Taxonomic characterization of
Ketogulonigenium vulgare gen. nov., sp. nov.
and Ketogulonigenium robustum sp. nov.,
which oxidize L-sorbose to 2-keto-L-gulonic acid

J. W. Urbance,1 B. J. Bratina,1† S. F. Stoddard2 and T. M. Schmidt1

Author for correspondence: T. M. Schmidt. Tel: +1 517 353 1796. Fax: +1 517 353 8957.
e-mail: tschmidt@msu.edu

Four bacterial strains that oxidize L-sorbose to 2-keto-L-gulonic acid, a key intermediate in the synthesis of vitamin C, were isolated from soils of geographically distinct locations. All were Gram-negative, facultatively anaerobic, chemoheterotrophic rods. Comparative analysis revealed nearly identical 16S rDNA sequences amongst them (99.7–100% identical) and identified them as members of the α subclass of the Proteobacteria. Phylogenetic analysis identified the closest taxonomically defined genus as Roseobacter (92.1–92.8% identical). On the basis of phylogenetic, phenotypic and genotypic analyses, a new genus is proposed, Ketogulonigenium gen. nov. Based upon these analyses, we also propose the reclassification of strain DSM 4025T, originally identified as Gluconobacter oxydans, to the genus Ketogulonigenium. Two species are proposed: the type species Ketogulonigenium vulgare gen. nov., sp. nov., consisting of strains 62A-12A, 266-13B and the type strain K. vulgare DSM 4025T, and Ketogulonigenium robustum gen. nov., sp. nov., consisting of the type strain K. robustum X6LT (= NRRL B-21627 = KCTC 0858BP). The species affiliation of the fifth strain (291-19p) remains unresolved.

Keywords: Ketogulonigenium, 2-keto-L-gulonic acid, ascorbic acid, vitamin C, Gluconobacter

INTRODUCTION

L-Ascorbic acid (vitamin C) can be synthesized chemically from D-glucose by the method of Reichstein & Grussner (1934). This method involves many steps, and on a commercial scale requires large amounts of environmentally toxic solvents. Alternatively, the exploitation of micro-organisms to synthesize vitamin C or vitamin C precursors has both economic and ecological advantages. A number of axenic and mixed-culture microbial processes have been described for the biosynthesis of various intermediates in L-ascorbic acid synthesis (Tengerdy, 1961a, b; Imai et al., 1990; Sugisawa et al., 1990; Yin et al., 1990; Nogami et al., 1995). One key intermediate in vitamin C synthesis is 2-keto-L-gulonic acid (2-KLG), which can be converted chemically to L-ascorbic acid by esterification followed by lactonization (Delić et al., 1989). Members of a number of bacterial genera have been identified that produce 2-KLG from the oxidation of L-sorbose (another intermediate of vitamin C synthesis). These include the acidophilic, γ-Proteobacteria Gluconobacter and Acetobacter, the γ-Proteobacteria Pseudomonas, Escherichia, Klebsiella, Serratia and Xanthomonas, the Gram-positive Bacillus and Micrococcus (Imai et al., 1990; Sugisawa et al., 1990; Yin et al., 1990; Nogami et al., 1995), and the genus ‘Pseudoglucono-bacter’ (Nogami et al., 1995). However, the yield of 2-KLG from these organisms is generally too low to be exploited industrially (for a review of vitamin C chemistry, natural biosynthesis and industrial synthesis, see Delić et al., 1989).

To search for naturally occurring micro-organisms capable of more efficient conversion of L-sorbose to 2-KLG, samples from a range of environments were used as inocula for media rich in L-sorbose. Those

1 Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824-1101, USA
2 Archer Daniels Midland Company, Decatur, IL 62526, USA
mixed-culture enrichments that demonstrated 2-KLG production were then screened for 2-KLG-producing isolates. We report here the polyphasic characterization of four isolates, representing a new, non-acidophilic genus of *z-Proteobacteria* that is characterized by efficient production of 2-KLG from *t*-sorbose, *Ketogulonigenium*, and the reclassification of patent strain DSM 4025\textsuperscript{T}, previously identified as *Glucobacter oxydans* (Yin *et al.*, 1990). Two species, *Ketogulonigenium vulgar* and *Ketogulonigenium robustum*, are proposed.

**METHODS**

**Enrichment and isolation.** Samples were taken of moist soil, sand, sediment, fruit, berries, humus and other environmental habitats from various regions of the midwestern, southern and southeastern United States. Following collection, each sample was immediately stored in a cool, ventilated and humidified container. For primary enrichment of *t*-sorbose-utilizing organisms, approximately 1 g of sample was added to 30 ml Enrichment Medium A [EM-A; 20 g *t*-sorbose l\(^{-1}\), 2 g glucose l\(^{-1}\), 0.1 g FeSO\(_4\) \(\cdot\) 7H\(_2\)O l\(^{-1}\), 1.7 g Yeast Nitrogen Base (Difco) l\(^{-1}\), 6 g CaCO\(_3\) l\(^{-1}\), 5 mg cycloheximide l\(^{-1}\), and 10 g corn steep liquor solids l\(^{-1}\)]. pH 7.2 in a baffled 250 ml flask and incubated with shaking (200–240 r.p.m.) for 2 d at 28–30 °C. Secondary enrichments were established by using 0.50–0.75 ml of the primary enrichments to inoculate 30 ml of Enrichment Medium B (EM-B; same composition as EM-A except with 50 g *t*-sorbose l\(^{-1}\) and no glucose). These were incubated as described above for 3–4 d, after which portions were analysed for 2-KLG production and cryogenically preserved. For cryogenic preservation, 2 ml broth culture was added to 1 ml sterile 40% (w/v) glycerol and stored at −70 °C.

Culture media were screened for the presence of 2-KLG using TLC on Whatman LK5 Silica Gel 150 plates (0.25 mm thick). The plates were spotted with 5 µl centrifuged broth culture and developed for 5–6 h in solvent (157 ml n-propanol, 39 ml deionized water, 4 ml 1% (v/v) phosphoric acid and 0.4 ml glacial acetic acid). The plates were air-dried, sprayed with a solution of tetrazolium blue chloride (0.125 g dissolved in 25 ml methanol and 25 ml 6 M NaOH) and then baked at 60 °C for 5 min. *t*-Sorbose and 2-KLG were visualized as purple spots on the finished plates and identified by comparison with a standard containing 10 g l\(^{-1}\) each of *t*-sorbose and 2-KLG.

Production of 2-KLG was quantified by HPLC. Samples were prepared by 1:10 dilution in a solvent mobile phase (1:1 ml ACS grade sulfuric acid diluted to 4 l using Milli-Q water), followed by filtration through a 0.45 µm porous membrane. Samples of 100 µl each were loaded onto two 2 mm × 300 mm × 7.8 mm Aminex HPX-87H columns (Bio-Rad) arranged in series to provide a total column length of 600 mm, preceded by a guard column of the same resin. The column was run at 55 °C at a flow rate of 0.6 ml min\(^{-1}\). *t*-Sorbose and 2-KLG were detected using a Waters model no. 410 differential refractometer, and were identified by comparison with a standard containing 2-KLG and *t*-sorbose.

Mixed-culture enrichments that produced 2-KLG were then screened for 2-KLG-producing organisms. The frozen, glycerol stocks were serially diluted in EM-A and used to inoculate spread plates of EM-A agar. Plates were incubated at 30 °C for 24 h and colonies were picked after examination under ×8 and ×40 magnification. The plates were then incubated for an additional 24 h and re-examined for slower growing colonies. The individual colonies were transferred to EM-A agar for isolation. Several colonies of each type and size were subcultured. Each strain was further streaked for isolation one to three times on either EM-A or PYM plates (10 g peptone l\(^{-1}\), 10 g yeast extract l\(^{-1}\), 0.5 g glycerol l\(^{-1}\), 30 g mannitol l\(^{-1}\), 20 g agar l\(^{-1}\)). Pure cultures were then cryogenically preserved in PYM broth with 20% glycerol at −70 °C and screened for their ability to convert *t*-sorbose to 2-KLG. To account for the possibility that 2-KLG production might require combined activity of two or more micro-organisms, each new isolate was tested in pairwise combination with all strains originating from the same enrichment, as well as in pure culture. Each strain or strain pair was grown in 30 ml modified EM-B (15 g corn steep liquor solids l\(^{-1}\), no cycloheximide) in 250 ml shake flasks at 30 °C for 3 d and then screened for 2-KLG production using TLC and HPLC as described above.

**Maintenance media and growth conditions.** The four new *Ketogulonigenium* isolates and strain DSM 4025\textsuperscript{T} were maintained on either Trypticase Soy Agar (TSA; Difco), Trypticase Soy Broth (TSB; Difco), or a complex medium [10 g Soytone (Difco) l\(^{-1}\), 10 g yeast extract l\(^{-1}\), 20 g mannitol l\(^{-1}\), 5 g NaCl l\(^{-1}\), 2.5 g K\(_2\)HPO\(_4\) l\(^{-1}\), 2% equine serum and 1.5% agar] designated here as SYM agar. Stock cultures were stored at −80 °C in TSB supplemented with 25% glycerol. *Roseobacter* strains were maintained on Marine Agar 2216 (MA; Difco) or Marine Broth 2216 (MB; Difco) and stored at −80 °C in MB supplemented with 25% glycerol.

Characterization of isolates was used for modifications of a defined basal medium designated here as DM (amounts are in mg l\(^{-1}\) (yeast extract, 500; mannitol, 10000; 21 amino acids (includes hydroxyproline: 100 each), bases (adenine, uracil, guanine, thymine, cytosine: 100 each), MgCl\(_2\) \(\cdot\) 6H\(_2\)O, 1000; Na\(_2\)SO\(_4\), 100; NaCl, 5000; KCl, 350; K\(_2\)HPO\(_4\), 140; Ca\(_2\)Cl\(_2\) \(\cdot\) 2H\(_2\)O, 75; NaBr, 50; H\(_3\)BO\(_3\), 20; Na\(_2\)WO\(_4\) \(\cdot\) 2H\(_2\)O, 3; nitritriacetate, 1.5; FeSO\(_4\) \(\cdot\) 7H\(_2\)O, 14; CuSO\(_4\) \(\cdot\) 5H\(_2\)O, 1; MnSO\(_4\) \(\cdot\) H\(_2\)O, 0.305; CoCl\(_2\), 6H\(_2\)O, 0.305; NiCl\(_2\), 6H\(_2\)O, 0.20; Na\(_2\)SO\(_4\) \(\cdot\) 2H\(_2\)O, 0.001; pyridoxine \(\cdot\) HCl, 0.10; p-amino-benzoic acid, 0.05; nicotinic acid, 0.05; dl-calcium pantothenate, 0.05; thiamin \(\cdot\) HCl, 0.05; dl-6,8-thiathionic acid, 0.05; riboflavin, 0.04; biotin, 0.02; folic acid, 0.02; vitamin B\(_6\), 0.001; 250 mM MOPS; pH 7.2, filter-sterilized) or TSB as described below. The ability to grow anaerobically was tested in 20 ml serum vials containing 10 ml anoxic TSB supplemented with 1 g glucose l\(^{-1}\) (final glucose concentration was 19 mM). Motility was tested using an inoculating needle to stab plates of TSB containing 0.2% agarose. Unless otherwise stated, the pH of all media was 7.2–7.5, all cultures were incubated at 28–30 °C and broth cultures were incubated on rotary shakers (200–240 r.p.m.). All characterizations were performed on cultures that were transferred no more than twice from frozen stocks.

The newly isolated strains have been deposited in the USDA Agricultural Research Service Culture Collection with the following accession numbers: NRRL B-21627 (strain X6L\textsuperscript{pp}), NRRL B-30035 (291-19\textsuperscript{pp}), NRRL B-30036 (266-13B\textsuperscript{pp}), NRRL B30037 (62A-12A\textsuperscript{pp}). Strain X6L\textsuperscript{pp} was also deposited in the Korean Collection for Type Cultures under the accession number KCTC 0858BP.
Phenotypic characterization

Colony and cell morphology. Colony morphology was evaluated after 48 h incubation on TSA. Cultures used for light microscopy were grown on either TSB or DM. For visualization of flagella, cells were harvested from motility plates by stabbing the edge of the zone of motile growth with a sterile Pasteur pipette and gently transferring the plug of agarose to a Formvar grid coated lightly with carbon. After 1–2 min, the agarose plug was wicked off from the side of the grid using filter paper and the grid was then negative-stained for 1 min with a solution of 1% (w/v) phosphotungstic acid and 0.01% BSA (pH 6), after which the excess stain was also wicked off. Samples used for preparing thin sections were grown in TSB. Aliquots of the broth cultures were collected at 24 and 60 h and fixed by incubating them overnight in paraformaldehyde and glutaraldehyde [final concentration 2% (w/v) each] at 4°C. The fixed cells were washed three times in fresh TSB, stained for 1 h at room temperature with TSB containing 1% (w/v) osmium tetroxide, and then washed three times in double-distilled water. After centrifugation, the cell pellets were dehydrated in a graded ethanol series, washed three times with 100% acetone and infiltrated at room temperature with acetone/EPON resin (Embed 812, Electron Microscopy Sciences) mixtures. Thin sections were made using a Reichert Ultracut S ultramicrotome and counterstained with 10% (w/v) uranyl acetate in methanol (10 min) followed by Sato’s lead stain (5 min). Images were viewed and collected using a JEOL 1200EX scanning and transmission electron microscope (STEM).

Biochemical tests. Cytochrome oxidase was tested colorimetrically using a commercially available system (Dryslide; Difco) and catalase activity was tested for using H2O2 as described by Koneman et al. (1979).

Carbon sources. Carbon utilization was tested using a base medium of modified DM with reduced yeast extract (0.1 g l−1), amino acids (0.02 g l−1), bases (0.01 g l−1) and no mannitol. This medium supported growth of the isolates to very low cell yields. Carbon sources were added to the base medium at 20 g l−1. Inocula for carbon utilization tests were grown overnight in the base medium. An aliquot (100 μl) of these overnight cultures was used to inoculate tubes containing 6 ml base medium plus a carbon source (each carbon source was tested in triplicate). Growth was monitored visually at regular intervals over 72 h and compared to growth in the base medium without added carbon source.

Temperature range and optima. Duplicate 250 ml flasks with 50 ml TSB at the required growth temperature were inoculated with an overnight culture (grown in TSB) to a final OD660 of 0.002–0.007. Growth of the isolates was monitored by measuring the OD660 in samples taken at regular intervals (1–4 h, depending on the growth rate) until the cultures reached stationary phase. Growth rate determinations were based upon a minimum of four OD measurements taken during exponential growth. Growth rates were determined at 4, 10, 15, 25, 28, 30 and 37 °C.

pH range and optima. Inocula for pH experiments were grown in unbuffered DM to mid-exponential phase. Aliquots of 125 μl were used to inoculate duplicate test tubes containing 6 ml DM buffered with either 250 mM MES (pH 6.0–6.5) or 250 mM MOPS (pH 6.5–8.5). The range of pHs tested was 6.0–8.5 at intervals of 0.25 pH units, and pH 4.5. Growth was monitored visually at regular intervals over 72 h.

Salt tolerance. Inocula for salt tolerance experiments were grown to mid-exponential phase in modified DM (0% added NaCl, unbuffered). Aliquots of 125 μl were used for inoculation. Salt tolerance was tested in duplicate test tubes containing 6 ml DM with the following modifications: NaCl concentration was varied from 0 to 4% at 0.5% intervals and the MOPS concentration was reduced to 25 mM to reduce the Na⁺ concentration of the basal medium to 31.2 mM. OD660 was measured at regular intervals using a Spec20 (Milton Roy).

BIOLOG metabolic experiments. Each isolate was grown in 20 ml TSB to an OD660 of 1.0 or greater. The cells were harvested by centrifugation at 10,000 g and resuspended in 20 ml unbuffered saline (0.85% NaCl). BIOLOG GN MicroPlates were inoculated using 150 μl of the cell suspension per well. Plates were sealed with Parafilm and incubated at 30°C. Metabolic activity in the wells was measured as a change in OD590 using a EL 312e microplate reader (Bio-Tek Industries). Measurements were taken immediately after inoculating the wells and approximately 2, 4 and 6 h following inoculation. The capacity for substrate oxidation was scored based on optical density readings after 6 h. The results were scored as negative (OD590 < 0.01), weakly positive (OD590 0.01–0.5) or positive (OD590 > 0.5).

Cellular fatty acids. Fatty acid profiles were quantified and identified by comparison to a commercial database, the Sherlock Microbial Identification System (MIS) by Microbial ID (MIDI) using the manufacturer’s protocols. Isolate identifications were attempted by comparison to the MIDI TSBA (version 3.9) microbial database. The isolates were compared to the library using covariance matrix, principal component analysis and pattern recognition software.

MIC testing. The MICs of selected antibiotics were determined using a microbroth dilution technique in accordance with the procedure described in the National Committee for Clinical Laboratory Standards (NCCLS) M31-T document. This procedure involved using a commercially available microtitre tray (Pasco Laboratories). Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 29213 were used as standards for quality control. The microtitre trays were incubated at 37°C for 24–48 h. The MIC for each drug/bacterium combination was recorded as the lowest concentration of the drug that completely inhibited the growth of the bacterial isolates as determined visually. In some cases, antibiotics were tested in combination to reflect common clinical usage.

Genotypic characterization

DNA preparation. Cells harvested from TSB cultures were suspended in 10 ml TE, pH 8 (Sambrook et al., 1989a) containing 0.5% SDS and 0.1 mg proteinase K l−1, and lysed by incubation at 37 °C for 1 h. Nucleic acids were purified by three extractions with TE-saturated phenol (pH 7), three extractions with TE-saturated phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and two extractions with chloroform, followed by ethanol precipitation. Nucleic acid precipitants were twice washed with 80% ethanol and redissolved in TE pH 8. Chromosomal and plasmid DNAs were further purified and separated by centrifugation in CsCl gradients (Sambrook et al., 1989b). After harvesting the DNA bands from the gradients, excess ethidium bromide was removed by repeated water-saturated butanol extraction and the final DNA preparations purified using Centricron 30 columns (Amicon) as per the manufacturer’s recommendations. The concentration and purity of DNA samples were quantified spectrophotometrically. Agarose gel electro-
analysed on an ABI 373a DNA sequencer. A collection of 12 Elmer 9600 thermal cycler using ABI dye terminator

amplified product was purified using Millipore MWCO

cycling profiles were as described by Eden

mendation. Cycle sequencing was performed on a Perkin

columns (Ultrafree-MC) as per the manufacturer's recom-

primer corresponded to positions 8–27 of

regions of bacterial small subunit rDNA. The forward

primer (rDNA) were amplified by PCR from genomic DNA using

hybridization studies (below).

DNA–DNA hybridization. Genomic DNA similarities were
determined by a membrane hybridization method. DNA
was immobilized on nylon membrane (MSI) using a slot-

sheared by 20 passages through a 30 gauge needle, denatured

was immobilized on nylon membrane (MSI) using a slot-

determined by this analysis was subsequently used to calculate

hybridization temperature used in the DNA–DNA

Genomic DNA similarities were
determined by the same method (Breznak & Canale-Parola, 1975), it was used as a reference to verify the system. The
genomic denaturation temperature (T\text{\text{D}}) determined by this analysis was subsequently used to calculate the

DNA–DNA hybridization temperature used in the DNA–DNA

hybridization studies (below).

Phylogenetic analysis. The 16S-rRNA-encoding genes
(rDNA) were amplified by PCR from genomic DNA using

oligonucleotide primers designed to anneal to conserved regions of bacterial small subunit rDNA. The forward
primer corresponded to positions 8–27 of E. coli 16S rRNA,

while the reverse primer corresponded to positions 1492–1510 (Eden et al., 1991). Reaction conditions and
cycling profiles were as described by Eden et al. (1991). The

amplified product was purified using Millipore MWCO

columns (Ultrafree-MC) as per the manufacturer's recommen-
dation. Cycle sequencing was performed on a Perkin

Elmer 9600 thermal cycler using ABI dye terminator chemistry (PE Applied Biosystems) and products were
analysed on an ABI 373A DNA sequencer. A collection of 12
primers (MicroSeq 16SrRNA Gene kit; PE Applied Bio-
systems) was used for sequencing, which provided an average
redundancy of 3:0 per nucleotide position. Consensus
sequences generated from assembled contigs were used for
analysis. Sequences generated in the present study were
deposited in GenBank (accession numbers AF136846-
AF136850).

The consensus sequences were aligned against the most

similar sequences in the ARB small subunit rRNA database

using the alignment algorithm in ARB (www.mikro-

biologie.tu-muenchen.de). Additional 16S rRNA sequences from
closely related bacteria were retrieved from GenBank

following a BLAST search (Altschul et al., 1990). Sequence

alignments were adjusted manually based upon elements

of primary sequence and secondary structure. Ultimately,
phylogenetic analysis was based on 1197 nucleotide

positions that were present in all sequences compared and

unambiguously aligned. Phylogenetic analyses were

performed by the neighbour-joining (Saitou & Nei, 1987)

and distance (DeSoete, 1983) methods from within the ARB

environment and by a maximum-likelihood method using

the program fastDNAm (Olsen et al., 1994). Bootstrap

values were based on 100 trees generated using the program

fastDNAm.boot.

RESULTS

Enrichment and isolation

Approximately 270 environmental samples were

screened as described above. Thirty-three of these

enrichments produced 2-KLG in amounts ranging

from 1.8 to 9.3 g l\text{−1}. Of those, only the 11 most

productive enrichments were screened for 2-KLG-

producing isolates. Each enrichment yielded 4–15
isolates, which were then screened individually and in

pairwise combinations for their ability to convert l-
sorbose to 2-KLG (a total of 118 isolates). Appreciable

(> 3 g l\text{−1}) 2-KLG production was demonstrated in 4
of the 118 isolates (each from a different sample). The

four isolates also came from geographically distinct

locations: 266-13B was isolated from forest soil in

Louisiana, 62A-12A was isolated from forest soil in

western Tennessee and X6L was isolated from cotton field

soil in Alabama. Also included in the present study was

DSM 4025\text{TP}, a patent strain originally deposited at the Deutsche Sammlung von

Mikroorganismen (DSM) as Gluconobacter oxydans
(Yin et al., 1990).

Morphology

Strains X6L\text{TP} and DSM 4025\text{TP} formed visible,

pinpoint colonies 24–36 h after incubation at 30 °C on

TSA. Strains 266-13B\text{FP}, 291-19\text{FP} and 62A-12A\text{FP}

usually required 48 h incubation before colonies were

visible. Older cultures of all strains produced tancoloured, smooth, circular, entire, raised to convex
colonies that were 1–2 mm in diameter. In broth culture, 62A-12A\text{FP} differed from the other strains in
producing a flocculent turbidity. A brown, diffusible,
water-soluble pigment with a single absorbance maxi-
mum of 310 nm was visible in the agar around colonies
after 3–4 d incubation and in broth cultures after
1–2 d. Pigment production was pH-dependent. The
amount of pigment produced increased with increasing

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Table 1. Phenotypic characteristics of Ketogulonigenium strains and selected relatives

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain/species</th>
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<tbody>
<tr>
<td></td>
<td>DSM 4025&lt;sup&gt;TP&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cell shape</td>
<td>Ovoid to rods</td>
</tr>
<tr>
<td>Cell size (µm):</td>
<td></td>
</tr>
<tr>
<td>Length</td>
<td>0.8–1.0&lt;sup&gt;¶&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diameter</td>
<td>0.5–0.7&lt;sup&gt;¶&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>+</td>
</tr>
<tr>
<td>Non-diffusible pigment</td>
<td>–</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
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<tr>
<td>Catalase</td>
<td></td>
</tr>
<tr>
<td>Motility</td>
<td>–</td>
</tr>
<tr>
<td>Growth at 4ºC</td>
<td>–</td>
</tr>
<tr>
<td>Growth at 37ºC</td>
<td>–</td>
</tr>
<tr>
<td>Optimum pH range</td>
<td>7.5–8.0</td>
</tr>
<tr>
<td>Growth at pH 4.5</td>
<td>–</td>
</tr>
<tr>
<td>Optimum [Na]&lt;sup&gt;+&lt;/sup&gt; [mM]</td>
<td>31±2</td>
</tr>
<tr>
<td>Growth in 4% (w/v) NaCl</td>
<td>+</td>
</tr>
<tr>
<td>Carbon sources:†</td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>–</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+ (–)</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>+</td>
</tr>
<tr>
<td>Succinate</td>
<td>+ (–)</td>
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<tr>
<td>Antibiotic MICs: (µg ml&lt;sup&gt;–1&lt;/sup&gt;)‡</td>
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<tr>
<td>Floxicetin</td>
<td>10</td>
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<td>Gentamicin</td>
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<td>Sarafloxacin</td>
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<tr>
<td>Tilmycosin</td>
<td>80</td>
</tr>
<tr>
<td>G+C content (mol%)</td>
<td>540</td>
</tr>
<tr>
<td>Plasmids present</td>
<td>–</td>
</tr>
</tbody>
</table>

* Growth in DM broth.
† Production of pigment weaker and more variable than with the other Ketogulonigenium strains.
‡ Motility demonstrated in one instance (see text).
§ Lowest Na<sup>+</sup> concentration tested (Na<sup>+</sup> concentration of DM with no added NaCl).
| Data shown only for those substances that differed between strains; see text for further information. Data are shown for both conventional and BIOLOG methods; BIOLOG data are shown in parentheses when they differed from those for conventional methods.
‡ Data shown only for those substances that differed between strains; see text for further information.
# Strain 291-19<sup>TP</sup> contained one 8–9 kb plasmid; strain X6L<sup>TP</sup> contained multiple plasmids.
** Data based upon present study.

*initial pH of the medium up to approximately pH 7.2, above which pigment production was more uniform. Little or no pigment was produced when the starting pH of the medium was below 6.75 or in unbuffered media (starting pH 7–7.3) containing carbon sources which resulted in significant acid production (e.g. glucose). Pigment production of strain X6L<sup>TP</sup> was weaker and more variable than that of the other strains. When grown on inositol, all strains produced a bright yellow compound and no brown pigment was observed.

The cell morphology of all strains was similar when grown in DM broth (Table 1). However, there were considerable differences in morphology between strains when grown on TSB (Fig. 1c). While X6L<sup>TP</sup> looked similar in both media, in the TSB medium cells of strains DSM 4025<sup>TP</sup> and 62A-12A<sup>TP</sup> were somewhat longer (2–3 µm) while cultures of strain 266-13B<sup>TP</sup> and 291-19<sup>TP</sup> contained mostly elongated, odd-shaped cells, 7–30 µm in length after 24–36 h. Also, the development of chains of cells by all the strains was more common in TSB than in DM. Fig. 1(a) is a negatively stained electron micrograph of X6L<sup>TP</sup> illustrating the presence of flagella. Filaments were observed in TEM micrographs of thin sections of X6L<sup>TP</sup> and 291-19<sup>TP</sup> (Fig. 1b). TEM of thin sections was not performed on the other strains. All strains were Gram-negative, although X6L<sup>TP</sup> differed from the other strains in a tendency to resist decolorization.
Physiological and biochemical characteristics

Phenotypic traits of *Ketogulonigenium* strains and selected relatives are summarized in Table 1. All five strains grew anaerobically in TSB supplemented with 1 g l\(^{-1}\) glucose. Growth was slower anaerobically than aerobically (5–6 d for visible turbidity) and resulted in much lower cell yields. All strains were cytochrome-oxidase- and catalase-positive. Only the proposed *K. robustum* isolate (X6L\(^{TP}\)) was consistently positive in motility experiments. 62A-12A\(^{PP}\) demonstrated motility in only one experiment (18 replicates total). The other isolates were non-motile. Only X6L\(^{TP}\) grew at 37 °C, although growth was poor and cells had a slightly elongated and swollen appearance at that temperature. *Ketogulonigenium* strains grew throughout the pH range tested (6–0–8–5). The optimum pH for 291-19\(^{PP}\) and the proposed *K. vulgare* isolates (DSM 4025\(^{TP}\), 266-13B\(^{PP}\) and 62A-12A\(^{PP}\)) ranged from 7-2 to 8-0 and is similar to the reported optima for *Roseobacter* species (7–0–8–0). In contrast, X6L\(^{TP}\) grew faster and had better cell yields at higher pHs (8–0–8–5).

This is in marked contrast to *Gluconobacter*, whose reported optimum pH ranges from 5-5 to 6-0. Although *Ketogulonigenium* strains grew at pH 6-0, growth was poor at that low pH. In contrast to *Gluconobacter*, none of the *Ketogulonigenium* strains grew at pH 4-5.

X6L\(^{TP}\) also differed from the other *Ketogulonigenium* strains in its response to NaCl concentration (Table 1). While all strains grew throughout the range of 0–4% added NaCl tested (31-2–716-0 mM Na\(^{+}\)), DSM 4025\(^{TP}\), 62A-12A\(^{TP}\), 266-13B\(^{PP}\) and 291-19\(^{PP}\) had fastest growth rates at 0% added NaCl (31 mM Na\(^{+}\) and greatest cell yields from 0 to 2% added NaCl (31–373 mM Na\(^{+}\)). The growth rates of these strains consistently declined with increasing salt concentration. In contrast, the maximum growth rate of strain X6L\(^{TP}\) was roughly consistent over the entire range of salt concentrations tested. However, the shortest lag periods and maximum cell yields for X6L\(^{TP}\) were observed between 0-5 and 2-5% added NaCl (117–459 mM Na\(^{+}\)).
The whole-cell fatty acid (FAME) compositions of the strains are shown in Table 2. The two main fatty acids were saturated hexadecanoic acid (16:0) (31.9–39.9% of total cellular fatty acids) and summed feature 7 (18:1 ω7c/ω9t/ω12t) (41.4–54.9% of total cellular fatty acids). When compared to the MIDI databases, fatty acid profile matching indexes for the Ketogulonigenium strains ranged from 0.144–0.228, well below the recommended threshold for reliable identification by MIDI (0.500).

MICs (µg ml⁻¹) of the following antibiotics were identical for all five strains: amikacin (2.0), amoxicillin/clavulanic acid (1/0.5), ampicillin (0.5), cefazolin (1.0), cefoxitin (1.0), cefotaxime (2.0), ceftiofur (0.06), cephalothin (1.0), enrofloxacin (0.03), imipenem (1.0), kanamycin (4.0), tetracycline (0.25), ticarcillin (8.0), trimethoprim/sulfamethoxazole (4/76). Antibiotic MIC differences between strains are listed in Table 1. All of the Ketogulonigenium strains tested were inhibited by relatively low concentrations of the antibacterial agents tested and would normally be considered ‘sensitive’ to the antibiotics, with the exception of the trimethoprim/sulfamethoxazole combination. Clinical isolates inhibited by those concentrations of trimethoprim/sulfamethoxazole would normally be considered to be ‘resistant’. However, because most of the Ketogulonigenium strains do not grow at 37 °C, these tests could not be performed in accordance with NCCLS standardized procedures and NCCLS interpretive criteria cannot be applied reliably.

Genotypic analyses

Four of the Ketogulonigenium strains had almost identical chromosomal G+C content (53.4–54.0 mol%; Table 1). Only X6L<sub>TP</sub> differed slightly from the others (52.1 ± 0.3%). The Ketogulonigenium G+C values are lower than the reported values.
for *R. litoralis* (56.3–58.1 mol %), *R. denitrificans* (59.6 mol %), and *G. oxydans* (56–64 mol %). Our calculated value of 60.7 mol % G+C for *Spirochaeta aurantia* strain J1T was consistent with the published value of 60.0 mol % (Breznak & Canale-Parola, 1975) and served as a measure of accuracy for our experimental results. The CsCl gradients and agarose gel electrophoresis revealed that strains X6LT and 291-19Pp contained plasmids (Table 1).

The similarities in small subunit rDNA sequence among the five *Ketogulonigenium* strains ranged from 99.7 to 100 % . Strains 266-13Bp, 291-19Pp and 62A-12Ap had identical 16S rDNA sequences over 1354 comparable nucleotide positions. DSM 4025T differed from these three strains at a single nucleotide position. Strain X6LT differed from DSM 4025T at five nucleotide positions and from the other three strains at four positions. All of the phylogenetic analyses tested placed five strains within the *Rhodobacter group* of the Ribosome Database Project II (Maidak et al., 2000) in the α-subclass of the Proteobacteria (Fig. 2, assembly ‘B’). The most similar sequence in the public databases was that of strain LFR (93.7–94.2 % identical), a dimethyl-sulfiniopropionate (DSMP)-degrader isolated from the Sargasso Sea (Ledyard et al., 1993). The described genus most closely related to *Ketogulonigenium* was *Roseobacter* (Fig. 2; 92.1–92.8 % identical), a genus composed of photosynthetic, marine organisms (Lafay et al., 1995; Ruiz-Ponte et al., 1998; Shiba, 1991). More distantly related were the genera *Glucobacter* and *Acetobacter* (Fig. 2; 81.8–83.3 % identical and 81.2–82.1 % identical, respectively). The phylogeny is supported by an abbreviated stem–loop structure (helices 45 and 46; as numbered by Van de Peer et al., 1996) found in *Ketogulonigenium* (Fig. 2, assembly B) that is shared by all members of the *Rhodobacter group* and serves to distinguish this group from most of the other α-Proteobacteria (Fig. 2, assembly A).

The recently described acetic acid α-proteobacterium *Asaia bogorensis* (Yamada et al., 2000) was also more distantly related (82.8–83.1 % identical) and lacked the truncated stem–loop structure found in *Ketogulonigenium*. Branch lengths and branching order of the phylogenetic tree suggest the five strains represent a genus of α-Proteobacteria not currently represented in the rDNA databases.

Results of DNA reassocation experiments are listed in

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**Fig. 2.** Phylogenetic relationships and supporting structural elements of *Ketogulonigenium* species and selected relatives. The tree was produced by a maximum-likelihood method using fastDNamlloop (Olsen et al., 1994) and was rooted using *E. coli* 16S rRNA. Bootstrap values were calculated using fastDNaml.boot (Olsen et al., 1994). The nodes without bootstrap values represent either branching orders that were inconsistent between the phylogenetic tree and the consensus tree generated from 100 bootstrap trees or had bootstrap values of 50 % or less. The scale bar represents 0.1 changes per nucleotide position. The numbering of nucleotide positions in the rRNA stem–loop structures are based upon *E. coli* 16S rRNA. Bootstrap values were calculated using fastDNAml (Olsen, 1994) and was rooted using *E. coli* numbering and illustrate the abbreviated stem–loop structure found in *Ketogulonigenium*. This truncated stem–loop structure is shared by all members of the assemblage designated ‘B’ and serves to distinguish them from the genera *Glucobacter* and *Acetobacter* and some other members of the α-Proteobacteria (designated assemblage ‘A’).
Table 3. DNA–DNA reassociation values (%) among Ketogulonigenium isolates and Roseobacter type strains

Mean values for reciprocal DNA–DNA hyridizations (n = 1–5 replicates) among the proposed species are boxed: K. vulgare is represented by strains DSM 4025TP, 62A-12App and 266-13Bpp; K. robustum is represented by strain X6LTP. Strain 291-19PP was not assigned to either species in this study (see text). Reassociation between homologous DNAs was defined as 100%.

<table>
<thead>
<tr>
<th>Source of target DNA</th>
<th>DSM 4025TP</th>
<th>62A-12App</th>
<th>266-13Bpp</th>
<th>291-19PP</th>
<th>X6LTP</th>
<th>R. litoralis†</th>
<th>R. denitrificans‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSM 4025TP</td>
<td>100</td>
<td>92±3</td>
<td>93*</td>
<td>49±4</td>
<td>14±2</td>
<td>10±3</td>
<td>12±3</td>
</tr>
<tr>
<td>62A-12App</td>
<td>88±1</td>
<td>100</td>
<td>91*</td>
<td>42±8</td>
<td>18±3</td>
<td>11±2</td>
<td>14±1</td>
</tr>
<tr>
<td>266-13Bpp</td>
<td>94*</td>
<td>70*</td>
<td>100</td>
<td>63±10</td>
<td>11±2</td>
<td>10±1</td>
<td>13±2</td>
</tr>
<tr>
<td>291-19PP</td>
<td>36±17</td>
<td>31±9</td>
<td>52±10</td>
<td>100</td>
<td>9±2</td>
<td>7±0</td>
<td>8±1</td>
</tr>
<tr>
<td>X6LTP</td>
<td>38±9</td>
<td>37±5</td>
<td>40±3</td>
<td>18±4</td>
<td>100</td>
<td>4±2</td>
<td>4±2</td>
</tr>
<tr>
<td>R. litoralis†</td>
<td>15*</td>
<td>ND</td>
<td>17*</td>
<td>ND</td>
<td>ND</td>
<td>100</td>
<td>33*</td>
</tr>
<tr>
<td>R. denitrificans‡</td>
<td>18*</td>
<td>ND</td>
<td>12*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>100</td>
</tr>
</tbody>
</table>

* Based upon a single hybridization experiment.
† ATCC 49566T.
‡ ATCC 33942T.

Table 3. Based upon the phylogenetic definition of a species as strains with approximately 70% DNA–DNA relatedness (Wayne et al., 1987), we have divided the five strains into two species. The first consists of 266-13Bpp, 62A-12App and DSM 4025TP (with the proposed name K. vulgare sp. nov.) and the second consists of X6LTP (proposed name K. robustum sp. nov.). The species affiliation of strain 291-19PP was left undetermined (see discussion below).

DISCUSSION

Although bacteria from several genera oxidize l-sorbose to 2-keto-l-gulonic acid (Imai et al., 1990; Sugisawa et al., 1990; Yin et al., 1990; Nogami et al., 1995), the yield is generally insufficient to warrant commercial production of 2-KLG, a key intermediate in the chemical synthesis of vitamin C. We initiated a search for bacteria that generated higher yields of 2-KLG through the use of enrichment cultures supplemented with l-sorbose. A high concentration of l-sorbose was used in initial enrichments to provide sufficient substrate to achieve detectable levels of 2-KLG despite the potential for 2-KLG-degrading organisms to be active in the mixed-culture enrichments. This high sugar concentration also simulated the conditions envisioned for commercial production of 2-KLG and provided a degree of osmotic selection for organisms capable of growing under production conditions. Similarly, corn steep liquor was used in enrichments and isolation media to simulate the expected commercial production medium. Calcium carbonate was included to limit the potential acidification of the medium resulting from the oxidation of substrates to acidic end products. Considering the subsequent discovery that these organisms produce large amounts of organic acids from carbohydrates, and generally grow poorly at pHs below 7.0, the inclusion of calcium carbonate may have been critical for obtaining successful enrichment cultures. In screening the enrichments for individual microbes that produced 2-KLG, plates were examined under ×8 to ×40 magnification after 24 and 48 h of incubation. Attention to the smallest and slowest-growing colonies proved necessary to recover 2-KLG-producing strains from the dilution plates. Three previously described 2-KLG-producing strains yielded higher product concentrations when grown in co-culture with a second, ‘helper’ species (Imai et al., 1990; Yin et al., 1990; Nogami et al., 1995). It was reasoned that enrichments positive for 2-KLG production could have resulted from the presence of at least one helper strain. To allow for this possibility, strains we isolated were screened for 2-KLG production in all possible pairwise combinations as well as individually. All strains that produced 2-KLG did so both individually and in paired combinations.

Four strains isolated during this study, each from a different enrichment, converted l-sorbose to 2-KLG efficiently: strains 266-13Bpp, 291-19PP, 62A-12App and X6LTP. Although the four isolates were obtained from geographically distinct locations, their 16S rDNA sequences were nearly identical to each other (99–100% identical). Comparative 16S rDNA sequencing subsequently revealed that they were also nearly identical (99–99.9% identical) to the 16S rDNA sequence from DSM 4025TP, a patent strain originally identified as Gluconobacter oxydans (Yin et al., 1990). Together, the five sequences formed a monophyletic group within the α-Proteobacteria (Fig. 2). The low sequence similarity (92–94% identical) between the 16S rDNA sequences from these five strains and any previously named bacteria suggested the need for creation of a new bacterial genus. We propose the new genus be named Ketogulonigenium.
Based on the conspicuous production of 2-keto-L-gulonic acid from L-sorbose by members of the proposed taxon. The newly isolated strains and strain DSM 4025<sup>TP</sup> were distinguishable from *Roseobacter*, the nearest phylogenetic relative, and from *Gluconobacter* and *Acetobacter*, the other genera of α-Proteobacteria that oxidize L-sorbose to 2-KLG (Imai *et al.*, 1990; Sugisawa *et al.*, 1990; Yin *et al.*, 1990; Nogami *et al.*, 1995).

Although the isolates characterized in this study are similar to *Roseobacter* strains in that they are catalase-positive and oxidase-positive, there exist major phenotypic differences related to the energy metabolism of the two groups. The genus *Roseobacter* is comprised aerobic, photosynthetic, mobile, pink-pigmented, marine bacteria, which contain bacteriochlorophyll <i>a</i> (Lafay *et al.*, 1995; Ruiz-Ponte *et al.*, 1988; Shiba, 1991). Strains of the proposed genus *Ketogulonigenium* are facultatively anaerobic chemoheterotrophs, do not contain bacteriochlorophyll <i>a</i>, and produce a diffusible, brown pigment. In addition, the 2-KLG-producing strains have a lower G+C content (52–1–54.0 mol%) and a higher and narrower optimum temperature range (27–31 °C) than those of *Roseobacter* species (56–60 mol% and 20–30 °C, respectively; Shiba, 1992).

The strains characterized in this study were not closely related to *Gluconobacter* or *Acetobacter* (81–83% 16S rDNA identity), but they did share a number of important phenotypic traits characteristic of these α-proteobacteria. Like *Gluconobacter* and *Acetobacter*, the five proposed *Ketogulonigenium* isolates oxidize L-sorbose to 2-KLG (although with greater efficiencies) and glucose to 2-keto-gluconic acid. Nonetheless, there were numerous phenotypic traits that distinguish the new isolates from *Gluconobacter* and *Acetobacter*. Members of both *Gluconobacter* and *Acetobacter* grow at pH 4.5 and have pH optima in between 5.4 and 6.3 (De Ley & Swings, 1984; De Ley *et al.*, 1984). The strains described here were decidedly not acidophilic, growing optimally in the pH range of 7.2–8.5. All five strains grew at pH 6.0, though growth rates were slow and cell yields were low: no growth was observed at pH 4.5. In addition, cultures of *Gluconobacter* and *Acetobacter* accumulate dihydroxyacetone when glycerol is supplied as a carbon source (data not shown), whereas the five strains described here do not. *Gluconobacter* and *Acetobacter* also contain elements of primary 16S rDNA sequence (Fig. 2) that distinguish them and the other α-Proteobacteria from the five *Ketogulonigenium* strains and other members of the *Rhodobacter* group. Phylogenetic and phenotypic data strongly support the hypothesis that the newly isolated strains are not members of the genus *Gluconobacter* or *Acetobacter*. Furthermore, the data also contradict the original identification of strain DSM 4025<sup>TP</sup> as *G. oxydans* (Yin *et al.*, 1990) and support reclassification of the strain within the proposed genus *Ketogulonigenium*.

Although rRNA-based phylogeny suggested the creation of a new genus to accommodate these five strains, 16S rRNA sequences generally lack the variability required to make species-level distinctions. Therefore, DNA–DNA reassociation studies were undertaken to address species-level relationships. Based on DNA–DNA hybridization results, we propose dividing the five strains into two species. The first species would consist of strains 266-13B<sup>PP</sup>, 62A-12A<sup>PP</sup> and DSM 4025<sup>TP</sup> (with the proposed name *K. vulgare* sp. nov.) and the second would consist of strain X6L<sup>TP</sup> (proposed name *K. robustum* sp. nov.). The low DNA–DNA reassociation values between 291-19<sup>PP</sup> and the other strains (Table 3) suggest that this strain should also be given species distinction. However, the phenotypic data did not support distinction from *K. vulgare* (Tables 1 and 2). Also, DNA–DNA reassociation values greater than 70% were occasionally recorded with members of the *K. vulgare* group. Because of these findings, we have chosen to leave the species status of 291-19<sup>PP</sup> unresolved at this time.

The proposed creation of the species *K. robustum* (strain X6L<sup>TP</sup>) and *K. vulgare* (266-13B<sup>PP</sup>, 62A-12A<sup>PP</sup> and DSM 4025<sup>TP</sup>) is supported by phenotypic characterization of the strains (Table 1). Strain X6L<sup>TP</sup> was more robust than the other strains, routinely growing faster and with greater cell yields over a broader range of conditions. Strain X6L<sup>TP</sup> was also the only motile strain and the only one capable of growing at 37 °C. While cells of strains 291-19<sup>PP</sup>, 266-13B<sup>PP</sup>, 62A-12A<sup>PP</sup> and DSM 4025<sup>TP</sup> were frequently elongated when grown on some media (Fig. 1c), cells of strain X6L<sup>TP</sup> were consistently ovoid to rod-shaped and were unique amongst the strains in resisting decolorization during the Gram stain. The pH optimum of X6L<sup>TP</sup> was higher (8.0–8.5) than those of the other strains (7.2–8.0) and its response to Na<sup>+</sup> concentration also differed dramatically (Table 1). The G+C content of X6L<sup>TP</sup> was also lower (52.1 mol%) than that of the other strains (53.4–54.0 mol%).

Although patterns of carbon utilization and antibiotic sensitivity are frequently used to discriminate amongst species within a genus, differences in carbon utilization and antibiotic sensitivity patterns were subtle amongst the *Ketogulonigenium* isolates. The lack of differences in antibiotic sensitivity patterns amongst the five strains suggests that none of the plasmids present in strains X6L<sup>TP</sup> and 291-19<sup>PP</sup> expressed antibiotic resistance.

In summary, four environmental isolates and one culture collection strain that converted L-sorbose to 2-KLG were characterized phenotypically and genotypically. Our analyses suggest these strains represent a new genus within the α-Proteobacteria, for which we propose the name *Ketogulonigenium*. Two species were identified within the new genus with the proposed names *K. vulgare* and *K. robustum*.

**Description of *Ketogulonigenium* gen. nov.**

*Ketogulonigenium* (Ke.to.gu.lo.ni.geˈni.um. N.L. n. *acidum* ketogulonicum ketogulonic acid; N.L. neut. *robustum* L. neut. robustum robust.)

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Ketogulonigenium gen. nov.

Ketogulonigenium robustum (ro.bust’um. L. adj. robustum strong).

Cells are motile, resist decolorization when Gram-stained, and are 0.8–1.0 μm in length and 0.6–0.7 μm wide regardless of the medium used in this study. Colonies are visible on solid media after 24 h and pigment production is poor and sporadic. Growth is observed at 37 °C. The G + C content of the DNA is slightly lower (52.1 ± 0.3 mol %) and the pH optimum is slightly higher (8.0–8.5) than those of the other members of the genus (53.4–54.0 mol % and 7.2–8.0 respectively). The one identified strain of this species grows faster and with greater cell yields than other members of the genus so far described. Other properties are as described for the genus. The type and only identified strain is X6LTP (= NRRL B-21627 = KCTC 0858BP).

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