Chromobacterium subtsugae sp. nov., a betaproteobacterium toxic to Colorado potato beetle and other insect pests

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Strain PRAA4-1^T, a motile, Gram-negative, violet-pigmented bacterium, was isolated from Maryland forest soil and found to be orally toxic to Colorado potato beetle larvae and other insects. Morphological, biological, biochemical and molecular characterization revealed that this strain was most similar to *Chromobacterium violaceum*, the type species and only currently recognized member of the genus *Chromobacterium*. DNA–DNA hybridization with *C. violaceum* ATCC 12472^T was 27 %. Phylogenetic analysis of 16S rRNA gene sequences revealed that strain PRAA4-1^T and *Chromobacterium violaceum* form a monophyletic clade, with the closest ancestral taxon *Vogesella indigofera* within the *Betaproteobacteria*. On the basis of phenotypic, genotypic and phylogenetic analyses, strain PRAA4-1^T (=NRRL B-30655^T=DSM 17043^T) is proposed as the type strain of a novel species of the genus *Chromobacterium*, *Chromobacterium subtsugae* sp. nov.

Strains of some bacterial species have insecticidal properties. For example, *Paenibacillus popilliae* (formerly *Bacillus popilliae*), which kills Japanese beetles (Dutky, 1940), *Bacillus thuringiensis*, which kills caterpillars, beetles, mosquitoes etc. (Schnepf *et al.*, 1998), *Serratia entomophila* and *Serratia proteamaculans*, which kill grass grubs (Jackson *et al.*, 1993), and *Photorhabdus luminescens*, which kills caterpillars (Forst & Nealson, 1996), have all been shown to be pathogenic or toxic to insects. Violet bacteria have infrequently been isolated from insects and are not considered as insect pathogens (Bucher, 1981). We have discovered a strain of violet bacteria that is orally toxic to Colorado potato beetle larvae and several other species of pest insects (Martin *et al.*, 2004).

Preliminary characterization, including positive protease and lecithinase activity and deep-violet colonies on nutrient agar, suggested that it was a member of the genus *Chromobacterium* (Gillis & Logan, 2005) rather than *Janthinobacterium*, another genus that contains species which produce violet pigment. *Chromobacterium violaceum*, the type species of the genus, produces a violet pigment, violacein (Hoshino *et al.*, 1987), synthesis of which is controlled by quorum sensing (McClean *et al.*, 1997). Violacein has been described as a trypanocide and having antibiotic properties (Durán *et al.*, 1994). *C. violaceum* also produces a variety of antibiotics (Durán & Menck, 2001). Furthermore, the sequence of the full genome of *C. violaceum* ATCC 12472^T suggests that insecticidal genes may be present (Brazilian National Genome Project Consortium, 2003). *Chromobacterium* strains are generally soil- and water-associated organisms (Hungria *et al.*, 2005).

Strain isolation

Strain PRAA4-1^T was isolated by suspending forest soil, rich in hemlock leaves, from the Catoctin Mountain region in central Maryland, in water and plating directly on Luria agar (L agar) without glucose (Atlas, 2004). The pH of the soil was 4.7 and the moisture content was 46.6 %. The original sample had a total aerobic microbial cell count of 1.75×10^6 c.f.u. (g soil)$^{-1}$. From this, approximately 80 violet colonies were recovered, one of which (PRAA4-1^T) was selected for further study because of its unusual colour. Since some pigmented bacteria, such as *Serratia marcescens*, are toxic to insects (Grimont & Grimont, 1978), we tested these bacteria for toxicity by feeding them to Colorado

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PRAA4-1^T is AY344056.

An electron micrograph of cells of strain PRAA4-1^T, photographs of colonies of strain PRAA4-1^T and *C. violaceum* ATCC 12472^T, results of Southern hybridization of genomic DNA from strain PRAA4-1^T and *C. violaceum* ATCC 12472^T and fatty acid profiles of strain PRAA4-1^T and related organisms are available as supplementary material in IJSEM Online.

potato beetle larvae on freeze-dried diet (Martin, 2004a). In the first bioassay, over 78 % of the 2nd instar beetle larvae fed diet containing PRAA4-1^T died within 3 days; this toxicity to Colorado potato beetle larvae led us to characterize the isolate further.

Cellular and colonial morphology

Strain PRAA4-1^T was found to be a facultatively aerobic, motile, Gram-negative betaproteobacterium. It was stained using a Gram stain kit (Difco). Cells were also shown to lyse in 30 % KOH. Unlike other *Chromobacterium* strains, cells had polar flagella. Cells were tapered rods, averaging 0.7 ± 0.1 μm wide and 2.4 ± 0.2 μm long as determined by electron microscopy. Occasional cells were curved (Supplementary Fig. S1 available in IJSEM Online). Colonies were deep-violet, smooth, regular and raised at 2–3 days (Supplementary Fig. S2b). Colonies formed initially at 24 h as small and cream coloured, and gradually turned light to dark violet from the centre outward during the next 24 h. In the presence of limiting oxygen as in a sealed container, violet pigment was not formed.

Phenotypic characterization

For phenotypic characterization, PRAA4-1^T and *C. violaceum* ATCC 12472^T were grown or tested at the same time under identical conditions. All tests other than temperature testing were performed at 25 °C. We used a variety of peptone-based media. TBAB (Difco) with 5 % sheep blood (Waltz Farms) was used for haemolysis testing; a clear or semi-clear zone around the colony indicated a positive test. *Bacillus cereus* agar base (without polymyxin B; Oxoid) with egg yolk (less than 24 h old) was used for lecithinase and lipase testing. A precipitate around a colony was a positive test for a lecithinase and a clear zone was a positive test for a lipase. TSA + Blood (Biolog) was used for carbon oxidation testing and M-9 (Atlas, 2004) for assessment of growth on minimal media. Bacterial growth was monitored by measuring the OD_{600} of liquid cultures. Growth in various concentrations of NaCl (1–3 %) at 25 °C was tested in L-broth. The effect of pH on growth was evaluated in L-broth adjusted to the appropriate pH with NaOH or HCl and growth was measured after 72 h at 25 °C by OD_{600}. The optimum growth temperature was evaluated on L agar. Growth was measured every 24 h for 6 days. Substrate utilization was evaluated using Gram-negative Biolog microtitre plates (Biolog version 3.5) and API 20 NE strips (bioMérieux).

Cell size was determined by measuring, at random, 50 stationary-phase cells (from a colony grown on L agar for 48 h) by electron microscopy ($\times 10\,000$ magnification) taken from 10 separate fields. To prepare cells for electron microscopy, cells of strain PRAA4-1^T were fixed for 2 h at room temperature by immersion in 3 % glutaraldehyde/0.05 M sodium cacodylate buffer (pH 7.0) and stored overnight at 4 °C. This was followed by washing in a sodium cacodylate buffer rinse, with six changes over 1 h, post-fixation in 2 % buffered osmium tetroxide for 2 h, dehydration in an ethanol series and infiltration with Spurr's low-viscosity embedding resin. Gold sections (10 nm) of the tissue were cut on a Reichert/AO Ultracut microtome with a Diatome diamond knife and mounted onto 200 mesh nickel grids. They were stained with 4 % uranyl acetate and 3 % lead citrate and viewed in an H-7000 Hitachi microscope at 75 kV. Alternatively, whole cells were measured from freeze-fracture scanning electron microscopy (Wergin & Erbe, 1991).

Motility was confirmed by the hanging drop method. Location of flagella was determined by staining with a modified Leifson stain (Forbes, 1981).

Colonies grew well on peptone-based media and on minimal medium, where the colonies were a very faint violet. On MacConkey agar, the colonies were also light violet. Strain PRAA4-1^T grew optimally at 25 °C, pH 6.5–8.0 and with 0–1.5 % (w/v) NaCl. Growth was barely detectable at 10 °C and at 40 °C and at pH 5.0 and 9.0. No growth was detected on media containing 3 % NaCl. Strain PRAA4-1^T was found to hydrolyse casein and produce a lecithinase and lipase on egg-yolk agar. The strain was not haemolytic on sheep blood. In the presence of oxygen, the strain was found to produce a violet pigment, matching the spectral and solubility properties of violacein (Hoshino *et al.*, 1987).

FAME

Fatty acid analysis was accomplished with the Sherlock Microbial Identification System (MIDI, Inc.). Both PRAA4-1^T and *C. violaceum* ATCC 12472^T were grown on TSA under identical conditions at 25 °C and analysis was repeated three times. Fatty acids were identified by GLC using the TSBA40 method (MIDI, 2002). Fatty acid identifications were confirmed by GC-MS using an Agilent 5890 GC with an Agilent 5970 mass spectrometer. Bacteria were identified by comparing the fatty acid profiles to the TSBA40 database of organisms provided with the Sherlock software.

The predominant fatty acid of PRAA4-1^T was $C_{16:1}\omega 7c$, representing 41.9 % of the total peak area. *C. violaceum* ATCC 12472^T had three fatty acids, cyclo-$C_{17:0}$, i-$C_{16:0}$ and $C_{12:1}$ 3-OH, that were not detected in PRAA4-1^T when compared under identical conditions. The MIDI System identified strain PRAA4-1^T as *Pseudomonas syringae* pv. *coronafaciens* with a low similarity index (0.403–0.650). Strain ATCC 12472^T was identified as *C. violaceum* with a similarity index of 0.470 (Supplementary Table S1).

DNA analysis

For determination of G+C content, DNA was degraded into nucleosides by the method of Mesbah *et al.* (1989) using *Escherichia coli* ATCC 11775^T as the calibration standard. DNA was released from cells using a French pressure cell. HPLC analyses were performed on an HP1100 apparatus using a Phenomenex Luna C18 column (3×250 mm).

Calculations were based on the ratio of deoxyguanosine to thymidine.

DNA for DNA–DNA hybridization was isolated by using a French pressure cell and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA reassociation was performed under optimal conditions (2× SSC plus 10 % formamide at 68 °C) with a Cary 100 Bio UV/VIS spectrophotometer (Varian) equipped with a Peltier-thermostatted 6×6 multi-cell changer and a temperature controller with *in situ* temperature probe (Huß *et al.*, 1983).

DNA was purified from strain PRAA4-1^T and *C. violaceum* ATCC 12472^T by standard methods for genomic DNA extraction from bacteria (Moore, 1995) for use in PCR, sequencing and Southern hybridization analysis. The 16S rRNA gene was amplified using primers universal to prokaryotes, R16F0 and R16R0 (Lee *et al.*, 1993), and sequenced using Big Dye terminator chemistry on an ABI Prism model 310 sequencer (PE Applied Biosystems) using these primers and chromo16SF1 (5′-AACGCTGGCGGC-ATGCTTTACAC-3′) and chromo16SF2 (5′-GAGGAAA-TCCCGCTGGTTA-3′), designed on the basis of the 16S rRNA gene sequence of *C. violaceum* ATCC 12472^T (GenBank accession no. M22510).

For hybridization analysis, digoxigenin-labelled (Roche) genomic DNA from *C. violaceum* ATCC 12472^T was used as a hybridization probe. Five micrograms of genomic DNA from PRAA4-1^T and *C. violaceum* ATCC 12472^T was digested separately with restriction endonucleases *Age*I and *Hin*dIII (Invitrogen) overnight at 37 °C, electrophoresed at low voltage through a 1 % agarose gel, transferred to nylon membrane and probed. The temperature for hybridization was 48 °C. Annealed DNAs were visualized using CSPD (Roche).

The 16S rRNA gene sequences of PRAA4-1^T, other *Chromobacterium* strains (Hungria *et al.*, 2005) and representative members of the *Betaproteobacteria* were aligned separately by the CLUSTAL V method with Lasergene software (DNASTAR). Phylogenetic analyses were performed using PAUP version 4.0b5 (Swofford, 2002). Uninformative characters were excluded from the analysis and the phylogenetic tree was constructed by the branch and bound algorithm to find the optimal tree(s). *E. coli* K-12 MG1655 was designated the outgroup to root the tree. Bootstrap analysis (10 000 replicates) was performed to estimate stability and support for the inferred clades.

The G+C content of strain PRAA4-1^T was 64.51 mol% (SD 0.14). The range of G+C content of the DNA of members of the genus *Chromobacterium* is 64–68 mol% (Gillis & Logan, 2005), and the G+C content for *C. violaceum* ATCC 12472^T by DNA sequencing was 64.83 mol% (Brazilian National Genome Project Consortium, 2003).

DNA–DNA hybridization with *C. violaceum* ATCC 12472^T revealed a mean relatedness of 27.2 %. Therefore strain PRAA4-1^T does not belong to the same species as *C. violaceum* ATCC 12472^T when the recommendations of a threshold value of 70 % DNA–DNA relatedness for the definition of bacterial species (Wayne *et al.*, 1987) are considered.

Phylogenetic analysis of 16S rRNA gene sequences revealed that strain PRAA4-1^T and *Chromobacterium* strains form a monophyletic clade, with closest ancestral taxon *Vogesella indigofera* (previously *Pseudomonas indigofera*; Grimes *et al.*, 1997), within the *Betaproteobacteria* (Fig. 1). Within the *Chromobacterium* clade, three potential subclades were identified. Strain PRAA4-1^T and two uncharacterized *Chromobacterium* strains isolated from the Brazilian Amazon region of Rio Preto (Hungria *et al.*, 2005) comprised one subclade and, on the basis of this analysis, may represent similar species from geographically distinct areas. Scholz *et al.* (2005) also found the *C. violaceum* species could be grouped into three distinct genotypes by PCR-RFLP analysis. The 16S rRNA gene sequence similarities between strains PRAA4-1^T and the two characterized members of *Chromobacterium*, *C. violaceum* ATCC 12472^T and *Chromobacterium* sp. MBIC3901, were respectively 97.4 and 98.3 %. However, the temperatures at which they grow were different, reflecting differences in strains isolated in the tropics compared with one isolated in a temperate climate. The 16S rRNA gene sequence similarity between strain PRAA4-1^T and *V. indigofera* ATCC 19706^T, the closest relative outside *Chromobacterium*, was 90.7 %.

Genomic similarity between strain PRAA4-1^T and *C. violaceum* ATCC 12472^T was also examined. A similarity coefficient (F) was calculated based on comparative analysis of their restriction patterns to be F = 0.64. The similarity coefficient was calculated by the method of Nei & Li (1979) as $F = 2N_{xy}/(N_x + N_y)$ where N_x and N_y are the total number of hybridized fragments in strains *x* and *y*, respectively, and N_{xy} is the number of hybridized fragment band positions shared by strains *x* and *y* (Supplementary Fig. S3).

Toxicity to insects

Bioassays were performed to assess toxicity of strain PRAA4-1^T to different insect species. Colorado potato beetles were reared from eggs to 2nd instar for bioassays on Insect Biocontrol Laboratory (IBL) potato leaf diet, a modification of the Forester diet (Gelman *et al.*, 2001). For bioassays, a freeze-dried version of this diet, without neomycin, was used as described by Martin (2004a). Final mortality was recorded at 120 h. The LC_{50} was calculated using the PROBIT procedure (SAS Institute, 2004). *C. violaceum* ATCC 12472^T was also tested against Colorado potato beetle larvae for toxicity. Corn rootworm assays were performed with field-collected adult beetles of mixed sexes as described by Schroder *et al.* (2001). Five adult beetles were added to each 100 cm Petri dish. Controls (without the bacteria) and each treatment were repeated five times. Mortality was recorded at 5 days after treatment. Corn rootworm larval assays were

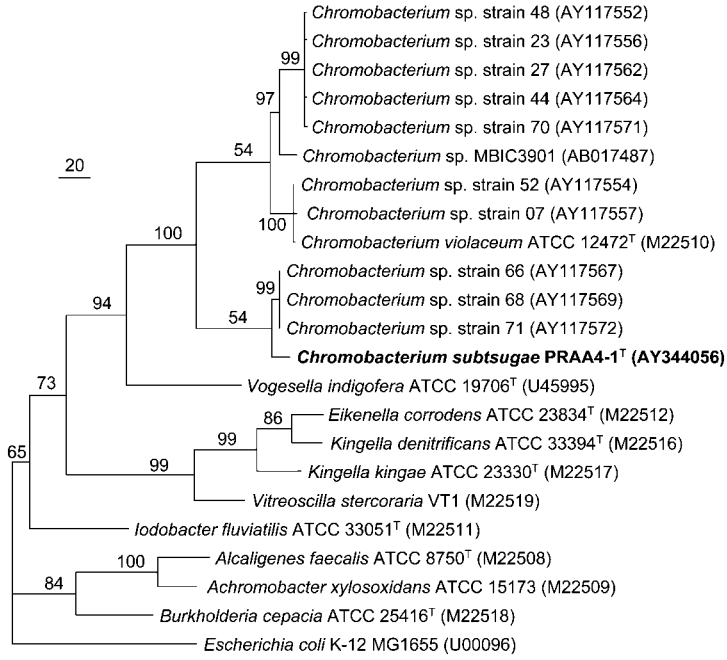

Fig. 1. Phylogenetic tree constructed by parsimony analysis (PAUP version 4.0b) of nearly full-length 16S rRNA gene sequences from representative *Chromobacterium* strains and other betaproteobacteria. 16S rRNA gene sequences of uncharacterized *Chromobacterium* strains 07, 23, 27, 44, 48, 52, 66, 68, 70 and 71, isolated from the Brazilian Amazon, were reported by Hungria *et al.* (2005). Sequences were aligned with CLUSTAL V (DNASTAR Lasergene software). *Escherichia coli* K-12 MG1655 was employed as the outgroup to root the tree. Bar, 20 inferred character state changes. Branch lengths are proportional to the number of inferred character state transformations. Bootstrap values greater than 50 % (measures of support for the inferred subclades) are shown as percentages on branches. GenBank accession numbers are shown in parentheses.

performed on freeze-dried corn rootworm diet (BioServe). Hive beetles were tested by combining PRAA4-1^T with field-collected pollen. Diamondback moth (*Plutella xylostella*) larval assays were performed on leaf discs of snap bean (*Phaseolus vulgaris* L. 'Roma II') as described by Farrar *et al.* (2001). Mortality was recorded at 7 days. Gypsy moth (*Lymantria dispar*) assays were the same as Colorado potato beetle except that gypsy moth diet was used (Bell *et al.*, 1981). Gypsy moth larvae were weighed at 7 days to determine sublethal effects. Gypsy moth egg masses were obtained from APHIS Otis Air Force Base and reared to 2nd instar on the same diet. *Culex pipiens* egg rafts were collected locally and mosquito larvae were raised in white enamel pans with 5 cm depth of de-ionized water and fed fish chow (Purina Mills). Ten 3rd instar larvae were tested in scintillation vials with 10 ml de-ionized water to which 100 µl PRAA4-1^T culture was added. Mortality was recorded at 48 h.

Toxicity to a variety of insects is summarized in Table 1. The LC_{50} for PRAA4-1^T to 2nd instar Colorado potato beetle larvae was $2.0 \pm 0.8 \times 10^8$ cells per diet pellet. The first indication of toxicity was cessation of feeding by the larvae. Less than 5 % mortality was observed in the water control or with *C. violaceum* ATCC 12472^T. Eighty per cent of the western or southern corn rootworm adult beetles that fed on PRAA4-1^T died within 5 days, compared with 8 % of controls. For diamondback moth 2nd instar larvae, the mortality was 90 % in 7 days. No control mortality occurred. None of the gypsy moth larvae died following

Table 1. Oral toxicity of strain PRAA4-1^T to various insect species

Insect	Stage	Mortality	Sublethal effects
Colorado potato beetle (*Leptinotarsa decemlineata*)	Adult	No	Feeding inhibition
Colorado potato beetle (*Leptinotarsa decemlineata*)	Larva	Yes	Feeding inhibition
Western corn rootworm (*Diabrotica virgifera*)	Adult	Yes	Unknown
Southern corn rootworm (*Diabrotica undecimpunctata*)	Adult	Yes	Unknown
Southern corn rootworm (*Diabrotica undecimpunctata*)	Larva	Yes	Feeding inhibition
Small hive beetle (*Aethina tumida*)	Larva	Yes	Feeding inhibition
Diamondback moth (*Plutella xylostella*)	Larva	Yes	Unknown
Gypsy moth (*Lymantria dispar*)	Larva	No	Feeding inhibition
Tobacco hornworm (*Manduca sexta*)	Larva	No	Feeding inhibition
Sweet potato whitefly (*Bemisia tabaci*)	Adult	Yes	Unknown
Sweet potato whitefly (*Bemisia tabaci*)	Nymph	Yes	Unknown
Southern green stink bug (*Nezara viridula*)	Adult	Yes	Unknown
Mosquito (*Culex pipiens*)	Larva	No	None observed

treatment with PRAA4-1^T, but the larvae which consumed PRAA4-1^T in their diet weighed 40 % less than the controls after 6 days (Martin, 2004b). There was no toxicity to mosquito larvae at 48 h.

Conclusions

In general, the morphological and biological characteristics of strain PRAA4-1^T, such as violacein production, lecithinase production and casein hydrolysis, were similar to those described by Gillis & Logan (2005) for the violet bacterium *C. violaceum* and as tested against the type strain ATCC 12472^T. Other characteristics in common include the absence of acid production from a variety of sugars including glucose, fructose, trehalose, sucrose, arabinose, mannose, xylose, salicin, mannitol, cellobiose and lactose. The Biolog assay based on substrate utilization identified PRAA4-1^T as *C. violaceum*, but with a low similarity index (0.604), suggesting that it was distinct from the type strain. Several differences from *C. violaceum* ATCC 12472^T were noticed during comparative characterization of toxic activity. Notably, the *C. violaceum* type strain was not toxic to Colorado potato beetle larvae, although it encodes a gene for an insecticidal protein, CV1887, with sequence similarity to that from *Photorhabdus luminescens* (Brazilian National Genome Project Consortium, 2003). Further, *C. violaceum* ATCC 12472^T grew, albeit poorly, at higher temperatures (42 °C) and used sucrose and citrate as sole carbon sources, while PRAA4-1^T did not (Table 2).

On the basis of 16S rRNA gene sequence similarity, phylogenetic analysis and genomic relatedness, strain PRAA4-1^T is clearly a member of the genus *Chromobacterium*. Stackebrandt & Goebel (1994) proposed that members of the same genus should be regarded as separate species if they have less than 97 % 16S rRNA gene sequence similarity. Wayne *et al.* (1987) also proposed that strains with less than 70 % DNA–DNA relatedness be considered separate species; the observed value of 27.2 % falls well below this threshold, and thus the strains are distinct at the species level. The designation of strain PRAA4-1^T within a species separate from the genus type species, *C. violaceum*, and other *Chromobacterium* strains is well supported. Further, fatty acid analysis profiles indicate a potentially closer relationship to the non-pigmented *Pseudomonas syringae* pv. *coronafaciens* than to the type species of the genus *Chromobacterium*. On the basis of polyphasic analysis, phylogenetic and phenotypic criteria and insect toxicity, we propose strain PRAA4-1^T as the type strain of a novel species of the genus *Chromobacterium*, *Chromobacterium subtsugae* sp. nov.

Description of *Chromobacterium subtsugae* sp. nov.

Chromobacterium subtsugae (sub.tsu′gae. L. pref. *sub* under; N.L. n. *Tsuga* scientific name of hemlocks; N.L. gen. n. *subtsugae* under *Tsuga*, because the type strain was found in soil under a hemlock tree).

Table 2. Comparison of biological characteristics of *C. violaceum* ATCC 12472^T and strain PRAA4-1^T

Data were derived in this study from a direct comparison of the two strains.

Characteristic	*C. violaceum* ATCC 12472^T	Strain PRAA4-1^T
Growth at/in:		
4 °C	−	+
45 °C	+	−
2 % NaCl	−	+
Oral toxicity to insects	−	+
Presence of fatty acids		
$C_{12:1}$ 3-OH, i-$C_{16:0}$, cyclo-$C_{17:0}$	+	−
$C_{17:1}\omega6c$	−	+
Use as a sole carbon source:		
Sucrose	+	−
Citrate	+	−
Acetate	−	+
Reduction of nitrate to nitrite	−	+
Hydrolysis of aesculin	+	−
Assimilation of:		
L-Mannose	−	+
Capric acid	−	+
DNA G+C content (mol%)	64.83	64.51
DNA–DNA hybridization with ATCC 12472^T (%)	100	27.2

Colonies start to form as cream coloured, but turn deep violet, starting from the centre, in 24–48 h in the presence of oxygen on peptone-based media. This colouration is due to the production of violacein, a violet di-tryptophan derivative. Colonies are smooth, regular and raised on L agar. Gram-negative, rod-shaped eubacteria. The mean cell dimensions are 0.7×2.4 μm. Acid is produced from fermentative metabolism of glucose, fructose and trehalose. While growth occurs in the presence of 2 % (w/v) NaCl, there is no growth in the presence of 3 % (w/v) NaCl. Optimal growth occurs at 25–28 °C, with sparse growth at 10 and 40 °C and no growth at 42 °C. The optimal pH range for growth is 6.5–8.0, and growth is inhibited below pH 4.5 and above pH 9.0. Casein is hydrolysed and a lecithinase and lipase are produced on egg-yolk agar. Not haemolytic on sheep blood. The predominant fatty acid is $C_{16:1}\omega7c$. The G+C content is 64.5 mol% (SD 0.1) (determined by HPLC). Using Biolog GN plates, the type strain oxidizes amino acids including D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, D-serine, L-serine and L-threonine. Sugars utilized included D-glucose, D-fructose and D-trehalose. Acids utilized included acetic acid, lactic acid, bromosuccinic acid and propionic acid. Other compounds found to be utilized as sole carbon sources are Tweens 40 and 80, *N*-acetylglucosamine, monomethyl succinate, inosine, glucose 1-phosphate and glucose 6-phosphate. Toxic to Colorado potato beetle, corn rootworm, small hive beetle, sweet potato whitefly, southern green stink bug and diamondback moth.

The type strain is PRAA4-1^T (=NRRL B-30655^T=DSM 17043^T), isolated from soil in the Catoctin Mountain region of Maryland, USA, from under a stand of hemlock trees (*Tsuga canadensis*).

Acknowledgements

The authors gratefully acknowledge S. Stone, C. Humphries, E. Patt, K. Moore, E. Soccer and H. Romani for assistance with characterization of strain PRAA4-1^T, M. Athanas and R. Farrar for insect bioassays, C. Taylor for electron microscopy, M. Sasser for use of MIDI database values in Supplementary Table S1, M. Pedroni for excellent technical assistance and A. Shropshire for coordinating studies and invaluable technical assistance.

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