Isolation from a shea cake digester of a tannin-degrading \textit{Streptococcus gallolyticus} strain that decarboxylates protocatechuic and hydroxycinnamic acids, and emendation of the species

Mohamed Chamkha,\textsuperscript{1} Bharat K. C. Patel,\textsuperscript{2} Alfred Traore,\textsuperscript{3} Jean-Louis Garcia\textsuperscript{1} and Marc Labat\textsuperscript{1}

Author for correspondence: Marc Labat. Tel: +33 4 9182 8575. Fax: +33 4 9182 8570. e-mail: labat@esil.univ-mrs.fr

INTRODUCTION

Tannins are a group of highly polar phenolic compounds found in plant extracts. These oligomeric compounds, with molecular weights of 500–3000 g mol\(^{-1}\), form insoluble complexes with proteins, cellulose, gelatin and pectin (Swain & Bate-Smith, 1962). Tannins are regarded as environmental pollutants. They are toxic to aquatic organisms, inhibit micro-organisms and enzymes involved in the decomposition of organic pollution (Scalbert, 1991) and, in some cases, are recalcitrant to biodegradation (Field & Lettinga, 1992).

Aerobic degradation of tannins has been widely studied. Soil fungi belonging to the genera \textit{Aspergillus} and \textit{Penicillium}, yeasts of the genus \textit{Pichia} and bacteria of the genera \textit{Klebsiella}, \textit{Bacillus}, \textit{Corynebacterium} and \textit{Achromobacter} are the most frequently observed degraders of hydrolysable tannins. The interest in anaerobic degradation of hydrolysable tannins has resulted from an increase in the application of anaerobic systems for treatment of tannin-containing wastewaters (Field & Lettinga, 1992).

A previous study of a continuous anaerobic digester fed with shea cake showed high tannin-removal rates and production of organic acids and methane (Ouattara, 1994). Isolation and characterization are reported of a strain of \textit{Streptococcus gallolyticus} capable of hydrolysing tannin acid and decarboxyl-
ating gallic acid to pyrogallol from the same digester. Both the novel isolate and the type strain, \( S. \) \textit{gallolyticus} ACM 3611\(^T\), decarboxylated protocatechuic acid to catechol and some \( p \)-hydroxybenzoic acid derivatives to their corresponding 4-vinyl derivatives. It is proposed to emend the description of \( S. \) \textit{gallolyticus} to include these newly described traits.

**METHODS**

**Source of strains.** Strain B7 was isolated from a continuous methanogenic digester located in Burkina Faso. This digester (20 l, 30 °C) was initially inoculated with anaerobic sludge from the pit of a slaughterhouse and fed for 6 months with shea cake. \textit{Streptococcus bovis} DSM 20480\(^T\) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). \( S. \) \textit{gallolyticus} ACM 3611\(^T\) was obtained from the Australian Collection of Microorganisms. \( S. \) \textit{gallolyticus} CIP 107089, \( S. \) \textit{gallolyticus} CIP 107090 and \( S. \) \textit{gallolyticus} CIP 107091 were obtained from the Collection of the Pasteur Institute.

**Culture media.** The anaerobic techniques of Hungate (Hungate, 1969; Macy \textit{et al}., 1972; Miller & Wolin, 1974) were used throughout this work. The basal medium contained \( 1 \) g tannic acid l\(^−1\), \( 0.4 \) g NH\(_4\)Cl, \( 0.5 \) g KH \(_2\)PO \(_4\), \( 0.4 \) g NaCl, \( 0.33 \) g MgCl\(_2\), \( 6 \) H\(_2\)O, \( 0.05 \) g CaCl\(_2\), \( 2 \) H\(_2\)O, \( 0.25 \) g cysteine hydrochloride, \( 2 \) g yeast extract (Difco), \( 1 \) ml trace-element mineral solution (Widdel & Pfennig, 1981) and \( 1 \) mg resazurin. The pH was adjusted to 7 with \( 10 \) M KOH. The medium was then boiled under a stream of O\(_2\)-free N\(_2\) gas and cooled to room temperature. Aliquots of \( 5 \) ml were dispensed into Hungate tubes, degassed under N\(_2)/CO\(_2\) (80:20\%, v/v) and subsequently sterilized by autoclaving at 110 °C for 45 min. Prior to inoculation, \( 0.05 \) ml 10 \( % \) (w/v) NaHCO\(_3\) and \( 0.05 \) ml 5 \( % \) (w/v) Na\(_2\)SO\(_4\) were injected from sterile stock solutions. Substrates were injected from concentrated anaerobic sterile stock solutions to obtain the desired final concentration.

Strain B7 and the four strains of \( S. \) \textit{gallolyticus} were routinely grown on basal medium containing 5 mM glucose. The type strain of \( S. \) \textit{bovis} was grown aerobically at 37 °C using a medium containing \( 1 \) g tannic acid \( 1 \) g casen peptone-tryptic digest, \( 5 \) g yeast extract, \( 5 \) g glucose and \( 5 \) g NaCl. The pH was adjusted to 7-7.4 with \( 10 \) M KOH.

**Enrichment and isolation procedure.** The digester wastewater sample (0.5 ml) was inoculated into 5 ml basal medium containing \( 1 \) g tannic acid \( 1 \) g casen peptone-tryptic digest, \( 5 \) g yeast extract, \( 5 \) g glucose and \( 5 \) g NaCl. The pH was adjusted to 7-7.4 with \( 10 \) M KOH.

**Morphology and sporulation test.** Light and electron microscopy were performed as described by Fardeau \textit{et al}. (1997). For testing heat resistance, cells grown in basal medium containing glucose were exposed to 80, 90 and 100 °C for 10 min. The cultures were cooled quickly to ambient temperature and inoculated into fresh glucose-containing medium and growth was recorded after 24 h incubation at 37 °C. Conditions for sporulation that were tested included growth in the presence of glucose or tannic acid and without added carbon sources.

**Growth parameters.** For all experiments, basal medium containing 0.2 % yeast extract and 10 mM glucose was used. The pH of the pre-reduced anaerobic medium was adjusted with \( 5 \) % NaHCO\(_3\), \( 5 \) % NaCO\(_3\), or 0.1 M HCl to obtain a pH range between 5-0 and 9-5. Different amounts of NaCl were weighed directly in Hungate tubes prior to dispensing 5 ml medium to obtain the desired NaCl concentration (range 0-50 g l\(^−1\)). The temperature range for growth was determined between 10 and 50 °C (5 °C intervals).

**Electron acceptors.** Sulfate, thiosulfate, sulfite, fumarate (all at \( 20 \) mM) and elemental sulfur (2%, w/v) were tested as electron acceptors in basal medium containing 10 mM glucose.

**Substrate utilization.** Experiments were performed in duplicate with an inoculum subcultured at least once under the same test conditions. The substrates tested for utilization were injected from pre-sterilized and concentrated stock solutions into Hungate tubes that contained 5 ml pre-sterilized basal medium. The following substrates were used: \( 20 \) mM carbohydrates (arabitol, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, melibiose, melizitose, \textit{myo}-inositol, raffinose, ribose, sorbitol, sorbose, trehalose and xylose); \( 20 \) mM organic acids (acetate, adipate, butyrate, citrate, crotonate, formate, fumarate, isobutyrate, lactate, malate, propionate, succinate and valerate); \( 20 \) mM alcohols (butanol, ethanol, glycerol, isobutanol, 2-propanol, methanol and 1-propanol); \( 10 \) g peptides \( 1 \) [Biotryptase (Difco), Casamino acids (Sigma), gelatin (Sigma), peptone (Difco)] and \( 20 \) mM phenylalanine; and \( 5 \) mM aromatic compounds. Concentrated stock solutions were prepared, neutralized if necessary, rendered anaerobic by gassing with O\(_2\)-free N\(_2\) and sterilized by filtration (pore size 0.2 µm; Millipore). Aromatic compounds were tested with or without \( 5 \) mM glucose. Autotrophic growth was tested using H\(_2)/CO\(_2\) (20:80%, v/v) at a final pressure of 2 bar. An increase in OD\(_{580}\) in tubes containing added substrates, compared with control tubes lacking substrate, was considered as positive growth.

**Analytical techniques.** Bacterial growth was monitored by measuring the OD\(_{580}\) directly from anaerobic Hungate tubes inserted into the cuvette holder of a spectrophotometer (Shimadzu UV 160A). Aromatic compounds, carbohydrates, volatile fatty acids and alcohols were measured by HPLC as described by Meechichi \textit{et al}., (1999). H\(_2\) and CO\(_2\) were measured as described by Fardeau \textit{et al}., (1993).

**Determination of the G+C content and DNA–DNA hybridization.** The DNA G+C content was determined by the DSMZ. DNA was isolated and purified by chromatography on hydroxyapatite (Cashion \textit{et al}., 1977) and the G+C content was determined by HPLC as described by Meebah \textit{et al}., (1989). Non-methylated lambda DNA (Sigma) was used as the standard. DNA–DNA hybridization was performed at the DSMZ as described by De Ley \textit{et al}., (1970), with the modifications described by Escara & Hutton (1980) and Huß \textit{et al}., (1983), using a Gilford System model 2600 equipped with a Gilford model 2527-R thermostramp and plotter. Renaturation rates were computed with the TRANSFER.BAS program of Jahnke & Bahnweg (1986) and Jahnke (1992).

**DNA extraction and amplification of the 16S rRNA gene.** DNA was extracted from the isolate as described by Woo \textit{et al}. (1997). Amplification and sequencing of the 16S rRNA
was done as described by Redburn & Patel (1993) and Andrews & Patel (1996). The sequence data that were generated were aligned to an almost full-length consensus 16S rRNA gene sequence, assembled and checked manually for accuracy using the alignment editor ae2 (Maidak et al., 2000). These were compared with other sequences in the GenBank database using BLAST (Altschul et al., 1997) and in the Ribosomal Database Project, version 8.0, using SIMILARITY_RANK and SUGGEST_TREE (Maidak et al., 2000). Pairwise evolutionary distances based on 1235 unambiguous nucleotides were computed using the programs DNADIST (Jukes & Cantor, 1969) and NEIGHBOR that form part of the PHYLIP suite of programs (Felsenstein, 1993). TREECON was used extensively for bootstrap analysis (Van de Peer & De Wachter, 1994).

RESULTS AND DISCUSSION

Enrichment and isolation

Enrichment cultures developed in medium containing 1 g tannic acid l⁻¹ within 3 weeks of incubation at 37 °C, as shown by growth and pyrogallol production. After several transfers in the liquid medium, the enrichment developed a stable microbial population that degraded tannic acid. Several isolates were obtained using the roll-tube method (Hungate, 1969) and one of these cultures, designated strain B7, was studied further.

Morphology and physiology

Cells of strain B7 were non-motile cocci (< 2 μm in diameter) that occurred in pairs or short chains and stained Gram-positive. Spores were not observed and cells did not survive heat treatment of 10 min at 80 °C, indicating an absence of heat-resistant cells. Strain B7 was a facultatively anaerobic bacterium. The temperature range for growth was 15–48 °C, with optimal growth at 40 °C. No growth occurred at 10 or 50 °C. The pH range for growth was pH 5–9, with an optimum at pH 7–8. The NaCl concentration range for growth was 0–40 g l⁻¹. Yeast extract stimulated growth, but was not required. Strain B7 fermented a wide range of carbohydrates including cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, melibiose, raffinose and trehalose, producing predominantly lactate and minor amounts of acetate, formate and ethanol, but no gas. Malate and yeast extract were fermented into lactate. Isolate B7 did not utilize arabinol, melezitose, myo-inositol, ribose, sorbitol, sorbose or xylose, any of the organic acids tested, any of the alcohols tested, peptides, including Biotrypsin, Casamino acids, gelatin or peptone, phenylalanine or H₂/CO₂. Sulfate, thiosulfate, sulfate, elemental sulfur and fumarate could not be used as electron acceptors.

G+C content, 16S rRNA gene sequence analysis and DNA–DNA relatedness

The G+C content of strain B7 was 40.4 ± 0.3 mol%, as determined by HPLC. An almost complete 16S rRNA gene sequence was obtained for strain B7, consisting of 1535 nt. Phylogenetic analysis revealed that strain B7 was closely associated with members of the genus Streptococcus (Hardie, 1986) and, in particular, with S. gallolyticus ACM 3611T (similarity of 98%). S. bovis DSM 20480T, Streptococcus equinus NCDO 1037T, Streptococcus alactolyticus ATCC 43492T, Streptococcus macedonicus ACA-DC 206T and Streptococcus caprinus ACM 3970 (a synonym of S. gallolyticus; Sly et al., 1997), with a mean similarity of 98% (Fig. 1). S. gallolyticus, S. bovis, S. equinus, S. alactolyticus, S. macedonicus and S. caprinus form a cohesive rRNA cluster within the genus Streptococcus that is referred to as the S. bovis cluster (Farrow et al., 1984). DNA hybridization studies showed that S. gallolyticus, S. bovis, S. equinus, S. alactolyticus and S. macedonicus are distinct genospecies (Sly et al., 1997; Tsakalidou et al., 1998; Kawamura et al., 1998). The level of DNA–DNA relatedness was 29% between strain B7 and S. bovis DSM 20480T and 86% between strain B7 and S. gallolyticus ACM 3611T. According to phylogenetic analysis, strain B7 is a member of the genus Streptococcus and, in particular, the S. bovis group, which forms a cohesive rRNA cluster, with a mean similarity of greater than 97%. The level of DNA relatedness between strain B7 and S. gallolyticus ACM 3611T (86%) indicates that it should be regarded as a strain of S. gallolyticus. Phenotypically, strain B7 ferments mannitol, hydrolises tannic acid to release gallic acid and decarboxylates gallic acid to pyrogallol, traits it possesses in common with S. gallolyticus isolates (Sly et al., 1997; Brooker et al., 1994; Osaka & Sly, 1992; Osawa et al., 1993), but not with other members of the S. bovis group.

S. gallolyticus has so far been isolated from diverse habitats including the faeces of koalas, kangaroos, brushtail, possums, ringtail possums, cows, horses, pigs, dogs and guinea pigs, as well as animals with bovine mastitis, human clinical sources and the sheep rumen (Osawa et al., 1995; Sly et al., 1997). Nelson et al. (1998) reported that tolerance of and/or degradation of tannins by rumen bacteria was widespread. The isolation of strain B7 from an anaerobic digester fed with shea cake extends the known habitat for S. gallolyticus. The isolation of B7 from such an en-

Fig. 1. Phylogenetic position of strain B7 within the radiation of the S. bovis group. The S. bovis group is represented by S. gallolyticus, S. macedonicus, S. equinus, S. bovis and S. alactolyticus. The outgroup is represented by members of the Streptococcus mutans group, which includes S. mutans (AJ243965) and Streptococcus ratti (X58304). For phylogeny, 1235 unambiguous nucleotides were included in the analysis. GenBank accession numbers are provided in parentheses.
Table 1. Metabolism of aromatic compounds by strain B7 and *S. gallolyticus* ACM 3611<sup>T</sup>

Bacteria were grown in basal medium containing 0·2% yeast extract; aromatic compounds were used at 5 mM, with or without 5 mM glucose. Results were recorded after 2 days anaerobic incubation at 37 °C. For strain B7, the ΔOD<sub>540</sub> (difference between OD<sub>540</sub> after incubation at 37 °C and OD<sub>540</sub> prior to incubation) of the culture was 0·10–0·13 without added glucose and 0·54–0·66 with added glucose. For *S. gallolyticus* ACM 3611<sup>T</sup>, the ΔOD<sub>540</sub> of the culture was 0·11–0·13 without added glucose and 0·57–0·68 with added glucose.

<table>
<thead>
<tr>
<th>Aromatic acid metabolized</th>
<th>Compound produced</th>
<th>Amount of product (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without glucose</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>Pyrogallol</td>
<td>3·4</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>Pyrogallol</td>
<td>3·6</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>Catechol</td>
<td>1·8</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>Vinylphenol</td>
<td>4·5</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>Vinylcatechol</td>
<td>3·0</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>Vinylguaiacol</td>
<td>2·9</td>
</tr>
</tbody>
</table>

Environment after enrichment on tannic acid is not surprising, as the digester was rich in tannins and aromatic compounds and had been inoculated with anaerobic sludge from the pit of a slaughterhouse.

Metabolism of aromatic compounds

Strain B7 hydrolysed tannic acid (hydrolysable tannin) at 1 g l<sup>−1</sup> to release gallic acid, which was subsequently decarboxylated to pyrogallol. Glucose supplementation enhanced cell growth and markedly accelerated this conversion (Table 1). Tannin concentrations greater than 17 g l<sup>−1</sup> inhibited growth. Gallic acid, which is a monomer of tannic acid, was decarboxylated to pyrogallol, with or without added glucose (Table 1). Pyrogallol was the end product and the aromatic ring was not degraded after 1 month of incubation.

The following benzoic acid derivatives tested in the presence or absence of glucose were not metabolized: benzoate, hydroxylated benzoic acids (4-hydroxybenzoate, 3,5-, 2,4- and 2,6-dihydroxybenzoates and 2,4,6-trihydroxybenzoate), methoxylated benzoic acids (2,4-, 2,6-, 3,4- and 3,5-dimethoxybenzoates and 3,4,5-trimethoxybenzoate), mixed hydroxylated/methoxylated benzoic acids (4-hydroxy-3-methoxybenzoate, 3-hydroxy-4-methoxybenzoate and 4-hydroxy-3,5-dimethoxybenzoate) and 3,4-dimethylbenzoate. However, protocatechuic acid (3,4-dihydroxybenzoate) was decarboxylated to catechol. Again, the addition of glucose accelerated this conversion (Table 1).

Of a number of cinnamic compounds tested, namely cinnamic, α-, m- and p-coumaric, caffeic, ferulic, isoferulic and 3,4,5-trimethoxycinnamic acids, with or without added glucose, only p-coumaric, caffeic and ferulic acids were metabolized to their corresponding 4-vinyl (styrene) derivatives, respectively 4-vinylphenol (4-hydroxystyrene), 4-vinylcatechol (3,4-dihydroxybenzoate) and 4-vinylguaiacol (4-hydroxy-3-methoxystyrene) (Table 1). The mechanism involved decarboxylation of the carboxyl group in the unsaturated C<sub>2</sub>-aliphatic side chain. Supplementation with glucose increased the conversion yield.

Strain B7 did not decarboxylate phenylpropionic acids (hydrocinnamate and hydrocaffeate) or phenylactic acids (phenylacetate, p-hydroxyphenylacetate, 2,5- and 3,4-dihydroxyphenylacetates, p-methoxyphenylacetate and 3,4-dimethoxyphenylacetate), with or without added glucose, indicating a specific selection. In addition, pyrogallol and phloroglucinol were not metabolized.

The ratio of pyrogallol, catechol and 4-vinyl derivatives to their corresponding gallic, protocatechuic and 4-hydroxycinnamic acids in the medium increased during the course of the experiment until the utilization of the substrate was complete. Adding glucose increased cellular yield and markedly accelerated these conversions. With all aromatic compounds tested, and even after 1 month incubation, no ring cleavage was observed.

The metabolism of aromatic compounds by the type strain, *S. gallolyticus* ACM 3611<sup>T</sup>, and *S. gallolyticus* CIP 107089, *S. gallolyticus* CIP 107090 and *S. gallo**

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Emended description of *Streptococcus gallolyticus* (Osawa et al. 1995)


It is proposed that the description of *S. gallolyticus* be emended to include the novel characteristics described in this report.

**Emended description of Streptococcus gallolyticus**

**REFERENCES**


