**Rathayibacter agropyri** (non O’Gara, 1916) comb. nov., nom. rev., isolated from western wheatgrass (*Pascopyrum smithii*)

Brenda K. Schroeder,1 William L. Schneider,2 Douglas G. Luster,2 Aaron Sechler2 and Timothy D. Murray3,*

**Abstract**

*Aplanobacter agropyri* was first described in 1915 by O’Gara and later transferred to the genus *Corynebacterium* by Burkholder in 1948 but it was not included in the Approved Lists of Bacterial Names in 1980 and, consequently, is not recognized as a validly published species. In the 1980s, bacteria resembling *Corynebacterium agropyri* were isolated from plant samples stored at the Washington State Mycological Herbarium and from a diseased wheatgrass plant collected in Cardwell, Montana, USA. In the framework of this study, eight additional isolates were recovered from the same herbarium plant samples in 2011. The isolates are slow-growing, yellow-pigmented, Gram-stain-positive and coryneform. The peptidoglycan is type B2 containing diaminobutyric acid, alanine, glycine and glutamic acid, the cell-wall sugars are rhamnose and mannose, the major respiratory quinone is MK-10, and the major fatty acids are anteiso-15:0, anteiso 17:0 and iso-16:0, all of which are typical of the genus *Rathayibacter*. Phylogenetic analysis of 16S rRNA gene sequences placed the strains in the genus *Rathayibacter* and distinguished them from the six other described species of *Rathayibacter*. DNA–DNA hybridization confirmed that the strains were members of a novel species. Based on phenotypic, chemotaxonomic and phylogenetic characterization, it appears that strains CA-1 to CA-12 represent a novel species, and the name *Rathayibacter agropyri* (non O’Gara, 1916) comb. nov., nom. rev. is proposed; the type strain is CA-4 (=DSM 104101;=ATCC TSD-78)*.

The genus *Rathayibacter* belongs to the family *Microbacteriaceae* in the order *Actinomycetales* and the phylum *Actinobacteria*. Historically, species in genus *Rathayibacter* were classified as members of the genus *Corynebacterium* [1], and more recently of the genus *Clavibacter* [2]. Originally, plant pathogenic species in the genus *Corynebacterium* consisted of Gram-stain-positive, non-spore-forming, rod-shaped bacteria [1]; however, this was a heterogeneous genus [3–9] and Davis et al. [2] proposed establishing the genus *Clavibacter* comprising the Gram-stain-positive, coryneform bacteria containing 2,4-diaminobutyric acid (DAB) in their cell walls. At the time, plant pathogenicity was considered a distinguishing characteristic of the bacteria; however, the morphological and physiological characteristics, along with differences in menaquinone composition and phylogenetic analyses led to the discrimination of several coryneform species in separate genera [1, 10, 11]. Consequently, Zgurskaya et al. [12] proposed moving all of the species that contain B2y peptidoglycan with DAB in the cell wall, have MK-10 as the major menaquinone, phosphatidylglycerol and diphasphatidylglycerol as the major phospholipids, saturated anteiso-15:0, anteiso-17:0 and iso-16:0 as the major branched fatty acids, and rhamnose and mannose as the major cell-wall sugars, to the genus *Rathayibacter*. Sasaki et al. [13] subsequently proposed *Rathayibacter toxicus* comb. nov. based primarily on composition of its cell wall peptidoglycan content, phylogenetic analyses of 16S rRNA gene and menaquinone profiles, as well as other phenotypic characteristics. Currently, the genus *Rathayibacter* contains six validly described species including *Rathayibacter caricus* [14], *Rathayibacter festucae* [14], *Rathayibacter iranicus*, *Rathayibacter rathayi* (type species), *Rathayibacter tritici* [12] and *Rathayibacter toxicus* [13, 15]. In addition, there are 13 genome sequences deposited in NCBI GenBank including those of *R. rathayi* (NZ_OCNI0000000.1), *R. toxicus* (NZ_CP013292.1, NZ_CP010848.1, NZ_AUDP0000000.1, NZ_LBF101000000), *R. tritici* (NZ_CP015515.1) and one likely new species [16, 17], VKM Ac-2596 (NZ_CP015515.1), which has been provisionally named...
Aplanobacter agropyri [18] was first isolated from Agropyron smithii (now Pascopyrum smithii) collected in the Salt Lake Valley, Utah, USA in 1915 and described as Aplanobacter agropyri by O’Gara [18–20]. In 1923, Bergey et al. [20] transferred A. agropyri to Phytomonas agropyri; Burkholder [21] subsequently transferred it to the genus Corynebacterium as Corynebacterium agropyri. However, in 1980 when the Approved Lists of Bacterial Names [22] was published, neither A. agropyri, P. agropyri nor C. agropyri were included, because extant cultures were not available and C. agropyri and C. rathayi could not be distinguished on the basis of their original descriptions. Four bacterial strains resembling C. agropyri, CA-1 to CA-4T, were isolated in the 1980s (Table 1) [23]. Eight additional bacterial strains, CA-5 to CA-12, were isolated in the same way and later selected by O’Gara for CA-1 to CA-3 [23] from seed heads labelled as Agropyron species obtained from the Washington State University Mycological Herbarium exhibiting signs of dried bacterial ooze. Strain CA-4T was isolated from a fresh sample of Pascopyrum smithii in the same way and later selected as the type strain since it is the only isolate in this study obtained from freshly collected plant material and its characteristics are representative of other strains tested. No signs of nematodes were observed. Gram-stain reaction was determined on 48-hr-old bacterial cells as previously described [23] using the method described by Schaad [25].

Strains CA-1, CA-2 and CA-3 were cultivated on NBY agar, incubated at 28°C and 48-hr-old bacterial cells were visualized by scanning electron microscopy (SEM). For SEM, cells were harvested by scraping colonies from the agar surface, suspending them in water and filtering the suspension through Whatman 0.5 μm nucleopore filters (GE Healthcare Life Sciences). The filters containing bacterial cells were fixed in Karnovsky’s fixative, dehydrated through a graded ethanol series and critical-point dried in a Bomer critical-point bomb using CO2. Pieces of the filter were then placed on aluminum stubs, sputter-coated with gold and viewed with a Etec auto scan scanning electron microscope (Etec Systems). Strains were Gram-stain-positive and on NBY agar colonies were slow-growing, yellow-pigmented, round,  

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<tr>
<th>Strain</th>
<th>Host</th>
<th>Reference</th>
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<tr>
<td>Rathayibacter agropyri CA-1</td>
<td>Agropyron smithii* (now Pascopyrum smithii)</td>
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<tr>
<td>Rathayibacter agropyri CA-2</td>
<td>Agropyron trachycaulum* (now Elymus trachycaulus)</td>
<td>[23]</td>
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<tr>
<td>Rathayibacter agropyri CA-3</td>
<td>Agropyron riparium* (now Elymus lanceolatus)</td>
<td>[23]</td>
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<tr>
<td>Rathayibacter agropyri CA-4T (DSM 10410T, ATCC TSD-78T)</td>
<td>Pascopyrum smithii</td>
<td>This study</td>
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<tr>
<td>Rathayibacter agropyri CA-5</td>
<td>Pascopyrum smithii</td>
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<td>Rathayibacter agropyri CA-6</td>
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<td>Triticum aestivum</td>
<td>[4]</td>
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<td>Rathayibacter rathayi NCPPB 2980T</td>
<td>Dactylis glomerata</td>
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<td>Rathayibacter toxicus FH 79T (NCPPB 3552T)</td>
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<td>Rathayibacter toxicus FH 232 (FH 100)</td>
<td>Polygogon monspeliosis</td>
<td>[32]</td>
</tr>
<tr>
<td>Rathayibacter tritici NCPPB 18570T</td>
<td>Triticum aestivum</td>
<td>[4]</td>
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</table>

*Name given on original herbarium collection.
smooth and non-mucoid. The cells are non-motile, non-spore-forming coryneform rods with a size range of 0.36–0.69 × 0.65–1.57 μm (Fig. 1).

Phylogenetic analyses were completed using 16S rRNA gene sequences to confirm placement of bacterial strains CA-1 to CA-12 within the the Actinomycetales (Table S1, available in the online version of this article; Fig. 2), and subsequently in the Rathayibacter genus using 16S rRNA genes sequences of the type strain of the type species of the type genus of representative families. Efforts were also made to include sequences that were used in a previous phylogenetic analyses to solidify the phylogenetic placement of the Rathayibacter strains (26, 27) The 16S rRNA gene sequences were amplified from genomic DNA isolated using the Gram-positive protocol for Promega Wizard using primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) [28] and 1492 r(l) (5′-CCTTGTTACGACTTC-3′) [29] from strains CA-1 to CA-12 (Table 1) and sequenced by Elim Biopharmaceutical (Hayward, CA) using the GC rich protocol. 16S rRNA sequences for each strain obtained from two separate amplicons were assembled using Geneious software (Biomatters) and aligned with 16S rRNA gene sequences from bacteria in the order Actinomycetales (Table S1). CA strains having identical sequences were placed into haplotypes for analysis (Table S1). Neighbour-joining (NJ) and maximum-parisomony (MP) algorithms were completed in Geneious using PAUP* and MrBayes. Bootstrap analysis was completed with 1000 replicates. Trees generated by NJ and MP analyses were congruent and determined that strains CA-1 to CA-12 fell in a clade with Rathayibacter rathayi, separate from other genera in the Actinomycetales and supported by high posterior probabilities (0.56 or greater) (Fig. 2). Within the Rathayibacter clade, strains CA-1 to CA-12 formed two clades separate from R. rathayi, which suggests that the CA strains constitute a unique species. The 16S rRNA gene sequences of all CA strains, except CA-2 and CA-9, were identical and formed a monophyletic clade, supported by high posterior probabilities (Fig. 3). The 16S rRNA gene sequences of strains CA-2 and CA-9 differed from the other CA strains by one and two base pairs, respectively, and NJ and MP analyses placed them within the Rathayibacter clade but separate from the other CA strains (Fig. 2). NJ and MP analyses of the 16S rRNA gene sequences from the CA strains and other representative Rathayibacter species demonstrated that all CA strains fell into a strongly supported clade that included R. iranicus (Fig. 3); however, only strain CA-9 was in a subclade with R. iranicus. This is likely the result of the four nucleotide differences in the 16S rRNA gene sequences observed between R. iranicus and CA haplotype 1, CA-2 and CA-9 strains that are at the same nucleotide positions, as well as the one additional nucleotide position where CA-9 and R. iranicus are the same and different from CA-2 and CA haplotype 1 strains.

A preliminary multi-locus sequence analysis using rpoB, recA, gyrB and ppk gene sequences further supports the delineation of CA-1 to CA-12 strains from previously described Rathayibacter species (data not shown). Finally, DNA–DNA hybridization (DDH) was performed at DSMZ for strain CA-4T against Rathayibacter species strains R. rathayi (NCPPB 2980T), R. tritici (NCPPB 1857T), R. iranicus (NCPPB 2253T), R. carcis (DSM 15933T) and R. festucae (DSM 15932T) to confirm placement of the CA strains in the genus Rathayibacter. Based on DDH analysis, strain CA-4T exhibited 43.1±1.9% DNA relatedness with R. rathayi, 38.3±3.8% with R. tritici, 65.3±4.1% with R. iranicus, 40.0±5.3% with R. carcis, and 44.0±1.1% with R. festucae. Clearly, the placement of the CA strains as a separate Rathayibacter species is supported since all were below the 70% DDH threshold for unique species.

Bacterial strains CA-1 to CA-12 were evaluated for utilization and fermentation of specific carbon sources using the API Coryne, API20NE, API ZYMA and API 50 CH test strips (bioMérieux) with modifications to the protocols for API20NE and API CH test strips that included standardization of suspensions to 6 McFarland units, 1 ml of the suspension being added to the API 50 CHB/E medium, and results were recorded every 24 h for 4 days. Test results for strains CA-1 to CA-12 are recorded in Tables 2 and S2.
Bacterial strains CA-1 and CA-4<sup>T</sup> were evaluated for sensitivity to iranicin using the bacteriocin assay of Gross and Vidaver [30]. Strains were evaluated in triplicate against *R. iranicus* (NCPPB 2253<sup>T</sup>) and the assay was repeated four times (Table 2).

Analyses of the peptidoglycan, cell-wall sugars, respiratory quinones and polar lipids were performed by the Identification Service, Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany) for strains CA-1 and CA-4<sup>T</sup>. Thin layer chromatography (TLC) and two-dimensional silica gel thin-layer chromatography (2D-TLC) were used to determine that the peptidoglycan contained 2,4-diaminobutyric acid (DAB), alanine, glycine and glutamic acid. TLC was used to determine that whole cell-wall sugars of strains CA-1 and CA-4<sup>T</sup> contained rhamnose and mannose with lesser amounts of glucose (CA-1) and glucose and galactose (CA-4<sup>T</sup>). TLC on silica gel was used to separate the respiratory lipoquinones into different classes. The bands corresponding to the different classes of quinones were removed and analysed by high-performance liquid chromatography. The major respiratory quinones present for strains CA-1 and CA-4<sup>T</sup> were MK-10 (67%), MK-9(H8) (14%), and MK-11 (6%). Two dimensional-TLC determined that strains CA-1 and CA-4<sup>T</sup> contained three major polar lipids: diphosphatidylglycerol, phosphatidylglycerol and glycolipid. Fatty acids were extracted following the method by Sasser [31] and analysed using gas chromatography with a temperature ramping program (170 °C increasing 5 °C every minute until 270 °C was reached) for strains CA-1 to CA-12, as well at *R. iranicus*, *R. rathayi* and *R. tritici* (Table 1) grown for 48 h on tryptic soy broth agar at 28 °C. Sherlock MIS Software [31] was used to identify three prevalent fatty acids including anteiso-15 : 0 (approx. 50% of the total fatty acids), anteiso 17 : 0 (approx. 23%) and iso-16 : 0 (approx. 15%) characteristic of the genus *Rathayibacter*.

Therefore, based on this phenotypic, chemotaxonomic and phylogenetic study it is proposed that strains CA-1 to CA-12 should be placed in the genus *Rathayibacter* as a novel and unique species with strain CA-4<sup>T</sup> serving as the type...
Since neither *Aplanobacter agropyri* nor *Corynebacterium agropyri* were included in the Approved Lists [22], they were not validly published. Furthermore, because cultures from O’Gara’s work are not available for comparison and because the description provided by O’Gara differs in Gram-stain reaction (negative) and cell shape, we cannot conclude that the bacterium described in this study represents the same species described by O’Gara [18]. Regarding cell-shape and Gram-stain reaction, O’Gara noted in his original description [18] that ‘The organism is a short rod with rounded ends’ and ‘Many efforts at staining were made with Gram’s method but the results hardly warrant a conclusion. As a rule the stain was never completely lost nor was it ever retained in the original intensity…The mounts washed in alcohol lost a great deal of their intensity as compared with those not treated with alcohol and consequently the organism must be regarded as Gram-negative’, respectively. In Bergey’s sixth edition, Burkholder [21] describes *Corynebacterium agropyri* as ‘Gram variable’. Considering that the bacteria used in this study were isolated from the same plant species (some of which were collected over 60 years ago), with symptoms similar to those in O’Gara’s description [19], and collected from the same general geographic region, we propose naming this species *Rathayibacter agropyri* comb. nov., nom. rev. (non O’Gara, 1916) to recognize the pioneering work of O’Gara on bacterial plant pathogens and to indicate that this species is not the same as that described by O’Gara [18].

**DESCRIPTION OF RATHAYIBACTER AGROPYRI (NON O’GARA, 1916) COMB. NOV., NOM. REV.**

*Rathayibacter agropyri* (a.gro.py’ri) N.L. gen. n. *agropyri* from *Agropyron*, former generic name of western wheatgrass, the plant from which the type strain was isolated.

Basonym: *Aplanobacter agropyri* O’Gara 1916 [18]

The description for *Rathayibacter agropyri* comb. nov., nom. rev. differs from the original description notably in describing a coryneform, Gram-stain-positive bacterium. Several characteristics beyond those in the original description [18] and Burkholder’s [21] are provided.

Colonies on NBY agar are slow-growing, yellow-pigmented, round, smooth and non-mucoid. The Grain-stain-positive cells are non-spore-forming, non-motile, coryneform rods with a size range of 0.36–0.69×0.65–1.57 µm [23]. Reduced nitrates to nitrogen, oxidase-negative and catalase-positive. Utilizes glucose, arabinose, mannose, mannitol, maltose, potassium gluconate and malate as a sole carbon source. *N*-acetyl-glucosamine, capric acid, adipic acid, trisodium citrate...
and phenylacetic acid are not utilized as a carbon source. Acid is produced from D-glucose, D-xylene, D-mannitol, maltose, sucrose, glycerol, L-arabinose, methyl D-xylopyranoside, D-galactose, D-fructose, D-mannose, arbutin, salicin, cellobiose and inulin. Aesculin is hydrolysed, but not gelatin or urease. Indole production is positive but not arginine dihydrolase. Positive for pyrazinamidase, alkaline phosphatase, α-glucosidase and β-galactosidase enzymatic activity. No enzymatic activity of pyridoxal arylamidase, β-glucuronidase, β-galactosidase and N-acetyl-β-glucosaminidase. The cell-wall peptidoglycan is type B2γ and contains DAB, alanine, glycine and glutamic acid. Cell-wall sugars are rhamnose and mannose and to a lesser amount glucose. The major respiratory quinone is MK-10. The major fatty acids are anteiso-15:0, anteiso-17:0 and iso-16:0.

The type strain CA-4T (=DSM104101T, =ATCC TSD-78T) was isolated from Pascopyrum smithii collected near Cardwell, MT (N 45.749, W 111.849) in 1986.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

References


