Chitinilyticum aquatile gen. nov., sp. nov., a chitinolytic bacterium isolated from a freshwater pond used for Pacific white shrimp culture

Shu-Chen Chang,1,2 Wen-Ming Chen,3 Jih-Terng Wang4 and Ming-Chang Wu2

1Department of Food Science and Technology, Tajen University, 20 Weishin Rd, YanPu Shiang, Pingtung City 907, Taiwan, ROC
2Department of Food Science, National Pingtung University of Science and Technology, Pingtung, Taiwan, ROC
3Department of Seafood Science, National Kaohsiung Marine University, Kaohsiung, Taiwan, ROC
4Department of Biotechnology, Tajen University, Pingtung, Taiwan, ROC

Strain c14T, originally isolated from surface water of a freshwater pond located in Pingtung (southern Taiwan) used for culture of Pacific white shrimp (Litopenaeus vannamei), was subjected to a polyphasic taxonomic approach. The strain exhibited strong chitinolytic activity and was able to grow under aerobic and anaerobic conditions by utilizing chitin exclusively as the carbon, nitrogen and energy source. Phylogenetic analysis of the 16S rRNA gene sequence revealed a clear affiliation of the proposed bacterium to the Betaproteobacteria, most closely related to Chitinibacter tainanensis S1T, Deefgea rivuli WB 3.4-79T and Silvimonas terrae KM-45T, with 94.6, 93.6 and 92.9 % 16S rRNA gene sequence similarity, respectively. The predominant fatty acids detected in cells of strain c14T were C16 : 0, C18 : 1\(\Delta_7\)c and summed feature 3 (C16 : 1\(\Delta_7\)c and/or C15 : 0iso 2-OH). The G+C content of the genomic DNA was 69.5 (±1.0) mol%.

Biochemical, physiological, chemotaxonomic and phylogenetic analyses showed that strain c14T could not be assigned to any known genus of the Betaproteobacteria. Therefore, strain c14T is classified within a novel genus and species, for which the name Chitinilyticum aquatile gen. nov., sp. nov. is proposed. The type strain of Chitinilyticum aquatile is c14T (=LMG 23346T =BCRC 17533T).

Chitin is a common constituent of crustacean shells, cell walls of fungi, certain green algae and exoskeletons of insects and is the second most abundant polysaccharide in nature (after cellulose). It is an insoluble homopolymer composed of linear chains of \(\beta\)-1,4-linked \(N\)-acetyl-\(\beta\)-d-glucosamine (GlcNAc) residues, which are highly cross-linked by hydrogen bonds. Chitin and its partially deacetylated derivatives exhibit interesting properties and constitute a valuable raw material for biomedical, agricultural and cosmetic applications (Shigemasa & Minami, 1996). Annually, enormous amounts of chitin are synthesized in the biosphere, as much as 10\(^{10}\) to 10\(^{11}\) metric tons (Gooday, 1990), but only a tiny minority remains in the environment. The high turnover rate of the polysaccharide is attributed primarily to micro-organisms that degrade chitin, allowing carbon and nitrogen to return to the ecosystem (Gooday, 1990). It seems likely that shrimp culturing ponds could be a rich source of chitin-degrading bacteria. Since shrimp farming has contributed significantly to the economy of southern Taiwan, there are many types of shrimp ponds scattered around the Pingtung region. Some of them are very close to the coast, but some are far away from the sea, and that results in various water salinities in the ponds. Thus, the diverse culture types provide a variety of drastically different ecosystems in which micro-organisms can develop.

In our previous study, a strain of chitin-degrading bacteria, Chitinimonas taiwanensis c\(^f\), was isolated from a freshwater pond for shrimp (freshwater giant prawn, Macrobrachium rosenbergii) culture (Chang et al., 2004). In this study, a water sample was collected from a freshwater pond for Pacific white shrimp (Litopenaeus vannamei) culture located in the Pingtung countryside in
southern Taiwan. The temperature of the pond water was about 25 °C and the pH was approximately 7. The selected medium for isolation of chitin-degrading bacterium was enriched with 100 ml sample water with 0.5 % (w/v) autoclaved colloidal chitin. The initial culture was incubated at 25 °C with agitation at 125 r.p.m. Colloidal chitin was prepared from commercial chitin (Ohka Chemical) as described by Chang et al. (2004). After 5 days of incubation, the enrichment broth was diluted with sterile distilled water and spread onto chitin basal (CB) medium (Chang et al., 2004). A bacterial strain that showed large clear zones around the colonies resulting from chitin degradation was isolated and designated c14T. The colony morphology of strain c14T was clearly different compared with that of Chitinimonas taiwanensis cfT (Chang et al., 2004). Consequently, the organism was subjected to a polyphasic taxonomic study that demonstrated its status as a member of a novel genus and species.

Bacterial cells were observed by phase-contrast microscopy (Leica DM 2000) in the lag, exponential and stationary phases of growth to ascertain their morphology. The motility of cells was examined by the hanging drop method. Flagellum staining was performed using the Spot Test flagella stain (BD Difco). Gram staining was performed using the Gram stain set S (BD Difco) and the Ryu non-staining KOH method (Powers, 1995). Poly-β-hydroxybutyrate (PHB) granule accumulation was observed by light microscopy after Sudan black staining. For scanning electron microscopy observations, specimens were fixed in 25 % glutaraldehyde in phosphate buffer (pH 7) for 20 min and then washed with phosphate buffer. Secondary fixation was done with 4 % osmium tetroxide in phosphate buffer for 40 min, again followed by a buffer rinse. After the secondary fix, dehydration was accomplished through a series of ethanol solutions (50, 70, 80, 90, 95 and 100 %); each change was held for 10 min. The specimens were then critical-point dried (Hitachi E-1010) and examined with a digital scanning electron microscope (Hitachi S3500N).

The pH range for growth was determined by measuring optical densities (wavelength 595 nm) of the culture grown in tryptic soy broth (TSB; BD Difco). The pH of the medium was adjusted to pH 3–11 at intervals of 1.0 pH unit by using appropriate biological buffers: glycine/HCl, citrate/Na₂HPO₄, phosphate buffer and glycine/NaOH were used for pH values below 4, 4.0–8.0, 6.0–8.0 and 9.0–11.0, respectively. For the determination of the temperature range for growth, cells were incubated in CB broth at temperatures between 4 and 50 °C (4 °C and 10 to 50 °C at intervals of 5 °C) using an orbital water-bath shaker (125 r.p.m.). Tolerance of NaCl was determined by adjusting the salinity of CB medium to values between 0 and 3.0 % NaCl (w/v) (0, 0.25, 0.3, 0.5, 0.75, 1.2 and 3 %). Anaerobic cultivation was performed on CB and R2A media in the Oxoid AnaeroGen system. Culture supernatant of strain c14T was used to detect chitinolytic production by HPLC and by the hydrolysis of fluorogenic substrate analogues [(GlcNAc)ₙ, where n=1–6; Sigma] as described previously (Chang et al., 2004).

Light microscope investigations revealed that cells of strain c14T were Gram-negative, rod-shaped, 0.3–0.4 μm wide and 2.5–4.0 μm long, and contained PHB granules. Cells were motile by means of single polar flagella. A scanning electron micrograph of strain c14T is shown in Fig. 1.

Strain c14T formed visible colonies (approx. 1.0–1.5 mm in diameter) on CB agar after 3 days of incubation at 35 °C. Colonies were cream–white in colour, circular and convex with entire edges. Surrounding the colonies, a colourless clear zone was observed that extended to more than double the colony diameter, indicating that chitinolysis was performed by strain c14T during growth. With a medium containing only minerals and chitin, strain c14T still exhibited a reasonable growth rate under both aerobic and anaerobic conditions, indicating that the organism could use chitin as a sole carbon, nitrogen and energy source for growth. In addition, vitamins and yeast extract were not needed as supplements for growth in mineral medium. Strain c14T could also be grown on nutrient and tryptic soy media. Strain c14T grew at temperatures ranging from 15 to 40 °C, pH values between 7 and 11 and NaCl concentrations between 0 and 0.75 %. Optimal growth conditions were 30–35 °C, pH 8 and 0.25 % NaCl. When the chitinolytic products were examined by HPLC and hydrolysis of fluorogenic substrates, the main product that remained in the c14T culture medium was a disaccharide [chitodiose; (GlcNAc)₂], as shown in Supplementary Fig. S1, available in IJSEM Online. Clearly, strain c14T could be distinguished from Chitinimonas taiwanensis cfT, which produced chitotriose [(GlcNAc)₃] (Chang et al., 2004), and Chitinibacter taiwanensis S1T, which produced GlcNAc monosaccharides (Chern et al., 2004), as the major products of chitin cleavage.

Extraction of genomic DNA and PCR amplification and sequencing of the 16S rRNA gene were carried out as

![Fig. 1. Electron micrograph of cells of strain c14T. Bar, 1 μm.](http://ijs.sgmjournals.org)
described previously (Chen et al., 2001). Sequence reaction fragments were separated using a DNA sequencer (ABI PRISM 310 instrument; Applied Biosystems) and sequence assembly was done by using the Fragment Assembly System program from the Wisconsin Package 9.1 (Genetics Computer Group, 1995). The nearly complete 16S rRNA gene sequence of strain c14T was compared with corresponding sequences taken from the GenBank and Ribosomal Database Project II databases. Multiple-sequence alignment including strain c14T and its closest relatives was achieved using the BioEdit program (Hall, 1999) and MEGA version 3.1 (Kumar et al., 2004). Phylogenetic reconstruction was inferred by using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms. An evolutionary distance matrix was generated for the neighbour-joining algorithm using the distance model of Jukes & Cantor (1969) and bootstrap analysis (1000 resamplings).

A nearly complete 16S rRNA gene sequence (1455 nt) of strain c14T was obtained and revealed that strain c14T belonged to the Betaproteobacteria. A phylogenetic tree inferred on the basis of evolutionary distances by using the neighbour-joining method (Fig. 2) indicated that strain c14T formed a distinct lineage within the family Neisseriaceae. Sequence similarity calculations using pairwise alignment obtained from the EzTaxon database (Chun et al., 2007) showed the greatest degree of similarity to Chitinibacter tainanensis S1T (94.6 % similarity) (Chern et al., 2004), Deefgea rivuli WB 3.4-79T (93.3 %) (Stackebrandt et al., 2007), Silvimonas terrae KM-45T (92.9 %) (Yang et al., 2005), Formivibrio citricus DSM 6150T (92.4 %) (Hippe et al., 1999), Iodobacter fluviatilis ATCC 33051T (92.2 %) (Logan, 1989) and Chitinimonas taiwanensis cfT (92.0 %) (Chang et al., 2004). The sequence similarity with other species belonging to the Betaproteobacteria was less than 92 %. The overall topologies of the phylogenetic trees obtained with the neighbour-joining, maximum-likelihood and maximum-parsimony methods were similar.

Fluorometric DNA–DNA hybridization experiments were performed with photobiotin-labelled probes as described by Ezaki et al. (1989). Hybridization was conducted in 50 % formamide at 50 °C. Each experiment was done in triplicate and the data are displayed as means of triplicate experiments. Strain c14T showed relatively low DNA–DNA hybridization with its closest phylogenetic neighbour, Chitinibacter tainanensis BCRC 17254T (21 ± 3 %).

A DNA sample was prepared and degraded enzymically into nucleosides as described by Moshbah et al. (1989). The nucleoside mixture obtained was then separated by reversed-phase HPLC equipped with a Cosmosil 5C18 column (Waters). Non-methylated lambda phage DNA (Sigma) was used as the calibration reference. Each experiment was conducted in triplicate and the data are displayed as the mean of triplicate experiments. The G + C content of strain c14T was 69.5 (± 1.0) mol%.

Cellular fatty acids were analysed in cells grown on TSB agar for 2 days. The cellular fatty acids were saponified and degraded enzymically into nucleosides as described by Moshbah et al. (1989). The nucleoside mixture obtained was then separated by reversed-phase HPLC equipped with a Cosmosil 5C18 column (Waters). Non-methylated lambda phage DNA (Sigma) was used as the calibration reference. Each experiment was conducted in triplicate and the data are displayed as the mean of triplicate experiments. The G + C content of strain c14T was 69.5 (± 1.0) mol%.

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Fig. 2. Neighbour-joining phylogenetic tree of strain c14T and its relatives in the Betaproteobacteria based on 16S rRNA gene sequence comparisons. Bar, 1 % sequence dissimilarity (1 substitution per 100 nucleotide positions). Bootstrap values (%) are indicated at branches from 1000 resamplings; only values > 50 % are shown. GenBank accession numbers are shown in parentheses. Filled circles indicate generic branches recovered by using maximum-parsimony.
methylated and then extracted according to the standard protocol of the Microbial Identification System (Sasser, 1990; MIDI Inc., 1999). The fatty acids analysed by GC (Hewlett Packard 6890) were identified by the Microbial Identification software package. The major cellular fatty acids of strain c14T were C16:0, C18:1ω7c and summed feature 3 (C16:1ω7c and/or C15:0 iso2-OH). The detailed fatty acid compositions of strain c14T and its close phylogenetic neighbours are shown in Table 1. The fatty acid profile of strain c14T was similar to those of Chitinibacter tainanensis S1T, S. terrae KM-45T, D. rivuli WB 3.4-79T and F. citricus DSM 6150T, which all contained predominantly C16:0, C18:1ω7c and summed feature 3, but differed in the proportions of fatty acids (Table 1).

Strain c14T was characterized biochemically by using Biolog GN2 and API 20NE and API ZYM (bioMérieux) microtest systems according to the manufacturers’ instructions. Catalase activity was determined by bubble production in a 10% (v/v) H2O2 solution. Oxidase activity was determined on filter paper moistened with a 1% (w/v) aqueous solution of N,N,N',N'-tetrathymethyl-p-phenylene-diamine. Strain c14T was also examined for a broad range of phenotypic properties using conventional methods (MacFaddin, 2000). Susceptibility to antimicrobial agents was determined by a disc diffusion assay. The cell suspension was diluted to 0.5 McFarland standard in sterile saline after reaching the exponential growth phase and then spread onto CB medium and incubated at 35°C. Antimicrobial discs (Difco) used in this experiment separately contained amikacin (30 μg), ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), novobiocin (30 μg), rifampicin (5 μg), penicillin G (10 U), streptomycin (10 μg) and tetracycline (30 μg). The effect of antibiotics on cell growth was assessed after 3 days of incubation at 35°C and susceptibility was scored based on the distance from the edge of the clear zone to that of the disc (>3 mm, susceptible; 1–3 mm, moderately susceptible; <1 mm, resistant).

The differences in phenotypic and biochemical characteristics between strain c14T and related phylogenetic neighbours are summarized in Table 2. Strain c14T can be clearly distinguished from Chitinibacter tainanensis S1T by the abilities to grow anaerobically, reduce nitrate, ferment glucose, hydrolyse gelatin and assimilate mannose. Differential phenotypic characteristics between strain c14T and S. terrae KM-45T include that strain c14T can hydrolyse gelatin and lacks activities of β-galactosidase and β-glucosidase. Strain c14T can be clearly distinguished from D. rivuli WB 3.4-79T by its abilities to ferment glucose, hydrolyse chitin, grow at 40°C and hydrolyse gelatin. While strain c14T is facultatively anaerobic, F. citricus DSM 6150T was described as strictly anaerobic (Tanaka et al., 1991).

It is now generally accepted that bacteria with a G+C content difference of more than 10 mol% should not be classified within the same genus (Wayne et al., 1987). Also, 16S rRNA sequence similarities between two bacteria of less than 95% are an indication of affiliation to different genera (Ludwig et al., 1998). Strain c14T most likely represents a species of a new genus, since the 16S rRNA gene sequence similarity to its closest relatives with validly published names, Chitinibacter tainanensis S1T, S. terrae KM-45T and D. rivuli WB 3.4-79T, is 94.4, 92.7 and 92.6%, respectively. The G+C content of c14T (69.5 mol%) is also significantly higher than those of Chitinibacter tainanensis S1T (56 mol%), S. terrae KM-45T (58 mol%) and D. rivuli WB 3.4-79T (48.5 mol%). Moreover, strain c14T can be

### Table 1. Comparison of fatty acid profiles of strain c14T and its phylogenetically closest relatives

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12:0</td>
<td>2.8</td>
<td>2.6</td>
<td>3.6</td>
<td>2.7</td>
<td>5.3</td>
</tr>
<tr>
<td>C12:0 3-OH</td>
<td>2.6</td>
<td>3.9</td>
<td>-</td>
<td>2.5</td>
<td>-</td>
</tr>
<tr>
<td>C14:1ω5c</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C14:1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.2</td>
</tr>
<tr>
<td>C14:0</td>
<td>2.5</td>
<td>0.5</td>
<td>6.4</td>
<td>2.0</td>
<td>1.6</td>
</tr>
<tr>
<td>C15:1ω8c</td>
<td>-</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C15:1ω6c</td>
<td>-</td>
<td>0.3</td>
<td>-</td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td>C15:0</td>
<td>-</td>
<td>0.8</td>
<td>-</td>
<td>2.9</td>
<td>-</td>
</tr>
<tr>
<td>C16:1ω5c</td>
<td>0.7</td>
<td>0.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C16:0</td>
<td>23.0</td>
<td>16.7</td>
<td>33.5</td>
<td>20.7</td>
<td>11.9</td>
</tr>
<tr>
<td>C16:0 3-OH</td>
<td>0.3</td>
<td>1.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>C17:0 cyclo</td>
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<td>0.3</td>
<td>15.3</td>
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<tr>
<td>C17:1ω6c</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>C17:0</td>
<td>-</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C18:1ω7c</td>
<td>23.3</td>
<td>19.9</td>
<td>14.7</td>
<td>3.4</td>
<td>10.8</td>
</tr>
<tr>
<td>C18:1ω5c</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C18:0</td>
<td>0.1</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>11-Methyl C18:1ω7c</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>10-Methyl C18:0</td>
<td>2.4</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Summed feature 3*</td>
<td>41.3</td>
<td>48.6</td>
<td>21.8</td>
<td>60.1</td>
<td>57.7</td>
</tr>
<tr>
<td>Summed feature 4*</td>
<td>4.3</td>
<td>-</td>
<td>3.4</td>
<td>2.0</td>
<td>9.3</td>
</tr>
</tbody>
</table>

*Summed feature 3 comprises C16:1ω7c and/or C15:0 iso 2-OH; summed feature 4 comprises C14:0 3-OH and/or C16:1 iso I.

Ubiquinone 8 (Q8) was the only isoprenoid quinone present in strain c14T.
readily distinguished from its nearest phylogenetic neighbours, *Chitinibacter tainanensis* S1\(^T\), *S. terrae* KM-45\(^T\), *D. rivuli* WB 3.4-79\(^T\) and *F. citricus* DSM 6150\(^T\), by fatty acid compositions and phenotypic and biochemical characteristics. Therefore, based on phenotypic and phylogenetic criteria, we are of the opinion that strain c14\(^T\) isolated from surface water of a freshwater pond for shrimp culture should be assigned to a novel species of a new genus, for which the name *Chitinilyticum* gen. nov., sp. nov. is proposed.

**Description of Chitinilyticum gen. nov.**

*Chitinilyticum* (Chi.ti.ni.ly’ti.cum. N.L. n. chitinum chitin; N.L. adj. lyticus -a -um dissolving, used as subst. dissolver; N.L. neut. n. *Chitinilyticum* chitin-dissolver).

Cells are Gram-negative rods, motile by means of single polar flagella. PHB granules are stored as reserve material. Endospores are not formed. Colonies on CB medium display a large clear zone around them, which is derived from degradation of chitin. No diffusible pigments are produced. Grow well by using chitin as the sole carbon, showing a distant relatedness to *Chitinibacter tainanensis*, *Silvimonas terrae*, *Deegrea rivuli* and *Formivibrio citricus*. The type species is *Chitinilyticum aquatile*.

**Description of Chitinilyticum aquatile sp. nov.**

*Chitinilyticum aquatile* (L. neut. adj. *aquatile* living in water).

Displays the following properties in addition to those given in the genus description. Cells are 2.5–4.0 \(\mu\)m long and 0.3–0.4 \(\mu\)m in diameter, occurring singly. Growth occurs at 15–40 °C, pH 7–11 and 0–0.75 % NaCl. Positive for glucose fermentation. Negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H\(_2\)S production (triple-sugar iron agar and sulfide indole motility medium), acetoin production (Voges–Proskauer test) and fermentation of mannitol, sucrose, arabinose and rhamnose. API 20NE tests show positive reactions for nitrate reduction, glucose fermentation, gelatin hydrolysis, assimilation of glucose, mannose, N-acetyl-D-glucosamine and gluconate and negative reactions for indole production, arginine dihydrolase, urease, aesculin hydrolysis, \(\beta\)-galactosidase and assimilation of arabinose, mannitol, maltose, caprate, adipate, malate, citrate and phenylacetate. In API ZYM tests, shows positive results for alkaline phosphatase, C4 esterase, C8 lipase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl-\(\beta\)-glucosaminidase and negative reactions for \(\alpha\)-glucosidase,

### Table 2. Difference in phenotypic and biochemical characteristics between strain c14\(^T\) and its phylogenetically closest relatives

<table>
<thead>
<tr>
<th>Characteristic</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Freshwater</td>
<td>Soil</td>
<td>Soil</td>
<td>Hard-water creek</td>
<td>Anoxic freshwater mud</td>
</tr>
<tr>
<td>Morphology</td>
<td>Rods, straight to slightly curved</td>
<td>Facultatively anaerobic</td>
<td>Rods, straight to slightly curved</td>
<td>Rods, single or in pairs</td>
<td>Curved rods, single or in pairs</td>
</tr>
<tr>
<td>Relation to O(_2)</td>
<td>Catalase</td>
<td>Chitin hydrolysis</td>
<td>Growth at 40 °C</td>
<td>API 20NE results</td>
<td>DNA G+C content (mol%)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>69.5</td>
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<td>+</td>
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<td>+</td>
<td>61</td>
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</table>
β-glucuronidase, α-galactosidase, β-galactosidase, β-glucosidase, C14 lipase, valine arylamidase, cystine arylamidase, α-mannosidase, α-fucosidase, trypsin and α-chymotrypsin. The following carbon sources are oxidized (Biolog GN2): dextrin, glucose, D-fructose, sucrose, cellobiose, mannose, trehalose, turanose, gluconate, D-glucuronic acid, acetic acid, α-ketobutyric acid, α-ketovaleric acid, DL-lactate, N-acetyl-D-glucosamine, α-hydroxybutyric acid, glucose 6-phosphate and L-alanine. The following substrates are not oxidized: trehalose, turanose, gluconate, D-glucuronic acid, acetic acid, α-ketobutyric acid, α-ketovaleric acid, α-hydroxybutyric acid, glucose 6-phosphate, α-mannosidase, α-fucosidase, trypsin and α-chymotrypsin.

Acknowledgements

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citramalate, citrate, mesaconate, and pyruvate by a gram-negative
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