mM creatinine were degraded resulting in a ratio of 1:1, giving rise to the following products: 29 mM monomethylamine, 22.5 mM acetate and 55 mM ammonia. Sarcosine was found in low concentrations (0.3 mM) at the end of the exponential growth phase, indicating a role of sarcosine as an intermediate. Small amounts of glycine (0.02 mM)

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**Fig. 3.** Substrate utilization, product formation, growth and pH changes in cultures of strain KRE 4^T. (a) Utilization of creatine (○) and formate (■), and production of ammonia (△), acetate (×) and monomethylamine (□). (b) Growth (●) and pH changes (■).
**Table 2.** Enzymes involved in creatinine metabolism in *T. creatinophila* strain KRE 4°

<table>
<thead>
<tr>
<th>Enzyme*</th>
<th>EC no.</th>
<th>Specific activity [units (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatine reductase</td>
<td>1.4.4. -</td>
<td>0.035†/0.011†</td>
</tr>
<tr>
<td>Sarcosine reductase</td>
<td>1.4.4. -</td>
<td>0.072†/0.017†</td>
</tr>
<tr>
<td>Glycine reductase</td>
<td>1.4.4. -</td>
<td>0.029†/0.003†</td>
</tr>
<tr>
<td>Creatine amidohydrolase</td>
<td>3.5.3.3</td>
<td>0.140</td>
</tr>
<tr>
<td>Creatinine deiminase</td>
<td>3.5.4.21</td>
<td>ND</td>
</tr>
<tr>
<td>N-Methylhydantoin amidohydrolase</td>
<td>3.5.2.14</td>
<td>ND</td>
</tr>
<tr>
<td>N-Carbamoylsarcosine amidohydrolase</td>
<td>3.5.1.59</td>
<td>ND</td>
</tr>
<tr>
<td>Methylene-THF reductase§</td>
<td>1.5.1.20</td>
<td>3.0</td>
</tr>
<tr>
<td>Methylene-THF dehydrogenase§</td>
<td>1.5.1.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Formyl-THF synthetase§</td>
<td>6.3.4.3</td>
<td>1.1</td>
</tr>
<tr>
<td>Formate dehydrogenase§</td>
<td>1.2.1. -</td>
<td></td>
</tr>
<tr>
<td>MV</td>
<td></td>
<td>4.2</td>
</tr>
<tr>
<td>BV</td>
<td></td>
<td>4.2</td>
</tr>
<tr>
<td>CO dehydrogenase§</td>
<td>1.2.99.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not detectable.

* MV, methyl viologen as electron acceptor; BV, benzyl viologen as electron acceptor.
† Result from reductase test of Meyer *et al.* (1995).
‡ Result from reductase test of Hormann & Andreesen (1989).
§ Enzyme activities determined in extracts of cells cultivated in the absence of formate.

were also detected. Thus, during degradation of creatinine via creatine and sarcosine, 2 mol ammonia was released from 1 mol creatinine, as would be expected from the activities of creatinine amidohydrolase and creatine amidohydrolase in combination with urease and sarcosine reductase, or by the coupled activities of creatine reductase and N-methylguanidine hydrolase (Fig. 2). The pH increased very slowly in the range of 1.1 units, because the formation of acetate was counteracted by ammonia and methylamine (Fig. 3).

**Enzymic studies**

The activities of enzymes potentially involved in creatinine degradation were measured in crude extracts (Table 2). For all three reductive deamination reactions potentially involved in creatinine metabolism, activities were detected using two different assays. In general, the activity of a sarcosine reductase was the highest, that of a glycine reductase the lowest.

The activity of creatine reductase in combination with N-methylguanidine hydrolase led to the products acetyl phosphate, ammonia, carbon dioxide and methylamine. The same products would be formed by the enzymes of creatinine amidohydrolase, urease and sarcosine reductase (Fig. 2). These two different metabolic pathways should be distinguishable by the influence of hydroxylamine on the activity of creatine- and sarcosine reductase. In contrast to sarcosine and glycine, creatine cannot form a Schiff base with a carbonyl group of the substrate-specific protein to activate the C–N bond, as also observed for betaine (Meyer *et al.*, 1995). Hydroxylamine would form an oxime with the potential carbonyl group, thereby rendering the respective reductase sensitive. The course of inhibition by hydroxylamine was comparable for sarcosine- and creatine reductase (Fig. 4). The determined activity of creatine reductase might be composed of the activities of both creatine amidohydrolase and sarcosine reductase. This suggestion...
was supported by the fact that formation of the N-containing product of creatine reductase, N-methylguanidine, was never detected in strain KRE 4T. In addition, no hydrolysis of N-methylguanidine by growing cells was observed. In contrast, urea hydrolysis did occur, indicating the presence of an active urease.

The enzyme creatine amidinohydrolase, forming urea and sarcosine, was present in crude extracts of strain KRE 4T at relatively high activity (Table 2). Despite its lability, this enzyme was further studied. It was most stable at pH 7.8. Above 30 °C the activity decreased significantly: at 40 °C, only 11% of enzyme activity was left after 30 min incubation. This instability was also obvious after storage for 24 h at 24 °C when only 34% of the initial activity remained. Dialysis or column chromatography abolished activity completely. Using crude extract without creatine amidinohydrolase activity, no creatine reductase activity could be determined, again pointing to the fact that no genuine creatine reductase activity was present and that the creatine reductase activity measured was due to the combined action of creatine amidinohydrolase, urease and sarcosine reductase. No activity of

\[ \text{Creatine deiminase, N-methylhydantoin amidohydrolase or N-carbamoylsarcosine amidohydrolase was detected (Table 2). These results were substantiated by the fact that N-methylhydantoin was never detectable as an intermediate by HPLC or enzymic analysis.} \]

In the absence of formate, strain KRE 4T had to oxidize (part of) the intermediate sarcosine to obtain reducing equivalents for the reductase reaction. Methanol was never detected as a free intermediate. Therefore, the methyl group might be transferred to THF to give methyl-THF and glycine. At least, all enzymes involved in the oxidation of methyl-THF to CO₂ (methylene-THF reductase, methylene-THF dehydrogenase, methenyl-THF cyclohydrolase, formyl-THF synthetase and formate dehydrogenase) were present in extracts of strain KRE 4T in sufficient high amounts for catabolic processes (Table 2). Glycine might become oxidized by glycine decarboxylase to deliver reducing equivalents or might be reduced by glycine reductase present in the extract.

**DISCUSSION**

**Physiological and biochemical aspects**

Under anaerobic conditions the usual degradation pathway for creatinine involves the formation of N-methylhydantoin, N-carbamoylsarcosine and sarcosine (Gauglitz, 1988; Hermann et al., 1992; Szulmajster, 1958a; b; ten Krooden & Owens, 1957). In contrast, the newly isolated strain KRE 4T degraded creatinine via creatine, sarcosine and glycine to the products acetyl phosphate, monomethylamine, ammonia and carbon dioxide. However, growth of strain KRE 4T on compounds other than creatinine or creatine was not possible. Even the intermediates sarcosine and glycine were not utilized, indicating a quite specific transport system. Thus, strain KRE 4T is very specialized in its substrate degradation. A similar phenomenon was described for *E. acidaminophilum* which could not use the intermediate pyruvate as carbon source though growth could be detected after addition of sarcosine to a pyruvate-containing medium (Granderath, 1988). In contrast to *E. acidaminophilum*, supplementation of the medium with sarcosine and/or glycine did not stimulate growth of strain KRE 4T on creatinine.

Creatine reductase, sarcosine reductase and glycine reductase activities were measured in crude extracts of strain KRE 4T in the range 29–72 mU (mg protein)⁻¹ (Table 2). The reducing equivalents were supplied by the oxidation of formate present as cosubstrate. The glycine reductase of all species studied, such as *Clostridium sticklandii*, *Clostridium sporogenes*, *E. acidaminophilum*, *Clostridium histolyticum* involve selenocysteine-containing proteins, explaining the selenium-dependence of growth (Andreesen, 1994; Kreimer & Andreesen, 1995). The reduction of betaine, sarcosine and glycine in *E. acidaminophilum* involves only substrate-specific proteins that differ in sensitivity towards hydroxylamine (Andreesen, 1994; Meyer et al., 1995). In comparison to *E. acidaminophilum*, the reductase activities in strain KRE 4T were quite low in extracts. These low enzyme activities in strain KRE 4T could be explained by a high sensitivity to disruption of the electron flow in the reductase systems. Compared to sarcosine reductase, the glycine reductase seems to play a subordinate role in the creatinine metabolism of strain KRE 4T because the observed equimolar ratio in the formation of monomethylamine to the utilized creatinine indicates that during growth on creatinine and formate only sarcosine reductase, not glycine reductase, is active to obtain redox balance.

Creatine is an important natural substance (Shimizu et al., 1986) which might allow the evolution of a specific reductase of its own, as is found for glycine, sarcosine and betaine (Hormann & Andreesen, 1989; Meyer et al., 1995) but not for dimethylglycine (Andreesen, 1994). For this reason, highly specific conditions were chosen for enrichment with creatinine and formate as substrates. However, the reductase activities measured in crude extracts could not be discriminated by a different sensitivity towards hydroxylamine as anticipated. It was concluded from the other experiments also that no genuine reductase was produced by strain KRE 4T. The creatine reductase activity observed was due to the combined action of creatine amidinohydrolase and sarcosine reductase. So far, a creatine reductase activity has not been described. Its putative product, N-methylguanidine, has been reported for *Pseudomonas stutzeri* (van Eyk et al., 1968) and a specific methylguanidine amidohydrolase seems to occur in *Alcaligenes* sp. N-42 (Nakajima et al., 1980). Creatine amidinohydrolase from strain KRE 4T could
be measured in crude extract at relatively high activity (Table 2). This enzyme was shown to be very unstable with reference to temperature, storage, dialysis or column chromatography. The instability of the creatine amidinohydrolase is well-described in the literature, particularly for Pseudomonas putida (Kaplan & Naugler, 1974; Schumacher et al., 1993; Schumann et al., 1993; Yoshimoto et al., 1976).

Classification of strain KRE 4T

By its physiological and morphological characteristics, strain KRE 4T did not resemble any of the described amino-acid-degrading anaerobes. Based on 16S rRNA analysis, the non-spore-forming isolate was clearly identified as a member of the genus Tissierella, where the bacterium formed a new and distinct subtype. A 16S rRNA gene sequence divergence value of approximately 5% with currently described Tissierella spp. demonstrates that the bacterium represents a new species, for which the name Tissierella creatinophila sp. nov. is proposed.

The first species described in the genus Tissierella was T. praeacuta, formerly classified as Bacteroides praeacutas (Collins & Shah, 1986). T. praeacuta is now allocated to the Clostridium subphylum of the Gram-positive bacteria (Farrow et al., 1995) and is embraced within Clostridium cluster XII (nomenclature: Collins et al., 1994). This classification was unexpected as T. praeacuta stains Gram-negative and does not produce spores (Collins & Shah, 1986). T. creatinophila also belongs to Clostridium cluster XII which includes purine- and glycine-utilizing bacteria such as Clostridium purinolyticum (Farrow et al., 1995). In Clostridium cluster XI or XIII, organisms such as C. litorale, C. sticklandii or Peptostreptococcus magnus and Peptostreptococcus micros are listed (Farrow et al., 1995); all of these are able to conserve energy by substrate-level phosphorylation via reductive deamination of glycine, sarcosine or betaine. So far, these enzyme systems seem to be specific to anaerobic bacteria (Andreesen, 1994).

T. creatinini, which was originally isolated from a wastewater pool at a sugar refinery, exhibited > 95% sequence relatedness to T. praeacuta (Farrow et al., 1995). T. creatinini and T. creatinophila differ from T. praeacuta by being clearly Gram-positive. The cell wall of T. creatinini contains D-ornithine as dibasic amino acid whereas T. praeacuta has a wall composed of meso-diaminopimelic acid (Farrow et al., 1995). Despite these differences, T. praeacuta, T. creatinini and T. creatinophila show similarities in being rod-shaped and non-fermentative (carbohydrates are not degraded), lacking endospores and respiratory menaquinones, and containing a low G + C content of the DNA (28–32 mol%). T. creatinophila can be readily distinguished from T. creatinini in its different substrate utilization: in contrast to T. creatinini, growth of T. creatinophila on compounds other than creatinine or creatine was not possible.

Description of Tissierella creatinophila sp. nov.

Tissierella creatinophila (cre.at.in.o'phi. la. Gr. adj. kreatinos creatine, referring to meat; Gr. adj. philos loving; M.L. fem. adj. creatinophila creatine-loving).

Cells are rod-shaped (0.7–1.1 × 2–6 μm), mostly with rounded ends, occur singly or in pairs and show motility by means of peritrichous flagella. Gram-positive cell wall structure revealed by electron microscopy. Growth occurs by utilization of creatinine via creatine, sarcosine and glycine to acetyl phosphate, ammonia, monomethylamine and carbon dioxide. Doubling time is decreased by the addition of formate as electron donor (8.7 h). Hydrogen can replace formate. Selenite (10^-6 M) is required for growth on creatinine or creatine. N-methylhydantoin, N-carbamoylsarcosine, sarcosine, glycine, hydantoin, hydantoic acid, betaine, arginine, ornithine, trimethylamine, dimethylamine, choline, glucose, galactose, fructose, lactose, ribose, pyruvate, maltate, succinate, fumarate, aspartate, acetate, serine, adenine, cytosine, thymine, uric acid, guanidine, xanthine, isoleucine + glycine, alanine + glycine, leucine + sarcosine, valine + sarcosine, phenylalanine + sarcosine and leucine + ornithine are not utilized. Temperature range for growth 10–40 °C, optimum at 30–34 °C; pH range 6.5–8.5, optimum 7.4. Colonies on blood agar are small, circular, flat with rough surface, greyish and milky. Major metabolic end products in peptone/yeast extract glucose broth are acetate, ethanol and some butyrate. Menaquinones are not produced. Nitrate, sulfate and sulphur are not reduced. The G + C content of the DNA is 30 mol%. Habitat is anaerobic sludge in sewage plants. Adverse effects on animals and humans are not known. Type strain is KRE 4T (= DSM 6911T).

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