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

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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 4025^{TP}, 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^{PP}, 266-13B^{PP} and the type strain *K. vulgare* DSM 4025^{TP}, and *Ketogulonigenium robustum* gen. nov., sp. nov., consisting of the type strain *K. robustum* X6L^{TP} (= NRRL B-21627 = KCTC 0858BP). The species affiliation of the fifth strain (291-19^{PP}) 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 (Delic *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, α -*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 '*Pseudogluconobacter*' (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 Delic *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

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Abbreviation: 2-KLG, 2-keto-L-gulonic acid.

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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 *α-Proteobacteria* that is characterized by efficient production of 2-KLG from L-sorbose, *Ketogulonigenium*, and the reclassification of patent strain DSM 4025^{TP}, previously identified as *Gluconobacter oxydans* (Yin *et al.*, 1990). Two species, *Ketogulonigenium vulgare* 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 L-sorbose-utilizing organisms, approximately 1 g of sample was added to 30 ml Enrichment Medium A [EM-A; 20 g L-sorbose l⁻¹, 2 g glucose l⁻¹, 0.1 g FeSO₄ · 7H₂O l⁻¹, 1.7 g Yeast Nitrogen Base (Difco) l⁻¹, 6 g CaCO₃ 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 L-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. L-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 L-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. L-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 L-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 L-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^{TP} 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 K₂HPO₄ 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 conducted using 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), MgCl₂ · 6H₂O, 1000; NaSO₄, 1000; NaCl, 5000; KCl, 350; K₂HPO₄, 140; CaCl₂ · 2H₂O, 75; NaBr, 50; H₃BO₃, 20; Na₂WO₄ · 2H₂O, 3; nitrilotriacetate, 1.5; FeSO₄ · 7H₂O, 1.4; CuSO₄ · 5H₂O, 1; MnSO₄ · H₂O, 0.305; CoCl₂ · 6H₂O, 0.305; NiCl₂ · 6H₂O, 0.20; Na₂MoO₄ · 2H₂O, 0.001; pyridoxine · HCl, 0.10; *p*-aminobenzoic acid, 0.05; nicotinic acid, 0.05; DL-calcium pantothenate, 0.05; thiamin · HCl, 0.05; DL-6,8-thioctic acid, 0.05; riboflavin, 0.04; biotin, 0.02; folic acid, 0.02; vitamin B₁₂, 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 (200–250 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^{TP}), NRRL B-30035 (291-19^{PP}), NRRL B-30036 (266-13B^{PP}), NRRL B30037 (62A-12A^{PP}). Strain X6L^{TP} 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 OD₆₀₀ of 0.002–0.007. Growth of the isolates was monitored by measuring the OD₆₀₀ 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. OD₆₀₀ was measured at regular intervals using a Spec20 (Milton Roy).

BIOLOG metabolic experiments. Each isolate was grown in 20 ml TSB to an OD₆₀₀ of 1.0 or greater. The cells were harvested by centrifugation at 10000 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 0–0.1), weakly positive (OD₅₉₀ 0.1–0.5) 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 22 °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 precipitates 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 Centricon 30 columns (Amicon) as per the manufacturer's recommendations. The concentration and purity of DNA samples were quantified spectrophotometrically. Agarose gel electro-

phoresis (0.8% agarose in $1 \times$ TAE) was also used to assess the quality of the DNA preparations and for the visualization of plasmids.

G + C content. This was determined by the thermal denaturation (T_m) method of Marmer & Doty (1962) using a Perkin-Elmer Lambda 14 spectrophotometer with a water-jacketed cuvette holder connected to a circulating waterbath. The correlation between cuvette and waterbath temperatures was previously established using a thermister in a sealed cuvette. Approximately 50 µg genomic DNA in 1 ml $0.5 \times$ SSC was used for analysis. Because the G + C content of the chromosomal DNA from *Spirochaeta aurantia* strain J1^T has been previously determined by the same method (Breznak & Canale-Parola, 1975), it was used as a reference to verify the system. The genomic denaturation temperature (T_m) determined by this analysis was subsequently used to calculate the hybridization temperature used in the DNA–DNA 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-blot device (ABN VacuSystems; American Bionetics) to membrane saturation. Membrane saturation was empirically determined to be 4–5 µg per slot. Genomic DNAs were sheared by 20 passages through a 30 gauge needle, denatured for 10 min at room temperature in 0.4 M NaOH and then diluted in 9 vols $6.7 \times$ SSC (final concentration $6 \times$ SSC). Five micrograms of DNA were then deposited per slot. DNA was fixed to the membrane by UV cross-linking (1200 mJ) followed by baking at 80 °C for 2 h. Membranes were prehybridized in 8 ml hybridization buffer [0.9 M NaCl, 0.15 M Tris, 5 mM EDTA, 0.5% SDS, 100 mg Poly(A) ml⁻¹, $5 \times$ Denhardt's solution (Sambrook *et al.*, 1989c), pH 7.5] for 5 h at 68 °C ($T_m = 25$ °C). This was replaced with 3 ml fresh hybridization buffer and 1 µg ³²P-labelled probe was added. Sheared genomic DNAs were used to make nick-translated probes using a commercial kit (Gibco BRL) as per the manufacturer's instructions. Hybridizations were incubated for 18–24 h at 68 °C. Hybridized membranes were briefly rinsed twice with 80 ml wash solution (WS; $1 \times$ SSC, 1% SDS) and washed for 30 min at room temperature in 80 ml fresh WS. Membranes were then stringently washed for 15 min at 68 °C in 80 ml WS, transferred to 80 ml of fresh WS and washed for an additional 15 min at 68 °C. Membranes were finally washed overnight at room temperature in 200 ml WS to remove excess background counts. The specifically bound probe was quantified using a radioanalytic imaging system (AMBIS).

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 recommendation. 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 Biosystems) 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 (Altshul *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 fastDNAmI (Olsen *et al.*, 1994). Bootstrap values were based on 100 trees generated using the program fastDNAmI_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⁻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⁻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^{PP} and 291-19^{PP} were isolated from different samples taken from around a sugar refinery in Louisiana, 62A-12A^{PP} was isolated from forest soil in western Tennessee and X6L^{TP} was isolated from cotton field soil in Alabama. Also included in the present study was DSM 4025^{TP}, a patent strain originally deposited at the Deutsche Sammlung von Mikroorganismen (DSM) as *Gluconobacter oxydans* (Yin *et al.*, 1990).

Morphology

Strains X6L^{TP} and DSM 4025^{TP} formed visible, pinpoint colonies 24–36 h after incubation at 30 °C on TSA. Strains 266-13B^{PP}, 291-19^{PP} and 62A-12A^{PP} usually required 48 h incubation before colonies were visible. Older cultures of all strains produced tan-coloured, smooth, circular, entire, raised to convex colonies that were 1–2 mm in diameter. In broth culture, 62A-12A^{PP} differed from the other strains in producing a flocculent turbidity. A brown, diffusible, water-soluble pigment with a single absorbance maximum 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

Table 1. Phenotypic characteristics of *Ketogulonigenium* strains and selected relatives

Except where noted, data for *Roseobacter*, *Gluconobacter* and *Acetobacter* are based upon published results (Shiba, 1991, 1992; Lafay *et al.*, 1995; Ruiz-Ponte *et al.*, 1998; De Ley *et al.*, 1984; De Ley & Swings, 1984). ND, No data; d, inconclusive results; w, weak reaction; v, variable results.

Characteristic	Strain/species							
	DSM 4025 ^{TP}	62A-12A ^{PP}	266-13B ^{PP}	291-19 ^{PP}	X6L ^{TP}	<i>Roseobacter</i>	<i>Gluconobacter</i>	<i>Acetobacter</i>
Cell shape	Ovoid to rods	Ovoid to rods	Ovoid to rods	Ovoid to rods	Ovoid to rods	Ellipsoidal to rods	Ovoid to rods	Ellipsoidal to straight to curved rods
Cell size (µm):								
Length	0.8–1.0*	1.0–1.1*	0.9–1.1*	0.8–1.3*	0.8–1.0*	1.0–2.0	0.9–4.2	1.0–4.0
Diameter	0.5–0.7*	0.5–0.7*	0.6–0.7*	0.6–0.7*	0.6–0.7*	0.6–0.9	0.5–0.8	0.6–0.8
Anaerobic growth	+	+	+	+	+	—	—	—
Non-diffusible pigment	—	—	—	—	—	Pink–red	v	v
Diffusible pigment	Brown	Brown	Brown	Brown	Brown†	—	v	v
Oxidase	+	+	+	+	+	+	—	—
Catalase	+	+	+	+	+	+	+	+
Motility	—	—‡	—	—	+	+	v	v
Optimum temperature range (°C)	27–29	28–30	28–30	25–28	29–31	20–30	25–30	25–30
Growth at 4 °C	—	+	+	+	—	v	ND	ND
Growth at 37 °C	—	—	—	—	+	—	—	v
Optimum pH range	7.5–8.0	7.2–7.7	7.5–7.7	7.2–8.0	8.0–8.5	7.0–8.0	5.5–6.0	5.4–6.3
Growth at pH 4.5	—	—	—	—	—	—	+	+
Optimum [Na ⁺] (mM)	31–2§	31–2§	31–2§	31–2§	117–459	ND	ND	ND
Growth in 4% (w/v) NaCl	+	+	+	+	+	ND	ND	ND
Carbon sources:								
Fructose	+	—	+(w)	d(w)	+	ND	ND	ND
Raffinose	—	—(w)	—(w)	d	—	ND	ND	ND
Sucrose	+(—)	—(w)	+(—)	+(w)	+(—)	ND	ND	ND
Trehalose	+	—	+	+	+	ND	ND	ND
Pyruvate	+	d	d	d	+	ND	ND	ND
Succinate	+(—)	—	+(—)	d(—)	+(—)	ND	ND	ND
Antibiotic MICs: (µg ml ^{−1})¶								
Florfenicol	1.0	1.0	0.5	0.5	1.0	ND	ND	ND
Gentamicin	0.12	0.12	0.12	0.25	0.12	ND	ND	ND
Sarafloxacin	0.06	0.06	0.06	0.06	0.12	ND	ND	ND
Tilmicosin	8.0	4.0	4.0	4.0	2.0	ND	ND	ND
G + C content (mol %)	54.0	54.0	53.6	53.4	52.1	56.3–59.6	56–64	51–65
Plasmids present	—	—	—	+ #	+ #	—**	ND	ND

* 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⁺ concentration tested (Na⁺ 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^{PP} contained one 8–9 kb plasmid; strain X6L^{TP} 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 7.2–7.3) containing carbon sources which resulted in significant acid production (e.g. glucose). Pigment production of strain X6L^{TP} 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^{TP} looked similar in both media, in the TSB medium cells of strains DSM 4025^{TP} and 62A-12A^{PP} were somewhat longer (2–3 µm) while cultures of strain 266-13B^{PP} and 291-19^{PP} 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^{TP} illustrating the presence of flagella. Fimbriae were observed in TEM micrographs of thin sections of X6L^{TP} and 291-19^{PP} (Fig. 1b). TEM of thin sections was not performed on the other strains. All strains were Gram-negative, although X6L^{TP} differed from the other strains in a tendency to resist decolorization.

Fig. 1. (a) TEM of a negative stain of strain X6L^{TP} demonstrating flagella, (b) TEM of thin section of strain X6L^{TP} demonstrating fimbriae, (c) differences in cell morphology between strains when grown on TSB (overnight cultures). Bars: (a) 1 µm, (b) 0.2 µm, (c) 10 µm.

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^{PP}, 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⁺).

Table 2. Relative whole-cell fatty acid composition (%) of strains of *Ketogulonigenium* and *G. oxydans*

Analysis was performed using a commercial system (MIS, MicroID).

Fatty acid	Strain					
	DSM 4025 ^{TP}	62A-12A ^{PP}	266-13B ^{PP}	291-19 ^{PP}	X6L ^{TP}	<i>G. oxydans</i>
10:0	0.0	0.7	0.3	0.0	0.0	0.0
10:0 3-OH	3.6	3.7	3.6	3.1	4.3	0.0
12:0	0.0	0.6	0.7	0.9	0.0	0.0
12:0 3-OH	2.9	2.9	3.0	2.9	3.3	0.0
14:0	2.4	2.1	2.5	2.3	2.8	0.9
14:0 3-OH/16:1 iso I*	0.0	0.0	0.0	0.0	0.0	2.0
15:0	0.0	0.0	0.7	0.0	0.0	0.0
16:0	33.5	34.9	39.9	39.6	31.9	13.0
16:0 2-OH	0.0	0.0	0.0	0.0	0.0	7.8
16:0 3OH	0.0	0.0	0.0	0.0	0.0	2.5
16:1 ω 7c/15 iso 2-OH†	2.7	3.8	5.5	5.3	2.8	0.0
18:0	2.9	1.1	0.7	0.9	0.0	1.6
18:1 2-OH	0.0	0.0	0.0	0.0	0.0	1.7
18:1 ω 7c/ ω 9t/ ω 12t‡	52.0	49.5	41.4	43.5	54.9	65.8
un 18.846/18.858§	0.0	0.7	1.7	1.4	0.0	0.0
19:0 cyclo 8c	0.0	0.0	0.0	0.0	0.0	2.9
20:3 ω 6,9,12c	0.0	0.0	0.0	0.0	0.0	1.7

* Summed feature 3.

† Summed feature 4.

‡ Summed feature 7.

§ Summed feature 9; un, undetermined fatty acid.

There were only slight differences in carbon utilization between the five strains. All five *Ketogulonigenium* strains utilized arabinose, cellobiose, glucose, glycerol, inositol, lactose, maltose, mannitol, mannose, rhamnose, sorbitol and sorbose. Differences in carbon utilization between the strains are listed in Table 1. Carbon sources resulting in fastest growth and highest cell yields were the sugar alcohols mannitol, inositol, glycerol and sorbitol, along with lactose and arabinose. Although L-sorbose was used for the original enrichments and was utilized by all strains, cultures attained a lower turbidity on L-sorbose when compared to most of the other carbon sources tested. Carbon utilization and oxidation was tested by conventional methods and by BIOLOG, respectively. The BIOLOG carbohydrate data were in general agreement with that from conventional methods.

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 ω 7c/ ω 9t/ ω 12t) (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 (μ g ml⁻¹) of the following antibiotics were identical for all five strains: amikacin (2.0), amoxicillin/clavulonic acid (1/0.5), ampicillin (0.5), cefazolin (1.0), cefoxitin (1.0), ceftazidime (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^{TP} differed slightly from the others (52.1 $\pm$ 0.3%). The *Ketogulonigenium* G+C values are lower than the reported values

Fig. 2. Phylogenetic relationships and supporting structural elements of *Ketogulonigenium* species and selected relatives. The tree was produced by a maximum-likelihood method using fastDNAmL_loop (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* 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 *Gluconobacter* and *Acetobacter* and some other members of the α -Proteobacteria (designated assemblage 'A').

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 J1^T 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 X6L^{TP} and 291-19^{PP} contained plasmids (Table 1).

The similarities in small subunit rDNA sequence among the five *Ketogulonigenium* strains ranged from 99.7 to 100%. Strains 266-13B^{PP}, 291-19^{PP} and 62A-12A^{PP} had identical 16S rDNA sequences over 1354 comparable nucleotide positions. DSM 4025^{TP} differed from these three strains at a single nucleotide position. Strain X6L^{TP} differed from DSM 4025^{TP} at five nucleotide positions and from the other three strains at four positions. All of the phylogenetic analyses tested placed the 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, assemblage 'B'). The most similar sequence in the public databases was that of strain LFR (93.7–94.2% identical), a dimethyl-

sulfoniopropionate (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 *Gluconobacter* 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, assemblage 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, assemblage 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 rRNA databases.

Results of DNA reassociation experiments are listed in

Table 3. DNA–DNA reassociation values (%) among *Ketogulonigenium* isolates and *Roseobacter* type strains

Mean values for reciprocal DNA–DNA hybridizations ($n = 1-5$ replicates) among the proposed species are boxed: *K. vulgare* is represented by strains DSM 4025^{TP}, 62A-12A^{PP} and 266-13B^{PP}; *K. robustum* is represented by strain X6L^{TP}. Strain 291-19^{PP} was not assigned to either species in this study (see text). Reassociation between homologous DNAs was defined as 100%.

Source of target DNA	Source of target DNA						
	DSM 4025 ^{TP}	62A-12A ^{PP}	266-13B ^{PP}	291-19 ^{PP}	X6L ^{TP}	<i>R. litoralis</i> †	<i>R. denitrificans</i> ‡
DSM 4025 ^{TP}	100	92 ± 3	93*	49 ± 4	14 ± 2	10 ± 3	12 ± 3
62A-12A ^{PP}	88 ± 1	100	91*	42 ± 8	18 ± 3	11 ± 2	14 ± 1
266-13B ^{PP}	94*	70*	100	63 ± 10	11 ± 2	10 ± 1	13 ± 2
291-19 ^{PP}	36 ± 17	31 ± 9	52 ± 10	100	9 ± 2	7 ± 0	8 ± 1
X6L ^{TP}	38 ± 9	37 ± 5	40 ± 3	18 ± 4	100	4 ± 2	4 ± 2
<i>R. litoralis</i> †	15*	ND	17*	ND	ND	100	33*
<i>R. denitrificans</i> ‡	18*	ND	12*	ND	ND	ND	100

* Based upon a single hybridization experiment.

† ATCC 49566^T.

‡ ATCC 33942^T.

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 266-13B^{PP}, 62A-12A^{PP} and DSM 4025^{TP} (with the proposed name *K. vulgare* sp. nov.) and the second consists of X6L^{TP} (proposed name *K. robustum* sp. nov.). The species affiliation of strain 291-19^{PP} 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 $\times 8$ to $\times 40$ 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 266-13B^{PP}, 291-19^{PP}, 62A-12A^{PP} and X6L^{TP}. Although the four isolates were obtained from geographically distinct locations, their 16S rDNA sequences were nearly identical to each other (99.7–100% identical). Comparative 16S rDNA sequencing subsequently revealed that they were also nearly identical (99.6–99.9% identical) to the 16S rDNA sequence from DSM 4025^{TP}, 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^{TP} 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 *a* (Lafay *et al.*, 1995; Ruiz-Ponte *et al.*, 1998; Shiba, 1991). Strains of the proposed genus *Ketogulonigenium* are facultatively anaerobic chemoheterotrophs, do not contain bacteriochlorophyll *a*, 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-gulonic 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 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^{TP} 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^{PP}, 62A-12A^{PP} and DSM 4025^{TP} (with the proposed name *K. vulgare* sp. nov.) and the second would consist of strain X6L^{TP} (proposed name *K. robustum* sp. nov.). The low DNA–DNA reassociation values between 291-19^{PP} 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^{PP} unresolved at this time.

The proposed creation of the species *K. robustum* (strain X6L^{TP}) and *K. vulgare* (266-13B^{PP}, 62A-12A^{PP} and DSM 4025^{TP}) is supported by phenotypic characterization of the strains (Table 1). Strain X6L^{TP} was more robust than the other strains, routinely growing faster and with greater cell yields over a broader range of conditions. Strain X6L^{TP} was also the only motile strain and the only one capable of growing at 37 °C. While cells of strains 291-19^{PP}, 266-13B^{PP}, 62A-12A^{PP} and DSM 4025^{TP} were frequently elongated when grown on some media (Fig. 1c), cells of strain X6L^{TP} were consistently ovoid to rod-shaped and were unique amongst the strains in resisting decolorization during the Gram stain. The pH optimum of X6L^{TP} was higher (8.0–8.5) than those of the other strains (7.2–8.0) and its response to Na⁺ concentration also differed dramatically (Table 1). The G+C content of X6L^{TP} 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^{TP} and 291-19^{PP} 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.ge'ni.um. N.L. n. *acidum ketogulonicum* ketogulonic acid; N.L. neut.

suff. *genium* that which produces, N.L. neut. n. *Ketogulonigenium* that which produces ketogulonic acid).

Gram-negative, facultatively anaerobic, motile or non-motile, ovoid to rod-shaped cells, 0.8–1.3 µm long, 0.5–0.7 µm in diameter, with tapered ends, occurring as single cells, pairs and occasionally short chains. Some strains form elongated cells (up to 30 µm in length) on TSB. Flagella and fimbriae have been observed. Colonies are tan-coloured, smooth, circular, entire, raised to convex, 1–2 mm in diameter with a diffusible brown pigment after 48 h incubation. Oxidase and catalase reactions are positive. Optimum temperature range is 27–31 °C, optimum pH range is 7.2–8.5 and optimum Na⁺ concentration is 117–459 mM. Chemoorganotrophic. Carbon sources utilized include arabinose, cellobiose, fructose, glucose, glycerol, inositol, lactose, maltose, mannitol, mannose, rhamnose, sorbitol, sorbose, sucrose, trehalose, pyruvate and succinate. Favoured carbon sources are inositol, mannitol, glycerol, sorbitol, lactose and arabinose. All strains examined produce 2-keto-L-gulonic acid from L-sorbose. Major cellular fatty acids are 16:0 and 18:1 *ω*7c/*ω*9t/*ω*12t and the DNA G+C content is 52.1–54.0%. Small subunit rDNA sequence analysis places this genus in the α -subclass of the *Proteobacteria*. All strains isolated in the present study originated in soil. The DNA reassociation studies divide the genus into two species, *K. vulgare* and *K. robustum*, of which *K. vulgare* is the designated type species.

Description of *Ketogulonigenium vulgare* sp. nov.

Ketogulonigenium vulgare (vul.ga're. L. neut. adj. *vulgare* common).

Members of this species are non-motile and fail to grow at 37 °C. The optimum pH range is 7.2–8.0 and the G+C content of identified strains is 53.4–54.0 mol%. Strain 62A-12A^{PP} produces a flocculent turbidity in broth cultures. Other properties of this species are as described for the genus. Based upon DNA reassociation studies, three strains are included in this species; 266-13B^{PP}, 62A-12A^{PP} and DSM 4025^{TP}, of which DSM 4025^{TP} is the designated type strain.

Description of the type strain

The optimum pH range of DSM 4025^{TP} is 7.5–8.0 and its G+C content is 54.0 mol%. Pigment production in this strain is more pronounced than in other members of the species. Other properties of the type strain are as described for the species.

Description of *Ketogulonigenium robustum* sp. 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 X6L^{TP} (= NRRL B-21627 = KCTC 0858BP).

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REFERENCES

- Altshul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Breznak, J. & Canale-Parola, E. (1975). Morphology and physiology of *Spirochaeta aurantia* strains isolated from aquatic habitats. *Arch Microbiol* **105**, 1–12.
- De Ley, J. & Swings, J. (1984). Genus II. *Gluconobacter*. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 275–278. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- De Ley, J., Swings, J. & Gosselé, F. (1984). Genus I. *Acetobacter*. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 268–274. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Delić, V., Šunić, D. & Vlašić, D. (1989). Microbial reactions for the synthesis of vitamin C (L-ascorbic acid). In *Biotechnology of Vitamins, Pigments and Growth Factors*, pp. 299–336. Edited by E. J. Vandamme. London: Elsevier Applied Science.
- DeSoete, G. (1983). On construction of 'optimal' phylogenetic trees. *Z Naturforsch Sect C J Biosci* **38**, 156–158.
- Eden, P. A., Schmidt, T. M., Blakemore, R. P. & Pace, N. R. (1991). Phylogenetic analysis of *Aquaspirillum magnetotacticum* using PCR-amplified 16S rRNA-specific DNA. *Int J Syst Bacteriol* **41**, 324–325.
- Imai, K., Sakane, T. & Nogami, I. (1990). Biologically pure cultures of *Pseudomonas sorbooxidans* useful for producing 2-keto-L-gulonic acid. US Patent 4, 933,289.
- Koneman, E., Allen, S., Dowell, V. & Sommers, H. (1979). *Color Atlas and Textbook of Diagnostic Microbiology*, pp. 216–217. Philadelphia: Lippincott.

- Lafay, B., Ruimy, R., Rausch de Traubenberg, C., Breittmayer, V., Gauthier, M. J. & Christen, R. (1995). *Roseobacter algicola* sp. nov., a new marine bacterium isolated from the phycosphere of the toxin-producing dinoflagellate *Prorocentrum lima*. *Int J Syst Bacteriol* **45**, 290–296.
- Ledyard, K. M., Delong, E. F. & Dacey, J. W. H. (1993). Characterization of a DSMP-degrading bacterial isolate from the Sargasso Sea. *Arch Microbiol* **160**, 312–318.
- Maidak, B. L., Cole, J. R., Lilburn, T. G. & 8 other authors (2000). The RDP (Ribosomal Database Project) continues. *Nucleic Acids Res* **28**, 173–174.
- Marmer, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.
- Nogami, I., Shirafuji, H., Oka, M. & Yamaguchi, T. (1995). Method for producing 2-keto-L-gulonic acid. US Patent 5,474,924.
- Olsen, G. J., Matsuda, H., Hagstrom, R. & Overbeek, R. (1994). FastDNAml: a tool for the construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comput Appl Biosci* **10**, 41–48.
- Reichstein, T. & Grussner, A. (1934). Eine ergiebige synthese der l-ascorbinsäure. *Helv Chim Acta* **17**, 311–328.
- Ruiz-Ponte, C., Cilia, V., Lambert, C. & Nicolas, J. L. (1998). *Roseobacter gallaeciensis* sp. nov., a new marine bacterium isolated from rearings and collectors of the scallop *Pecten maximus*. *Int J Syst Bacteriol* **48**, 537–542.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989a). *Molecular Cloning: a Laboratory Manual*, 2nd edn, p. B.20. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989b). *Molecular Cloning: a Laboratory Manual*, 2nd edn, p. 1.42. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989c). *Molecular Cloning: a Laboratory Manual*, 2nd edn, p. B.15. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Shiba, T. (1991). *Roseobacter litoralis* gen. nov., sp. nov., and *Roseobacter denitrificans* sp. nov., aerobic pink-pigmented bacteria that contain bacteriochlorophyll *a*. *Syst Appl Microbiol* **14**, 140–145.
- Shiba, T. (1992). The genus *Roseobacter*. In *The Prokaryotes*, 2nd edn, pp. 2156–2159. Edited by A. Belows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Scheifer. New York: Springer.
- Sugisawa, T., Hoshino, T., Masuda, S., Nomura, S., Setoguchi, Y., Tazoe, M., Shinjoh, M., Someha, S. & Fujiwara, A. (1990). Microbial production of 2-keto-L-gulonic acid from L-sorbose and D-sorbitol by *Gluconobacter melanogenus*. *Agric Biol Chem* **54**, 1201–1209.
- Tengerdy, P. R. (1961a). Redox potential changes in the 2-keto-L-gulonic acid fermentation. I. Correlation between redox potential and dissolved oxygen concentration. *J Biochem Microbiol Technol Eng* **3**, 241–253.
- Tengerdy, P. R. (1961b). Redox potential changes in the 2-keto-L-gulonic acid fermentation. II. Correlation between redox potential and product formation. *J Biochem Microbiol Technol Eng* **3**, 255–260.
- Van de Peer, Y., Nicolai, S., De Rijk, P. & De Wachter, R. (1996). Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Res* **24**, 86–91.
- Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Yamada, Y., Katsura, K., Kawasaki, H., Widyastuti, Y., Saono, S., Seki, T., Uchimura, T. & Komagata, K. (2000). *Asaia bororensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the α -*Proteobacteria*. *Int J Syst Evol Microbiol* **50**, 823–829.
- Yin, G., Tao, Z., Yan, Z., Ning, W., Wang, C. & Wang, S. (1990). Fermentation process. US Patent 4,935,359.