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Tissierella creatinophila sp. nov., a Gram-positive, anaerobic, non-spore-forming, creatinine-fermenting organism

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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 4^T, 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 4^T 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 6911^T).

Keywords: *Tissierella creatinophila*, creatinine, creatine

INTRODUCTION

The concentration of creatinine in serum or urine is commonly measured in clinical laboratories for evaluating renal dysfunction (Shimizu *et al*., 1989). The alkaline sodium picrate method, based on Jaffé's reaction (Jaffé, 1886) is widely used for the determination of creatinine, although it is known to be subject to high interference by endogenous and exogenous substances (Blijenberg *et al*., 1994). In studies of microbial utilization of creatinine, much interest has been directed to the applicability of the enzymes involved in creatinine degradation for an enzymic assay of creatinine (Dubos & Miller, 1937; Kaplan & Naugler, 1974; Kaplan & Szabo, 1974; Kim *et al*., 1986a, b; Sugita *et al*., 1992; Tsuru *et al*., 1976). Creatine amidinohydrolase is highly significant in medical diagnostics because creatinine clearance is used to monitor the filtration rate of glomeruli in the kidney (Siedel *et al*., 1988).

Three metabolic pathways for the microbial degradation of creatinine have been described (Hermann *et al*., 1992). A strain classified as *Arthrobacter ureafaciens* was isolated which degraded creatinine via creatine and sarcosine and which was used in a bioassay for creatinine (Miller & Dubos, 1936; Kaplan & Naugler, 1974). The same metabolic pathway was found in many other bacteria, including *Pseudomonas*

Abbreviation: THF, tetrahydrofolate.

The GenBank accession number for the sequence of the 16S rRNA gene of strain KRE 4^T (DSM 6911^T) determined in this work is X80227.

aeruginosa, *Pseudomonas fluorescens* (Kopper, 1947), *Pseudomonas ovalis* (Appleyard & Woods, 1956), *Pseudomonas putida* (Tsuru *et al*., 1976) and *Alcaligenes denitrificans* subsp. *denitrificans* (Kim *et al*., 1986a). Another creatinine degradation pathway resulted in the formation of *N*-methylhydantoin, *N*-carbamoylsarcosine and sarcosine. *N*-Methylhydantoin could be detected as a characteristic intermediate in organisms of the genus *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Pseudomonas*, *Serratia* and *Bacillus* (Uwajima & Tereda, 1977; Yamada *et al*., 1985). A third possible pathway for creatinine utilization was described by van Eyk *et al*. (1968): in *Pseudomonas stutzeri*, creatinine was metabolized via creatine to acetate and methylguanidine, but methylguanidine amidohydrolase activity could not be detected. No other strains degrading creatinine via methylguanidine have been described in the literature.

Information on pure cultures of anaerobes utilizing creatinine or similar compounds is limited (Möller *et al*., 1986). '*Eubacterium sarcosinogenum*', a non-spore-forming, non-motile, Gram-positive bacterium, was reported to degrade creatinine to sarcosine, ammonia and carbon dioxide (Szulmajster & Kaiser, 1960; Szulmajster, 1960). *Clostridium paraputrificum* and *Clostridium perfringens* metabolized creatinine and creatine anaerobically to *N*-methylhydantoin (Szulmajster, 1958a, b; ten Krooden & Owens, 1957). The creatine- and sarcosine-utilizing *Eubacterium acidaminophilum* was shown to require selenite and suitable electron donors (Hormann & Andreesen, 1989; Zindel *et al*., 1988). Creatinine and *N*-methylhydantoin degradation was studied in several species of *Clostridium*, *Eubacterium* and *Peptostreptococcus* (Gauglitz, 1988). In *Eubacterium* strain BN 11^T, now classified as *Tissierella creatinini* (Farrow *et al*., 1995), creatinine was converted to acetate via *N*-methylhydantoin, *N*-carbamoylsarcosine and sarcosine. A strictly anaerobic, spore-forming, Gram-positive strain, *Clostridium* sp. FS23, which converted creatinine to *N*-methylhydantoin via a creatinine iminohydrolase has been isolated and characterized (Hermann *et al*., 1992).

In this paper, results are presented from studies on creatinine metabolism by a newly isolated, non-spore-forming, Gram-positive strain, KRE 4^T, which is able to degrade creatinine via creatine, sarcosine and glycine to the products acetate, monomethylamine, ammonia and carbon dioxide. The selenium-dependent growth of the bacterium was stimulated by formate, indicating the participation of a creatine reductase, sarcosine reductase and/or glycine reductase.

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 $MgSO_4$, 1 mM $CaCl_2$, 4·7 mM NH_4Cl and 1·5 mM KH_2PO_4. 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 & Andreesen, 1989). The following sterilized components were added separately to the autoclaved medium to give the indicated final concentrations: 50 mM $NaHCO_3$ and 2·7 mM Na_2S. 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^T and KRE 2, were deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) with the accession numbers DSM 6911^T 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^T and KRE 2 were initially characterized by using a variety of standard physiological tests directed to the *Eubacterium* species-complex (Andreesen, 1992; Zindel *et al*., 1988) and were found to be similar to *T. creatinini*, strain BN 11^T described by Gauglitz (1988). Key enzymes, such as glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, glutamate- and malate dehydrogenases, from strain KRE 4^T 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 Tomaoka & 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*-methylhydantoin 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 model 3920, equipped with a Chromosorb 101 column (80–100 mesh) and a Chromatopac C-R2AX integrator (Shimadzu). The injector temperature was 205 °C, the oven temperature 180 °C and the detector temperature 210 °C. The determination of methanol was carried out with a GC-Model F22 (Perkin Elmer) and an integrator model 3390A (Hewlett Packard). A Porapack QS column was used (80–100 mesh). The injector temperature was 120 °C, the oven temperature 80 °C and the detector temperature 120 °C.

Sarcosine and *N*-carbamoylsarcosine levels were determined in an enzymically coupled reaction with sarcosine oxidase and peroxidase (Gauglitz, 1988). The reaction solution contained in a final volume of 100 ml: 4·58 mg TES, 20·3 mg 4-aminoantipyrin and 375 mg 2,4,6-tribromo-3-hydroxybenzoic acid; the pH was adjusted to 7·8 with 10 M NaOH. For the quantification of sarcosine, 0·1 ml sample, 0·05 ml sarcosine oxidase (160 U ml^{-1}) and 0·01 ml peroxidase (520 U ml^{-1}) were added in a final volume of 1·05 ml reaction solution. It was neccessary to dilute the anaerobic samples at least 1:10 to avoid an inhibition of the sarcosine oxidase by lack of oxygen. The reaction was started by the addition of peroxidase. 10 min incubation at 24 °C, the increase in absorption was measured at 546 nm. By adding 0·1 ml *N*-carbamoylsarcosine hydrolase (10 U ml^{-1}; kindly provided by Boehringer Mannheim) to the test system, the determination of *N*-carbamoylsarcosine was also possible.

Harvesting of cells and preparation of cell-free extracts. The cells were anaerobically mass-cultured in 20 l 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 N_2, 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 [^{14}C]acetate from [1-^{14}C]creatine, [2-^{14}C]sarcosine or [2-^{14}C]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 amidinohydrolase 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 creatinine (85 mM, pH 7·4), was incubated for 30 min at 34 °C.

Determination of *N*-methylhydantoin hydrolase was performed using in a final volume of 3 ml: 90 mM glycine buffer, pH 8·9, 3 mM dithioerythritol, 20 mM *N*-methylhydantoin, 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*-methylhydantoin 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*-methylhydantoin hydrolase was also determined by incubating 100 μl crude extract with 100 μl *N*-methylhydantoin (100 mM in 50 mM potassium phosphate buffer, pH 7·5) for 30 min at 34 °C. After this 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 methylene-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 5-methyl-THF and the reduction of dichlorophenol/indophenol was followed at 522 nm ($\varepsilon_{522} = 8{\cdot}6$ 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 & Thauer, 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 ($\varepsilon_{600} = 13$ mM^{-1} cm^{-1}) (Spormann & Thauer, 1988).

Chemicals. [2-^{14}C]Sarcosine was custom made and [2-^{14}C]glycine was purchased from Amersham-Buchler. [1-^{14}C]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 4^T, 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 4^T, was studied in more detail. The rod-shaped cells of strain KRE 4^T 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 4^T was observed. In contrast to *T. creatinini*, peritrichous flagella were observed in strain KRE 4^T 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 4^T 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 4^T 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 4^T was 30 mol%. Comparative 16S rRNA gene sequencing demonstrated that strain KRE 4^T 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. praeacuta*, *T. creatinini* and *T. creatinophila* strain KRE 4^T

Organism	Major end products from PYG*	Metabolism†	MDH‡	GDH‡	G6PDH‡	6PGDH‡	Major long-chain fatty acids	Menaquinones	G+C content (mol%)
T. praeacuta§	B, A, iV	NF	−	−	−	+	iso-$C_{15:0}$	−	28
T. creatinini‖	ND	NF	ND	ND	ND	ND	$C_{18:1\ c11/12}$	−	32
Strain KRE 4^T	A, E, B	NF	+	+	−	−	ND	−	30

* A, acetate; B, butyrate; E, ethanol; iV, isovaleric acid. *T. creatinini* and strain KRE 4^T 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).

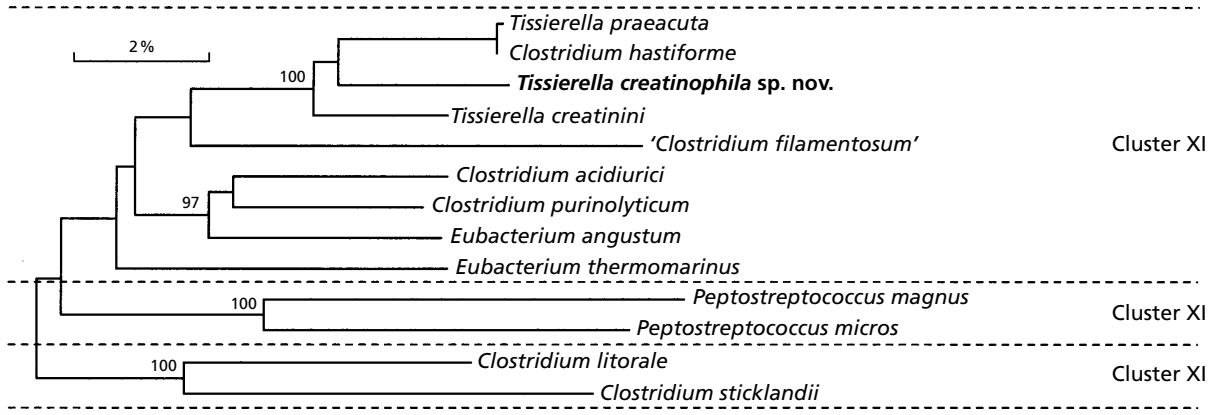

Fig. 1. Unrooted tree based on 16S rRNA gene sequences showing the phylogenetic position of *Tissierella creatinophila* sp. nov. (strain KRE 4^T) within the *Clostridium* subphylum of Gram-positive bacteria. The tree was constructed using the neighbour-joining method. Significant bootstrap values (calculated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

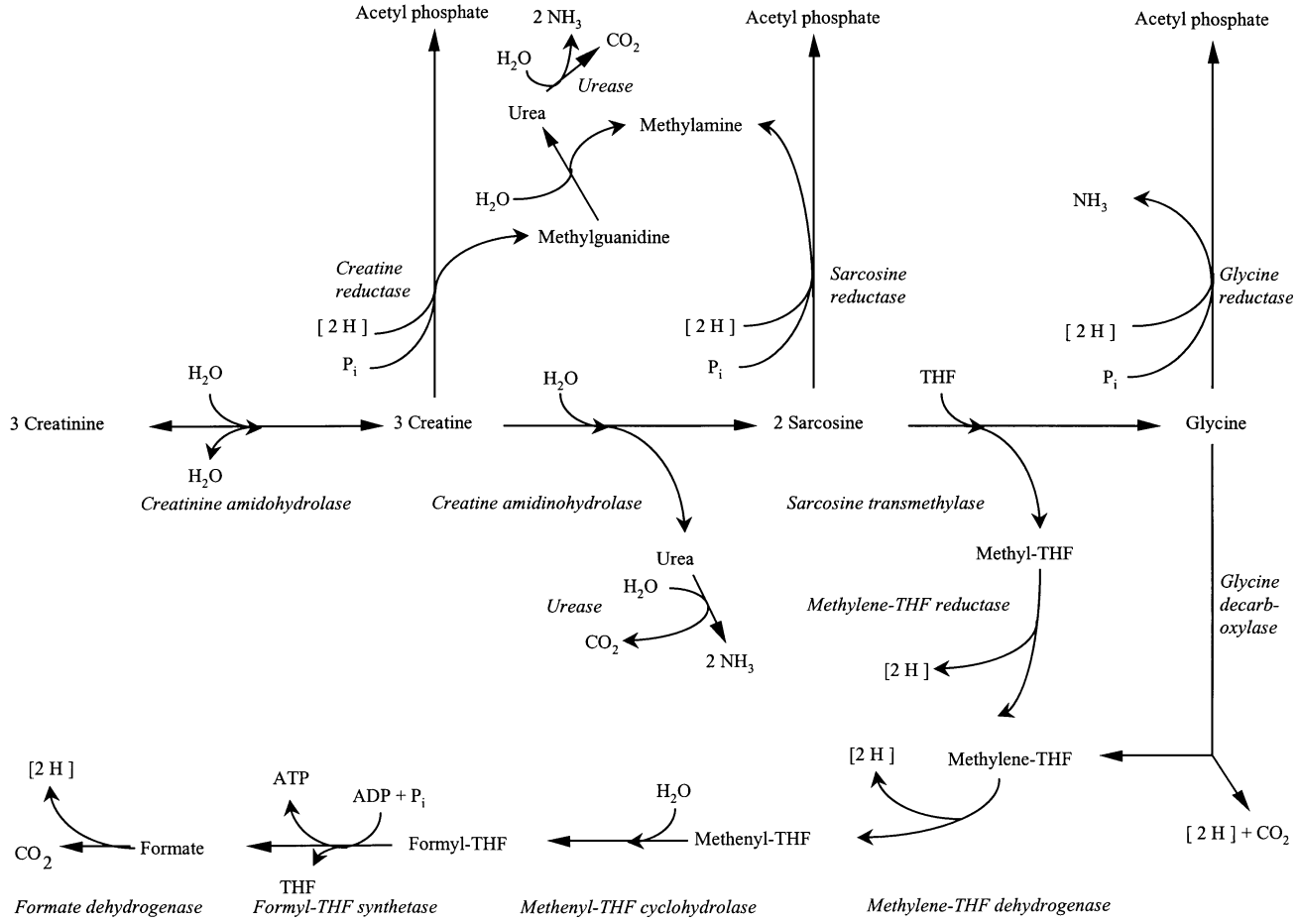

Fig. 2. Possible metabolic reactions during creatinine degradation in strain KRE 4^T discussed. Enzymes are shown in italics.

strain KRE 4^T is shown in Fig. 1 and confirmed the close affinity of the unknown isolate with the genus *Tissierella*. The biochemical characterization of strain KRE 4^T revealed differences with the two described species of *Tissierella*, *T. praeacuta* and *T. creatinini* (Table 1). In contrast to *T. praeacuta* (Farrow *et al.*,

1995), 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 only 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 20 mM), purines and pyrimidines (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^{-9} M Na_2SeO_3, 20 h at 10^{-8} M, 9 h at 10^{-7} M, 8·5 h at 10^{-6} M, 9·6 h at 10^{-5} M and 10 h at 10^{-4} M Na_2SeO_3. Thus, optimal growth of strain KRE 4^T was observed in the presence of 10^{-6} M selenite in the liquid medium, indicating an involvement of a creatine reductase, sarcosine reductase or glycine reductase in the creatinine degradation pathway.

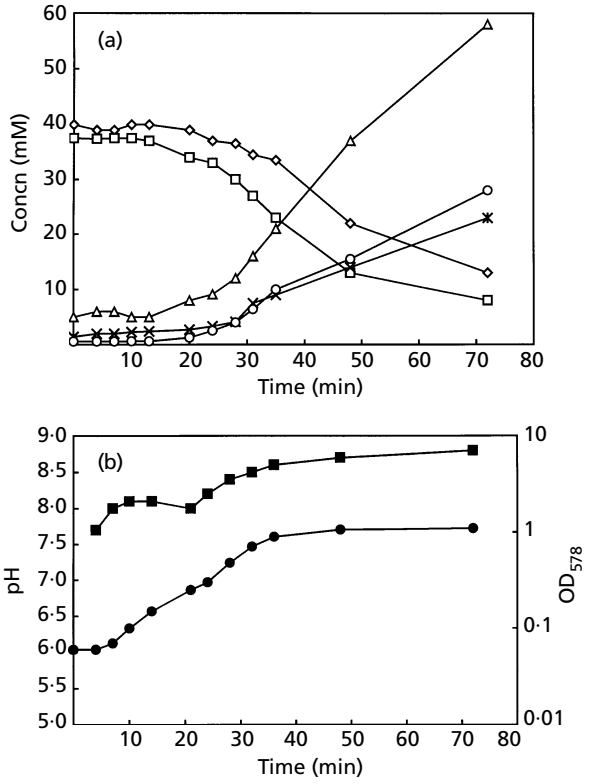

Fig. 3. Substrate utilization, product formation, growth and pH changes in cultures of strain KRE 4^T. (a) Utilization of creatine (◇) and formate (□), and production of ammonia (△), acetate (×) and monomethylamine (○). (b) Growth (●) and pH changes (■).

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)

Table 2. Enzymes involved in creatinine metabolism in *T. creatinophila* strain KRE 4^T

Enzyme*	EC no.	Specific activity [units (mg protein)$^{-1}$]
Creatine reductase	1.4.4.–	0·035†/0·011‡
Sarcosine reductase	1.4.4.–	0·072†/0·017‡
Glycine reductase	1.4.4.–	0·029†/0·003‡
Creatine amidinohydrolase	3.5.3.3	0·140
Creatinine deiminase	3.5.4.21	ND
N-Methylhydantoin amidohydrolase	3.5.2.14	ND
N-Carbamoylsarcosine amidohydrolase	3.5.1.59	ND
Methylene-THF reductase§	1.5.1.20	3·0
Methylene-THF dehydrogenase§	1.5.1.5	1·2
Formyl-THF synthetase§	6.3.4.3	1·1
Formate dehydrogenase§	1.2.1.–	
MV		4·2
BV		4·2
CO dehydrogenase§	1.2.99.2	
MV		ND

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 amidinohydrolase 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 creatine 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 creatine amidinohydrolase, 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 amidinohydrolase and sarcosine reductase. This suggestion

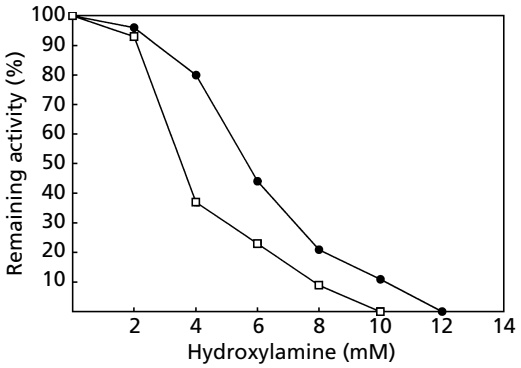

Fig. 4. Influence of hydroxylamine on the activity of sarcosine- and creatine reductase. The reductase assay was carried out as described in the Methods. Crude extract (2 mg) was preincubated for 10 min with different concentrations of neutralized hydroxylamine at 37 °C before starting with substrate. ●, Sarcosine reductase (100% activity corresponds to 72 mU mg^{-1}); □, creatine reductase (100% activity corresponds to 35 mU mg^{-1}).

was supported by the fact that formation of the N-containing product of creatine reductase, *N*-methylguanidine, was never detected in strain KRE 4^T. 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 4^T 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 creatinine 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 4^T 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 CO_2 (methylene-THF reductase, methylene-THF dehydrogenase, methenyl-THF cyclohydrolase, formyl-THF synthetase and formate dehydrogenase) were present in extracts of strain KRE 4^T 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 4^T degraded creatinine via creatine, sarcosine and glycine to the products acetyl phosphate, monomethylamine, ammonia and carbon dioxide. However, growth of strain KRE 4^T 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 4^T is very specialized in its substrate degradation. A similar phenomenom 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 4^T on creatinine.

Creatine reductase, sarcosine reductase and glycine reductase activities were measured in crude extracts of strain KRE 4^T 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 litorale* and *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 4^T were quite low in extracts. These low enzyme activities in strain KRE 4^T 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 4^T 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 4^T. 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 amidinohydrolase seems to occur in *Alcaligenes* sp. N-42 (Nakajima *et al*., 1980).

Creatine amidinohydrolase from strain KRE 4^T 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 4^T

By its physiological and morphological characteristics, strain KRE 4^T 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 subline. 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 praeacutus* (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, malate, 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 4^T (= DSM 6911^T).

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