8-1-2016

Ventosimonas gracilis gen. nov., sp. nov., a member of the Gammaproteobacteria isolated from Cephalotes varians ant guts representing a new family, Ventosimonadaceae fam. nov., within the order 'Pseudomonadales'

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INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

NOTE

Lin *et al.*, *Int J Syst Evol Microbiol* 2017;67:447–453

DOI 10.1099/ijsem.0.001650

MICROBIOLOGY SOCIETY

Saccharedens versatilis gen. nov., sp. nov., a sugar-degrading member of the *Burkholderiales* isolated from *Cephalotes rohweri* ant guts

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Abstract

Cephalotes 'turtle' ants host a core group of gut–associated symbionts, but their potential contributions to ant nutrition and disease resistance remain uncharacterized *in vitro*. To gain a better understanding of the metabolic capability of core symbionts belonging to the *Burkholderiales*, we cultivated and characterized strain CAG32^T from the guts of *Cephalotes rohweri* ants. Strain CAG32^T was rod-shaped, Gram-stain-negative, motile and formed pale-white colonies on trypticase soy agar. Optimum growth occurred under an atmosphere of 20 % O_2 supplemented with 1 % CO_2. Strain CAG32^T grew under NaCl concentrations of 0–2.0 %, temperatures of 23–47 °C and pH values of 4.0–8.0, and was capable of producing *n*-butyric acid and degrading carbohydrates for growth. The G+C content of the genomic DNA was 59.2±0.6 mol% and the major fatty acids were $C_{16:0}$, $C_{16:1}\omega7c/C_{16:1}\omega6c$, $C_{17:0}$ cylcopropane, $C_{12:0}$ and $C_{14:0}$ 3-OH/$C_{16:1}$ iso I. The only respiratory quinone detected was ubiquinone-8 (Q-8) and the major polar lipids were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. Based on phylogenetic analysis of the 16S rRNA gene sequence, strain CAG32^T shared 96.9 % nucleotide similarity with its closest cultivated neighbours *Bordetella petrii* Se-1111R^T and *Bordetella bronchiseptica* ATCC 19395^T. This, combined with differences in the phenotypic and biochemical profile from neighbouring strains, warrants the classification of strain CAG32^T as representing a novel species of a new genus within the *Burkholderiales* family *Alcaligenaceae*. The name *Saccharedens versatilis* gen. nov., sp. nov. is proposed. The type strain of *Saccharedens versatilis* is CAG32^T (=NCIMB 15010^T=DSM 100909^T).

Insects often harbour communities of gut-associated bacterial symbionts, many of which play crucial roles in host nutrition and disease resistance [1, 2]. Mutualistic relationships with gut microbiota are frequently the key drivers behind the radiation and ecological success of insects [2, 3]. Arboreal turtle ants (genus *Cephalotes*), for example, are a highly diverse and species-rich neotropical clade [4] that have clearly benefited from their gut symbionts [5]. Treatment with antibiotics is detrimental to the ants, indicating symbiosis with the gut community [6], and studies using PCR amplification and sequencing of the 16S rRNA gene have revealed that *Cephalotes* ants harbour a core, conserved gut microbiota [5, 7–9]. Bacteria in this core microbiota consistently include representatives from the *Burkholderiales*, *Rhizobiales*, *Xanthomonadales*, *Opitutales* and *Pseudomonadales* phylotypes [5]. These symbionts are stable, protected by a proventricular filter [10], and probably coevolved with their hosts [11], signifying their importance as autochthonous members of the gut community [8, 11].

Previous studies of the *Cephalotes* gut microbiota have been limited to culture-independent 16S rRNA gene surveys. As part of our ongoing efforts to determine the *in situ* physiology of these bacteria, we previously characterized representatives belonging to two of the five core clades from *Cephalotes* ant guts [12, 13]. However, more cultivated representatives of these core symbionts are needed for full investigation into their functional contributions to ant fitness. In this study, we report the cultivation and characterization of strain CAG32^T, a novel, sugar-degrading betaproteobacterium representing a novel species of a new genus within the *Burkholderiales* family *Alcaligenaceae*. We propose the name *Saccharedens versatilis* gen. nov., sp. nov. to accommodate strain CAG32^T.

Strain CAG32^T was isolated from the guts of *Cephalotes rohweri* ants on trypticase soy agar (TSA; Difco BD). *C. rohweri* ants were collected from Tucson Mountain Park, Tucson, AZ, USA (32.23° N 111.12° W) in 2010 and lab-reared as previously described [7] until degutting. The gasters (terminal

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Keywords: *Cephalotes rohweri*; symbiont; *Burkholderiales*; *Saccharedens versatilis*; gut microbiota; short-chain fatty acid.
Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SCFA, short-chain fatty acid.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAG32^T is KT258895.
Three supplementary figures are available with the online Supplementary Material.

abdominal segments containing most internal organs) of 12–15 ants were removed using sterile forceps and homogenized in a glass tissue homogenizer in sterile trypticase soy broth (TSB; Difco BD). Afterwards, the homogenate was serially diluted and spread onto TSA plates. The plates were incubated in air or in atmosphere-controlled glove boxes (Coy Labs) under hypoxic (2 % O_2, 5 % CO_2, 93 % N_2) or anoxic (5 % H_2, 5 % CO_2, 90 % N_2) conditions at room temperature for approximately 1 month. Resulting colonies were isolated, purified and then identified by 16S rRNA gene sequencing using the universal primers 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and 1389R (5′-ACGGGCGGTGTGTACAAG-3′) [14]. Strain CAG32^T was isolated from plates maintained under hypoxia but was routinely grown on TSA plates or TSB shaken at ~200 r.p.m. at 28–30 °C under atmospheric air (20 % O_2) supplemented with 1 % CO_2. Additional testing showed growth on brain heart infusion agar (Difco BD), non-haemolytic growth on blood agar [TSA containing 5 % (v/v) sheep blood], but no growth on nutrient agar (Difco BD) or R2A agar (EMD Millipore). Frozen stocks were prepared by suspending bacterial cultures in TSB supplemented with 20 % (v/v) glycerol and storing them in cryovials (Simport) at –80 °C.

A number of growth experiments and biochemical analyses were performed to characterize strain CAG32^T and its metabolic capabilities. Unless noted otherwise, all experiments were performed with 3–5 replicates under 1 % CO_2-supplemented air. To determine the optimum O_2 and CO_2 conditions for growth, cultures of strain CAG32^T were incubated in stoppered anaerobic culture tubes (Chemglass) containing 5 ml TSB and growth was monitored by spectrophotometry at a wavelength of 600 nm [15]. The TSB medium was deoxygenated by sparging the liquid with 100 % N_2. Under a starting headspace of 100 % N_2, culture tubes were injected with 100 % CO_2 or atmospheric air (20 % O_2). After injection, the overpressure was released to obtain final headspace concentrations of 0 or 1 % CO_2 with 0–20 % O_2. To test if the strain could grow under anoxic conditions by fermentation or anaerobic respiration, tubes containing 0 % O_2 with 0 or 1 % CO_2 were injected with a reducing solution of 0.05 % (final concentration) cysteine·HCl [16]. Several of these tubes were also supplemented with 10 mM (final concentration) sodium nitrate, sodium nitrite or sodium fumarate to test for the usage of alternative electron acceptors [17]. An increase in turbidity by at least 50 % of the optical density (OD_{600}) measured at the time of inoculation was considered evidence for growth.

Strain CAG32^T grew under atmospheres containing 1–20 % O_2 and did not require supplemental CO_2 for growth (Table 1). The generation time decreased as the O_2 concentration increased (Table 1), and growth for strain CAG32^T at 20 % O_2 and 1 % CO_2 was significantly faster compared to growth under all other conditions (P<0.05 by Student's t-test). Thus, optimum growth for strain CAG32^T occurred under an atmosphere of 20 % O_2 with 1 % CO_2. Strain CAG32^T did not grow in the absence of O_2 in any of the conditions tested (Table 1), suggesting an inability to grow by fermentation or anaerobic respiration under reducing conditions using any endogenous or alternative electron acceptors (nitrate, nitrite, fumarate) added to the medium. This, along with the positive correlation between the final cell yield (OD_{600}) and O_2 concentration (Table 1), strongly suggests that strain CAG32^T is an obligate aerobe. However, our *in vitro* tests for its ability to grow by anaerobic respiration were not exhaustive.

Table 1. Effect of headspace O_2 and CO_2 concentrations on the generation time and final optical density (OD_{600}) of *Saccharedens versatilis* CAG32^T

Growth was measured by spectrophotometry at a wavelength of 600 nm in stoppered anaerobic culture tubes without replacement of the atmosphere. Values are expressed as mean±SD generation times and OD_{600} and were obtained from 3–4 replicates. NG, No growth.

O_2 (%)	CO_2 (%)	Generation time (h)	Final OD_{600}
0	0	NG	–
0	1	NG	–
1	1	26.8±8.3	0.10±0.01
2	1	15.7±1.4	0.10±0.01
4	1	10.1±1.3	0.17±0.02
8	1	7.2±0.4	0.43±0.01
12	1	7.0±0.2	0.71±0.01
16	1	7.0±0.6	0.93±0.01
20	1	6.5±0.2	1.06±0.03
20	0	7.9±0.1	1.02±0.03

The NaCl concentration for growth was determined by incubating the bacteria in TSB media containing 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8 or 10 % (w/v) NaCl. Growth in 0 % NaCl was tested by incubating the bacteria on media containing all the components of TSA [1.5 % (w/v) peptone, 0.5 % (w/v) tryptone and 1.5 % (w/v) agar] but without NaCl. Optimum growth occurred at 0.5 % (w/v) NaCl, although growth was possible for strain CAG32^T from 0 to 2.0 % NaCl and was inhibited above 2.0 % NaCl. The pH tolerance was assessed by incubating the bacteria in stoppered anaerobic culture tubes with 5 ml TSB ranging in pH from 4.0 to 9.0 in 0.5 pH unit increments. The pH was buffered using 0.1 M citric acid (pH 4.0–4.5), 0.1 M succinate (pH 5.0–5.5), 0.1 M MOPS (pH 6.0–7.5) or 0.1 M Tris-HCl (pH 8.0–9.0). Growth was measured by spectrophotometry as before. Growth was possible within a pH range of 4.0–8.0, while optimum growth occurred between pH 7.0 and 7.5.

The temperature range for growth was determined by incubating the bacteria in stoppered 5 ml anaerobic culture tubes in temperature-controlled shakers (Benchmark) set at 23, 37, 40, 45 or 47 °C. Growth was measured by spectrophotometry as before. Strain CAG32^T was able to grow at 23–47 °C with optimum growth at 40 °C.

Substrate usage was determined by using GN2 microplates (Biolog) and API 20NE test strips (bioMérieux). GEN III microplates (Biolog) were used to characterize antibiotic susceptibility and the usage of additional carbon sources.

Enzyme activity was characterized by using the API ZYM system (bioMérieux). All substrate and enzyme assays were performed in duplicate according to the manufacturers' instructions. From GN2 microplates, strain CAG32^T was able to use adonitol, D-arabitol, D-fructose, D-galactose, D-glucose, *myo*-inositol, D-mannose, D-gluconic acid, DL-lactic acid, L-glutamic acid, proline, α-D-glucose 1-phosphate and D-glucose 6-phosphate. From GEN III microplates, strain CAG32^T was able to use 3-methyl glucose, D-fucose, glycerol, D-fructose 6-phosphate, glucuronamide, citric acid and D-serine. Strain CAG32^T grew in the presence of the antibiotics rifamycin SV, lincomycin, vancomycin, nalidixic acid and aztreonam. Examples of substrates that strain CAG32^T could not use included sucrose, cellobiose, raffinose, melibiose, L-fucose, α-D-lactose, lactulose, xylitol, pectin, formic acid, propionic acid, L-alanine, L-asparagine, L-aspartic acid, L-histidine, L-leucine and DL-carnitine.

Using the API ZYM system, strain CAG32^T tested positive for the enzymes alkaline phosphatase, esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Strain CAG32^T was negative for esterase (C4), lipase (C14), valine arylamidase, cysteine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Using the API 20 NE system, strain CAG32^T tested positive for the assimilation of D-glucose, D-mannose and potassium gluconate.

Many symbionts of insects are known to have beneficial functions, such as the ability to produce short-chain fatty acids (SCFAs) by incomplete oxidation of polysaccharides and organic acids [18]. To test for the ability of strain CAG32^T to produce SCFAs from the oxidation of glucose or lactate, the bacteria was incubated in 5 ml of (a) TSB or (b) phenol red broth base (Hardy Diagnostics) containing either 0.05 % (w/v) D-glucose or 10 mM sodium L-lactate. Cultures were shaken at ~200 r.p.m. at 28–30 °C for 4, 8, 24 or 48 h. After incubation, 1 ml of the culture fluid was removed and centrifuged at 10 000 ***g*** for 1 min. The supernatant was removed and filtered through a 0.22 µm filter, adjusted to pH 2–3 using 1.0 M HCl and stored at −20 °C until analysis. SCFAs in the supernatant were detected and quantified by GC as described in Zhao *et al.* [19] using a J&W fused silica column with a free fatty acid phase (DB-FFAP 125-3237; Agilent). Uninoculated media incubated under the same conditions were used as controls. No SCFAs were detected in the supernatant of strain CAG32^T grown in phenol red broth containing D-glucose or sodium L-lactate at all tested time periods, suggesting that the substrates were completely oxidized by the bacteria during growth. However, *n*-butyric acid was found in low concentrations (0.39±0.18 mM) in the culture fluid when strain CAG32^T was incubated in TSB media for 48 h. These concentrations were significantly higher than the concentrations detected in the uninoculated media (P<0.005 by Student's *t*-test, Fig. S1, available in the online Supplementary Material). Surprisingly, acetic acid was detected in the uninoculated TSB media (1.0±0.05 mM) but was absent in the culture fluid of the bacteria incubated in TSB under the same conditions (P<0.0001 by Student's *t*-test, Fig. S1), indicating that strain CAG32^T can consume acetic acid. To confirm this finding, the strain was incubated in phenol red broth base containing 10 mM sodium acetate under the same conditions above for 48 h and the culture fluid was analysed by GC. We found a significant decrease in acetic acid in the bacterial culture supernatant compared to the uninoculated broth (P<0.001 by Student's *t*-test, Fig. S2), providing evidence that strain CAG32^T uses acetic acid.

Catalase activity was determined by placing cells on a microscope slide and monitoring gas accumulation beneath a coverslip after the addition of 3 % H_2O_2. The presence of cytochrome c oxidase was indicated by a blue colour change of cells after the addition of oxidase diagnostic reagent (Becton Dickinson). Urease activity was tested by monitoring cells for growth and media colour change in urea broth (Difco BD) supplemented with TSB and trace mineral supplement (ATCC). Motility was tested by inoculating the bacteria in sulfide-indole-motility (SIM) medium (EMD Millipore). Strain CAG32^T was motile, positive for oxidase, and negative for catalase and urease.

For transmission electron microscopy, the bacteria were fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate solution and sent to the Michigan State University Center for Advanced Microscopy (East Lansing, MI, USA). Cells were post-fixed in 1 % osmium tetroxide in 0.1 M phosphate buffer for 1 h, dehydrated using a 30–95 % acetone graded series, embedded in Poly/Bed 812 (Polysciences) and cut into 70 nm ultrathin sections. The sections were then stained with uranyl acetate, lead citrate and examined under a Jeol 100 CXII transmission electron microscope. Strain CAG32^T was rod-shaped and ranged from 0.8 to 1.6 µm in length and 0.3 to 0.4 µm in width (Fig. 1). Strain CAG32^T appeared to have a Gram-negative type outer membrane and thin cell wall. Its Gram-negative status was determined by Gram staining followed by light microscopy.

To determine the cellular fatty acid composition, cultures were grown on TSA plates at 28–30 °C for 7 days and cells were pelleted in TSB containing 20 % (v/v) glycerol and frozen in rubber-sealed cryovials at −80 °C. Frozen cells were sent on dry ice to MIDI labs (Newark, DE, USA) for analysis using the Sherlock Microbial ID system (MIS). The analysis of cellular respiratory quinones and polar lipid composition from frozen cells was carried out by the identification service of the DSMZ (Braunschweig, Germany), according to Tindall *et al.* [20]. As shown in Table 2, the major fatty acids were $C_{16:0}$ (29.3 %), $C_{16:1}\omega7c$/$C_{16:1}\omega6c$ (21.1 %), $C_{17:0}$ cyclopropane (14.2 %), $C_{15:0}$ (8.0 %), $C_{12:0}$ (7.6 %) and $C_{14:0}$ 3-OH/$C_{16:1}$ iso I (5.3 %). The only respiratory quinone detected was ubiquinone-8 (Q-8) and the major polar lipids identified were phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG). An unidentified lipid, aminolipid and phospholipid were also detected (Fig. S3).

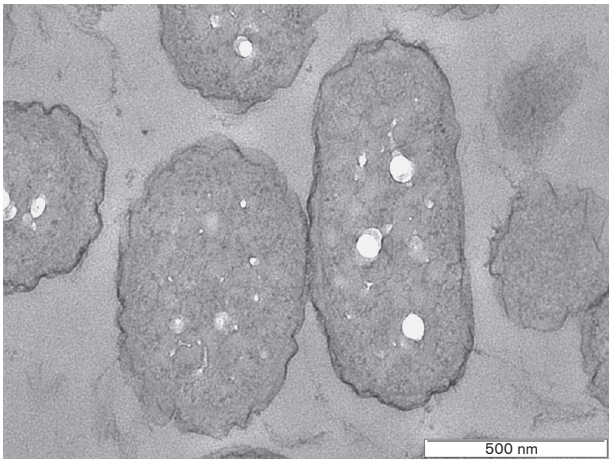

Fig. 1. Transmission electron micrograph of cells of *Saccharedens versatilis* CAG32^T. Bar, 0.5 µm.

To determine the genomic G+C content for strain CAG32^T, DNA was first extracted by using a Power Soil DNA extraction kit (MO BIO Laboratories) according to the manufacturer's instructions. The resulting DNA was digested by P1 nuclease and alkaline phosphatase and analysed by HPLC as described by Mesbah *et al.* [21] using a Zorbax normal-phase C18 column (880952-705; Agilent). The G+C content of the genomic DNA of strain CAG32^T was 59.2±0.6 mol%.

Table 2. Cellular fatty acid composition of *Saccharedens versatilis* CAG32^T compared to cultivated neighbours from the family *Alcaligenaceae*

Strains: 1, *Saccharedens versatilis* CAG32^T; 2, *Bordetella bronchiseptica* ATCC 10580; 3, *Bordetella bronchialis* LMG 28640^T (data from Vandamme *et al.* [26]); 4, *Pusillimonas harenae* B201^T (data from Park *et al.* [32]). Results are presented as percentages of total fatty acids. Major fatty acids (≥5.0 %) are highlighted in bold. –, Not detected; NR, not reported.

Fatty acid	1	2	3	4
$C_{12:0}$	**7.6**	0.7	1.0	**7.9**
$C_{12:0}$ 2-OH	–	2.3	**5.5**	–
$C_{14:0}$	3.3	4.8	2.0	1.7
$C_{14:0}$ 2-OH	–	–	**5.0**	NR
$C_{14:0}$ 3-OH/$C_{16:1}$ iso I	**5.3**	**5.7**	**15.8**	**8.8**
$C_{15:0}$	**8.0**	0.7	NR	0.7
$C_{15:0}$ 2-OH iso/$C_{16:1}\omega7c$	–	–	**8.5**	1.8
$C_{15:1}\omega6c$	4.3	–	NR	NR
$C_{16:0}$	**29.3**	**29.4**	**29.6**	**32.1**
$C_{16:1}\omega7c$/$C_{16:1}\omega6c$	**21.1**	**6.7**	NR	NR
$C_{17:0}$	2.4	1.8	NR	0.4
$C_{17:0}$ cyclopropane	**14.2**	**31.0**	**26.1**	**35.7**
$C_{18:0}$	0.7	**5.0**	3.3	0.6
$C_{18:1}\omega7c$	–	**6.5**	2.3	2.2
$C_{19:0}$ cyclopropane $\omega8c$	–	2.5	–	**6.0**

A cell lysate was made by mixing 2–3 loopfuls of bacteria grown on TSA plates for 7 days in 500 µl of sterile nanopure water. The mixture was vortexed, heated at 80 °C for 15–20 min and then incubated at –80 °C for 1 h. After thawing, the lysate was used for PCR amplification of the 16S rRNA gene using the general bacterial primers 63F, 334F, 786F, 519R, 939R and 1389R [14]. Amplification reactions were performed in a 25 µl mixture containing 2 µl cell lysate, 0.2 µM each primer, and a master mix containing 200 µM dNTPs, 1× *Taq* buffer, 1.5 mM $MgCl_2$ and 0.625 U *Taq* DNA polymerase (Empirical Bioscience). PCR was initiated with a 3 min denaturation step at 93 °C, followed by 30 cycles of denaturation at 93 °C for 30 s, primer annealing at 56 °C for 30 s, extension at 72 °C for 1 min and a final extension for 5 min. After the reaction, PCR products were purified using ExoSAP-IT (USB Corporation) and sent to the Michigan State University genomics core facility (East Lansing, MI, USA) for Sanger sequencing using an ABI 3730xl platform (Applied Biosystems). The sequences were trimmed and aligned with Sequencher v.5.4 (Gene Codes Corporation) and checked using the BLAST tool [22]. The resulting 16S rRNA gene sequence was used for phylogenetic analysis.

The 16S rRNA gene sequences were aligned using SINA [23]. Evolutionary distances between closely related strains were determined and phylogenetic analyses were performed in MEGA6 [24] using the maximum-likelihood, neighbour-joining and maximum-parsimony algorithms. Consistently across all three phylogenetic analyses, strain CAG32^T formed a monophyletic group within the *Burkholderiales* family *Alcaligenaceae* with 16S rRNA gene clones obtained from *Cephalotes varians* ants [8]. The placement of strain CAG32^T outside of the neighbouring genus *Bordetella* was robustly supported in all phylogenies with strong bootstrap values (Fig. 2). However, we found that the recently reclassified species *Verticiella sediminum* [25] occupied a unique phylogenetic position depending on the type of phylogenetic analysis used, as reported in Vandamme *et al.* [26]. Based on the 16S rRNA gene sequence, strain CAG32^T shared 96.9 % similarity with its closest cultivated neighbours *Bordetella petrii* Se-1111R^T [27] and *Bordetella bronchiseptica* ATCC 19395^T [28].

To compare the phenotypic characteristics of strain CAG32^T to its cultivated neighbours, the reference strain *Bordetella bronchiseptica* ATCC 10580 was grown on TSA under the same conditions and analysed for biochemical and cellular fatty acid properties as mentioned before. The phenotypic features of strain CAG32^T compared to other cultured relatives are summarized in Table 3. The results showed that strain CAG32^T has fatty acid ($C_{16:0}$ and $C_{17:0}$), respiratory quinone (Q-8) and polar lipid (PE, PG, DPG) profiles that are consistent with other members of the family *Alcaligenaceae* [29]. However, strain CAG32^T showed distinct characteristics from the members of its next closest clade, the genus *Bordetella*. First, strain CAG32^T was capable of degrading a number of sugar and sugar alcohol compounds (e.g. D-glucose and

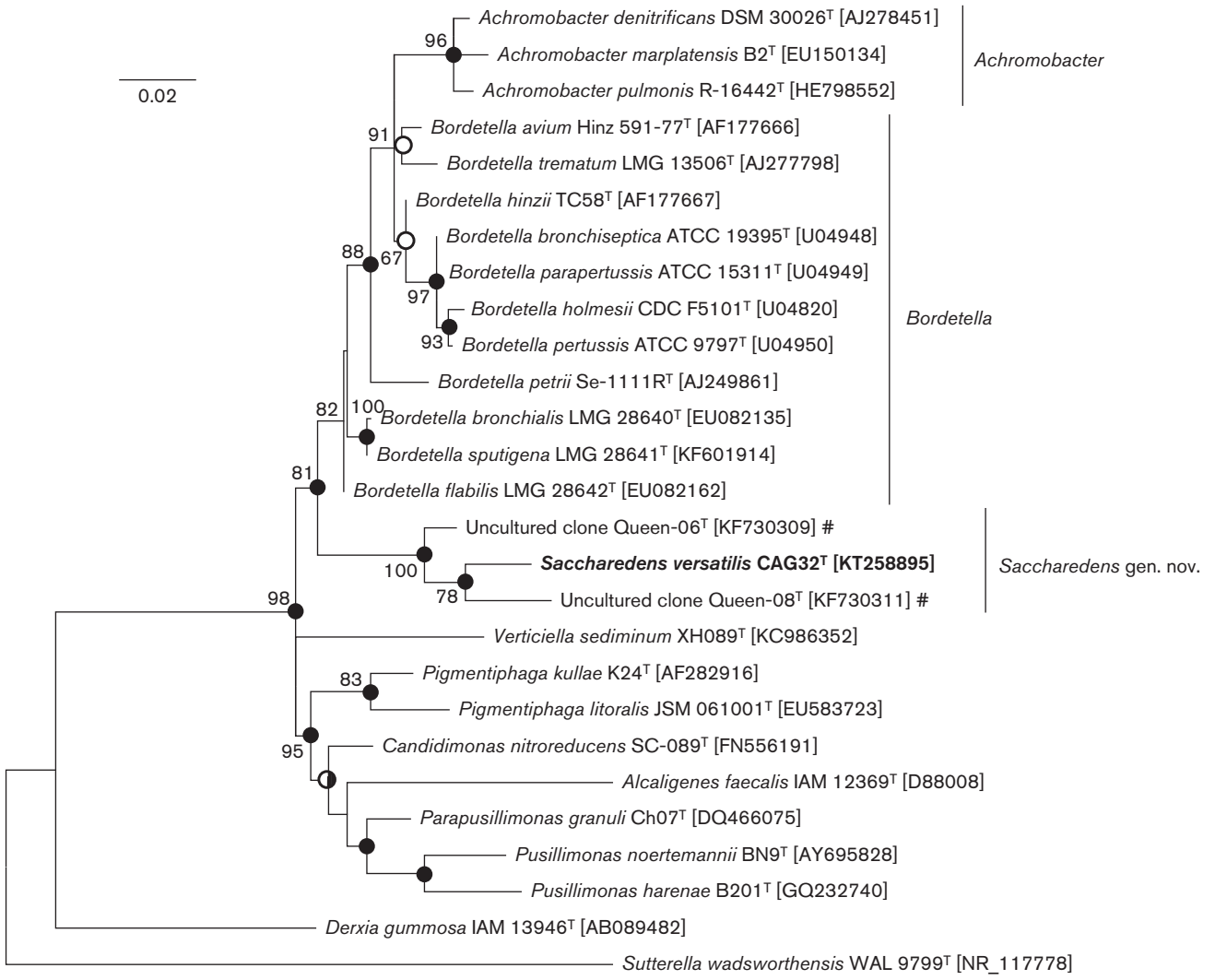

Fig. 2. Maximum-likelihood-based 16S rRNA gene phylogeny of strain CAG32^T. The alignment was generated using SINA v1.2.11. Phylogenetic analysis, including bootstrapping and tree visualization, was performed in MEGA6 [24] using the Tamura–Nei model and nearest neighbour interchange. Positions with less than 95 % coverage were eliminated, resulting in a total of 1363 shared nucleotide positions. The numbers at branch nodes indicate bootstrap support (1000 replicates) above 50 %. Closed circles at branch nodes represent conserved topologies across the three phylogenetic algorithms (maximum-likelihood, maximum-parsimony, neighbour-joining), partially closed circles represent conserved topologies across the maximum-parsimony and neighbour-joining algorithms, open circles represent conserved topologies across the neighbour-joining and maximum-likelihood algorithms, and the absence of circles at nodes indicates no conservation. Other members of the family *Alcaligenaceae* are given as reference species. GenBank accession numbers are shown in parentheses. #16S rRNA gene clones from *C. varians* guts. Bar, 0.02 changes per nucleotide.

D-arabitol). While members of the genus *Bordetella* can use organic and amino acids as carbon sources, they fail to use carbohydrates for growth [27, 30]. Second, strain CAG32^T had significantly different percentages of fatty acids in its cellular composition compared to strains from the genus *Bordetella* and its sister genus *Pusillimonas* (e.g. $C_{17:0}$ cyclopropane, Table 2). Third, the G+C content of the genomic DNA of strain CAG32^T was 59.2±0.6 mol%, which falls below the high range of 60–69 mol% that is characteristic for the genus *Bordetella* [27, 29]. Finally, strain CAG32^T shared 96.9 % 16S rRNA gene sequence similarity with its closest cultivated neighbours, which is below the 98.65 % cutoff recently proposed for species demarcation [31]. We believe that these biochemical and phylogenetic differences indicate that strain CAG32^T represents a novel species of a new genus within the family *Alcaligenaceae*. Accordingly, we propose the name *Saccharedens versatilis* gen. nov., sp. nov. to accommodate strain CAG32^T.

Interestingly, our analyses placed strain CAG32^T in a new sister genus to the genus *Bordetella*, a group that mainly consists of pathogenic or commensal host-associated strains that occasionally cause disease [30]. This close grouping of

Table 3. Differential characteristics between *Saccharedens versatilis* CAG32^T and other cultivated strains from the family *Alcaligenaceae*

Strains: 1, *Saccharedens versatilis* CAG32^T; 2, *Bordetella bronchiseptica* ATCC 10580 (G+C content from Daligault *et al.* [33]); 3, *Bordetella petrii* Se-1111R^T (data from von Wintzingerode *et al.* [27]); 4, *Bordetella bronchialis* LMG 28640^T (data from Vandamme *et al.* [26]); 5, *Pusillimonas harenae* B201^T (data from Park *et al.* [32]). Substrate usage and enzyme activity were determined using GN2 and GEN III microplates and API 20 NE test strips. +, Positive result; –, negative result; NR, not reported.

Characteristic	1	2	3	4	5
Isolation source	Ant gut	Dog lung	River sediment	Human lung	Beach sand
Cell shape	Rods	Rods	Rods	Rods	Rods
Cell width (μm)	0.3–0.4	0.2–0.5	0.4–0.7	0.2	0.5–0.7
Cell length (μm)	0.8–1.6	0.5–2.0	1.0–2.8	1.2	0.6–0.9
Colony colour	Pale white	Pale white	Cream white	Translucent	Ivory
Metabolism	Obligate aerobe	Obligate aerobe	Facultative	Facultative	Obligate aerobe
DNA G+C content (mol%)	59.2±0.6	68.2	63.8	67.5	53.1
Motility	+	+	–	+	+
Catalase	–	+	+	+	+
Urease	–	+	–	–	+
Temperature range (°C)	23–47	23–45	NR	28–47	15–45
pH range	4.0–8.0	5.0–9.0	NR	NR	5.0–9.0
NaCl range (%)	0–2.0	0–5.0	NR	0.5–3.0	0–6.0
D-Glucose	+	–	–	+ (weak)	–
D-Mannose	+	–	–	–	–

strain CAG32^T next to the genus *Bordetella* raises questions regarding its role in the ant gut and whether the isolate possesses characteristics that are detrimental to *Cephalotes* ants. However, previous studies have positioned *Burkholderiales* strains in monophyletic, *Cephalotes*-specific clusters, which is evidence that the bacteria share a stable and symbiotic association with *Cephalotes* ants [5, 7, 8]. Consistent with these findings, our phylogenetic analysis revealed that strain CAG32^T is most closely related to 16S rRNA gene clones obtained from *Cephalotes varians* ants (≥97.5 % similarity, Fig. 2) and forms a monophyletic cluster distinct from cultivated neighbours, signifying a long history of symbiotic coevolution with its host.

DESCRIPTION OF *SACCHAREDENS* GEN. NOV.

Saccharedens (Sac.char.e′dens. G. n. *saccharon* sugar; L. pres. part. *edens* eating; N.L. masc. n. *Saccharedens* a sugar-eating organism).

Cells are Gram-stain-negative and rod-shaped, and colonies on TSA are pale white. Metabolism is probably obligate aerobic. The major fatty acids are $C_{16:0}$, $C_{16:1}\omega7c/C_{16:1}\omega6c$, $C_{17:0}$ cylcopropane, $C_{12:0}$ and $C_{14:0}$ 3-OH/$C_{16:1}$ iso I and the major polar lipids are PE, PG and DPG.

DESCRIPTION OF *SACCHAREDENS VERSATILIS* SP. NOV.

Saccharedens versatilis (ver.sa′ti.lis. L. masc. adj. *versatilis* versatile).

Has the following characteristics in addition to those given for the genus. Cells are 0.8–1.6 μm in length and 0.3–0.4 μm in width. Growth is possible on brain heart infusion agar, blood agar (non-haemolytic), and on solid and in liquid trypticase soy medium at pH 4.0–8.0, with 0–2.0 % (w/v) NaCl, at 23–47 °C, and under atmospheres of 0–20 % O_2 with 0 or 1 % CO_2. Optimum growth occurs at pH 7.0–7.5, with 0.5 % (w/v) NaCl, at 40 °C, and under atmospheric conditions of 20 % O_2 with 1 % CO_2. The only respiratory quinone detected is Q-8. Cells are negative for catalase and urease and positive for oxidase, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Substrates used include adonitol, D-arabitol, D-glucose, D-fructose, D-galactose, D-glucose, 3-methyl glucose, D-fucose, *myo*-inositol, D-mannose, glycerol, acetic acid, citric acid, D-gluconic acid, DL-lactic acid, L-glutamic acid, glucuronamide, potassium gluconate, proline, D-serine, α-D-glucose 1-phosphate, D-glucose 6-phosphate and D-fructose 6-phosphate. Substrates not used include sucrose, cellobiose, raffinose, melibiose, L-fucose, α-D-lactose, lactulose, xylitol, pectin, acetic acid, formic acid, propionic acid, L-alanine, L-asparagine, L-aspartic acid, L-histidine, L-leucine and DL-carnitine. Produces *n*-butyric acid in TSB. Cells are resistant to rifamycin SV, lincomycin, vancomycin, nalidixic acid and aztreonam.

The type strain, CAG32^T (=NCIMB 15010^T=DSM 100909^T), was isolated from the gut of the arboreal ant *Cephalotes rohweri*, collected from Tucson, AZ, USA. The G+C content of the genomic DNA of the type strain is 59.2±0.6 mol%.

Funding information
This research was supported by a grant from the National Science Foundation (DEB-1442156 to J. T. W.) and a Calvin College Harvey Rozema Fellowship (to J. Y. L.).

Acknowledgements
We thank Scott Powell, Jon Sanders and Jacob Russell for the provision of *C. rohweri* ants, helpful discussions and advice. We acknowledge Alicia Withrow for transmission electron microscopy, Alanna Van Huizen for technical assistance and Scott Prentice for help with HPLC analysis. We are also grateful to Aharon Oren, Bernard Schink and David Noe for advice on nomenclature and construction of Latinized names.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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