

Research Paper

A glimpse into the genetic basis of symbiosis between *Hydrogenophaga* and their helper strains in the biodegradation of 4-aminobenzenesulfonate

Kangsan Kim^{1,2} and Han Ming Gan^{1,2,3} ✉

1. Genomics Facility, Tropical Medicine and Biology Platform, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, 47500 Selangor, Malaysia
2. School of Science, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, 47500 Selangor, Malaysia
3. Centre for Integrative Ecology, School of Life and Environmental Sciences, Deakin University, Pigdons Road, Waurm Ponds, Victoria 3216 Australia

✉ Corresponding author: Han Ming Gan, Email: han.gan@deakin.edu.au, Address: Room 4.338, Level 4, Building KA, School of Life and Environmental Sciences, Deakin University, Pigdons Road, Waurm Ponds, Victoria 3216 Australia

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Abstract

We report the whole genome sequences of *Hydrogenophaga intermedia* S1 and *Agrobacterium radiobacter* S2, the first reported bacterial co-culture capable of degrading 4-aminobenzenesulfonate (4-ABS), a recalcitrant industrial waste product. To gain insights into the genetic basis for the syntrophic interaction between this symbiotic pair and also another recently reported *Hydrogenophaga* associated co-culture, *Hydrogenophaga* sp. PBC and *Ralstonia* sp. PBA, we performed detailed genetic analysis of these four strains focusing on the metabolic pathways associated with biotin, *para*-aminobenzoic acid (pABA), and protocatechuate metabolism. Both assembled *Hydrogenophaga* draft genomes are missing a majority of the genetic components associated in the biosynthetic pathway of pABA and biotin. Interestingly, a fused pABA synthase was found in *R. sp. PBA* but not in *A. radiobacter* S2. Furthermore, using whole genome data, the taxonomic classification of *R. sp. PBA* and *A. radiobacter* S2 (both previously inferred from 16S rRNA gene) was re-investigated, providing new evidence to propose for their re-classification at the genus and species level, respectively

Key words: *p*-aminobenzoic acid, biotin, co-culture, phylogenomics, *Hydrogenophaga*, *Agrobacterium*, 4-aminobenzenesulfonate

Introduction

4-aminobenzenesulfonate (4-ABS) is a type of sulfonated aromatic amines that serve as key intermediates in the manufacturing of dyes, sulfa drugs, brighteners and pesticides. Presence of sulfonate moiety on aromatic ring renders the compound recalcitrant against biodegradation in activated sludge by aromatics-degrading bacteria [1-3]. In addition, the inhibitory effects of 4-ABS on prokaryotic folate metabolism presents another barrier to its utilization by common bacterial colonies

[4]. Not surprisingly, microbial degradation of 4-ABS is a rare occurrence among microbiomes found in natural soils, sediments and in activated sludge [5]. The first reported, and likely one of the most efficient microbial biodegradation of 4-ABS was reported in a co-culture consisting of *Hydrogenophaga intermedia* S1 and *Agrobacterium radiobacter* S2 isolated from Germany [1]. 15 years later, a co-culture consisting of another *Hydrogenophaga* strain but with a beta-proteobacterium, *Ralstonia* sp. PBA, was isolated

from textile wastewater in Malaysia similarly demonstrating efficient 4-ABS biodegradation ability [5, 6].

In strains S1/S2, it was demonstrated that the metabolites secreted by strain S2 e.g. p-aminobenzoate (*pABA*) and biotin, served as growth factors for strain S1, enabling the strain to degrade 4-ABS [6]. The first catabolic step of 4-ABS by S1 involves deamination of the compound to 4-sulfocatechol (4SC) [1]. The downstream reaction of 4-ABS degradation is characterized by ring cleavage of 4SC catalyzed by the combined actions of type II protocatechuate 3,4-dioxygenase alpha and protocatechuate 3,4-dioxygenase beta unit which are encoded by *pcaG2* and *pcaH2* respectively [7]. The catabolism of 4-SC involves ring cleavage and desulfonation catalyzed by PcaB2 (a modified 3-carboxy-cis,cis-muconate-lactonizing enzyme) and 4-sulfomuconolactone hydrolase, respectively, leading to the generation of maleylacetate that can be channeled into the tricarboxylic acid cycle [7-10].

The symbiotic relationship between *Hydrogenophaga* sp. PBC and *Ralstonia* sp. PBA is slightly different in that the helper strain PBA was not able to utilize 4-sulfocatechol as its sole carbon source and may depend on other compounds generated from the later step of 4-ABS metabolism to maintain growth [5]. Genomic resources for *Hydrogenophaga* sp. PBC and *Ralstonia* sp. PBA are now available [11, 12] and preliminary genomic analysis has shown that strain PBA contains two copies of *pabB* gene potentially allowing it to overproduce this growth factor. However, it remains unclear if similar genetic feature is present in *A. radiobacter* S2.

The species designation of strain S2 as *A. radiobacter* based on 16S rRNA phylotyping may be questionable given its atypical isolation source e.g. non-plant host. This is further complicated by the current lack of taxonomic consensus on the nomenclature and classification of *Agrobacterium* species [13]. Similarly, the genus designation of *Ralstonia* sp. PBA may benefit from additional investigation given its potentially unreliable classification based on 16S rRNA and also its substantially smaller genome size compared to other sequenced strains in the genus *Ralstonia* [14, 15].

In this study, we aim to (1) sequence the genome of *Hydrogenophaga intermedia* S1^T and *Agrobacterium radiobacter* S2, the first 4-ABS degrading co-culture strains to be reported, (2) understand the genetic basis for symbiotic relationship among 4-ABS degrading co-cultures, and (3) revisit the taxonomic status of *Agrobacterium radiobacter* S2 and *Ralstonia* sp. PBA using recent genomic concepts for taxonomic

demarcation.

Materials and Methods

Whole genome sequencing and genome assembly

Purified genomic DNA of strains S1 and S2 were tagged with Nextera XT (Illumina, San Diego, CA) and sequenced on the MiSeq desktop sequencer located at the Monash University Malaysia Genomics Facility using a 2 × 150 bp paired-end run configuration. Nextera adapter sequences in raw FASTQ sequence reads were trimmed using Trimmomatic software v0.35 [16], error-corrected and assembled *de novo* into contigs with SPAdes genome assembler v3.5.0 [17]. The assembled contigs were extended and scaffolded using SSPACE v3.0 [18]; gaps were removed with GapFiller v1.10 [19]. Average nucleotide identity (ANI) was calculated using JSpecies v1.2.1 [20].

Phylogenomic analysis and protein domain identification

Protein coding sequences were predicted from the assembled contigs using Prodigal v1.20, a gene prediction algorithm for prokaryotes [21] and subsequently utilized for phylogenomics tree construction with PhyloPhlAn. The percentage of conserved proteins (POCP) matrix was computed as previously reported [22]. Protein domain identification was performed using HMMsearch v3.1 [23] against selected TIGRFAM and Pfam profiles (Fig. 1A and B). For TIGRFAM profiles [24], significant hits were filtered using both trusted cutoff (the lowest-scoring known true positive that is above all known false positives) and noise cutoff (the highest-scoring known false positive) settings while for Pfam profiles [25], an E-value cutoff of 1e-5 was used [26].

Results and Discussion

Comparative analysis of genes involved in the biosynthesis of p-aminobenzoate

The absence of gene coding aminodeoxychorismate synthase (PabB) critical for the synthesis of *pABA* [27, 28] in both annotated genomes of strains S1 and PBC corroborates with their requirement for *pABA* to maintain growth in axenic culture (Figure 1A). Interestingly, two positive TIGRFam protein domain hits were detected from the annotated genes of strain PBA for PabB (TIGR00553) which catalyzes the formation of 4-amino-4-deoxychorismate from ammonia and chorismate [29]. However, the HMM scores for the TIGR03461 hits corresponding to PabC,

the catalytic enzyme involved in breakdown of 4-amino-4-deoxychorismate into *pABA* and pyruvate [30] were below the noise cutoff. Using an alternative HMM profile, PF01063 containing the amino-transferase class IV protein domain typically associated with but not specific to PabC, two significant hits were obtained of which one of them interestingly hit to a PabB protein (WP_009523382.1, contig AKCV01000025), indicating a potentially fused PabBC enzyme (Figure 1B). On the other hand, the second identified PabB (WP_009521599.1, contig AKCV01000019.1) was not genetically linked to any PF01063 domain-containing protein (Figure 1B).

Fused PabBC enzyme has been previously reported in several strains of gram-positive and gram-negative bacteria such as *Lactococcus lactis*, *Streptococcus pyogenes* and *Helicobacter pylori* [31]. Group of proteins that execute functionally reciprocal interactions incline to fuse together, giving rise to a protein modularity with defined domains of

biological functions [32, 33]. In addition, physical amalgamation of such kind endows selective advantage over allosteric gene regulation, metabolic features, protein size and many more – conferring a competitive edge over the survival of a strain [34]. In the case of fused PabBC in *Ralstonia* sp. PBA, this can potentially lead to the overproduction of *pABA* which can sustain the requirement of a *pABA* auxotroph partner e.g. *Hydrogenophaga* sp. PBC leading to the establishment of a stable symbiotic relationship. Further study is necessary however to confirm the function of both TIGR00553 domain-containing proteins and to measure the catalytic efficiency of the fused PabBC in *pABA* synthesis.

On the contrary, *A. radiobacter* S2 contains only one TIGRfam hit to PabB while the authentic PabC for this bacterium could not be confidently identified as none of the three genes coding for protein with hit to PF01063 was located on the same contigs as *pabB*. Surprisingly, subsequent search for TIGR03641 hits

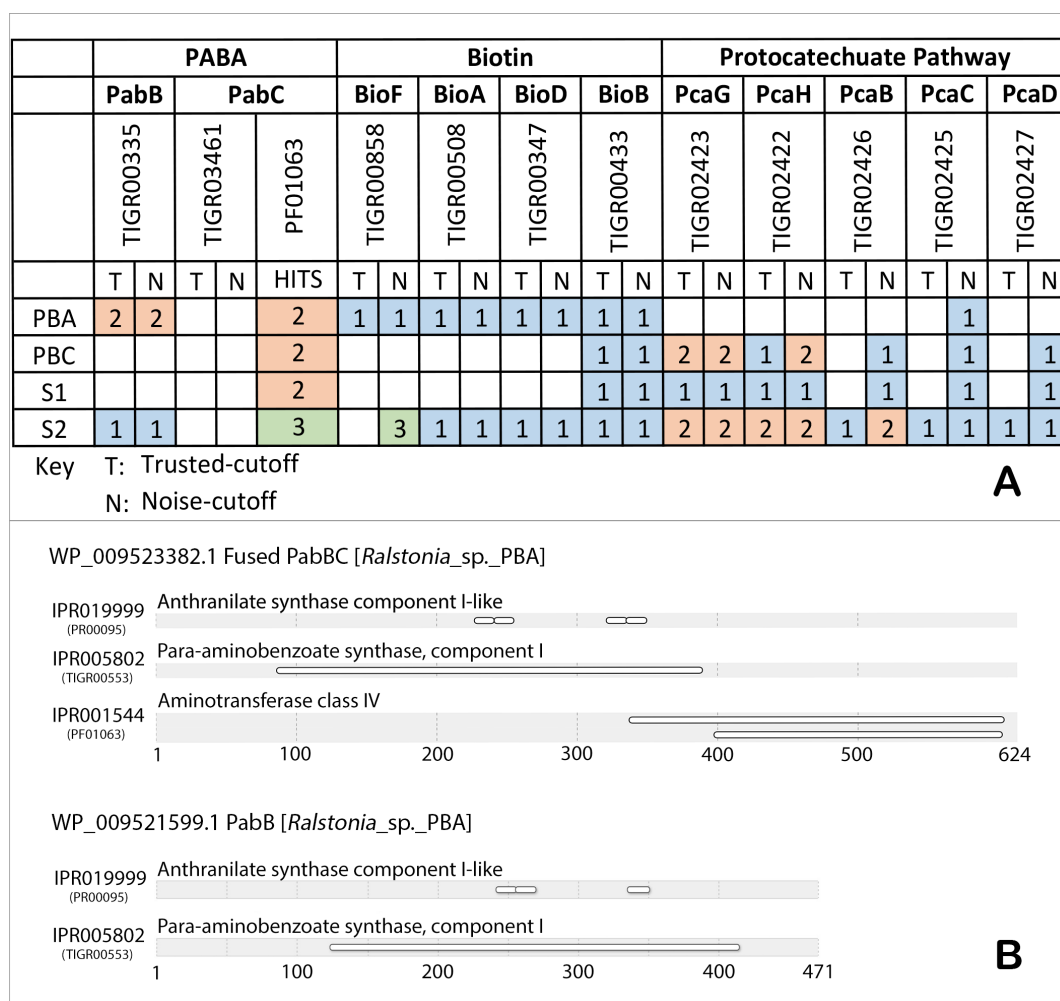


Figure 1. (A) Putative genetic catalogues of biosynthetic and metabolic pathways of *pABA*, biotin and protocatechuate in strain PBC, PBA, S1 and S2, as searched against TIGRFAM [24] and Pfam [25] databases. Number of hits designated to each of the strains was color annotated accordingly. (B) Fused PabBC and PabB protein domains identification in strain PBA, as searched against InterPro databases via InterProScan [26].

within the annotated genes of members from the genus *Agrobacterium* or even the known *pabC*-containing *Pseudomonas aeruginosa* strain PAO1 in Uniprot (as of 14 March 2017) returned zero hit (data not shown), suggesting the need to revise HMM scoring profile and threshold for TIGR03641 and/or the possible existence of alternative aminodeoxychorismate lyase as previously reported in *Chlamydia* [35]. Although we could not find genomic evidence to suggest enhanced *pABA* synthesis in strain S2, profligate synthesis of biotin instead has been demonstrated in wild-type *A. tumefaciens* (identical to *A. radiobacter* [36]) with biotin production per viable cell that is 20- and 60-fold greater than that of *E. coli* and *B. subtilis*, respectively. Such trait was subsequently suggested to be useful to *A. tumefaciens* in that the recipients of the cofactor will aid the growth and survival of *A. tumefaciens* [37].

Genes coding for enzymes involved in the biosynthesis of biotin are severely lacking in *Hydrogenophaga intermedia* S1 and *Hydrogenophaga* sp. PBC

Interestingly, while it was suggested that strains S1 and PBC were auxotrophs to biotin [5, 6], TIGRFAM search revealed the presence of a biotin synthase (BioB, EC:2.8.1.6) in their annotated genes (Fig 1A). Biotin serves as an integral cofactor in carbon fixation reactions [38], and BioB catalyzes the terminal-stage reaction of biotin synthesis through which it generates biotin from its precursor dethiobiotin [39]. Nonetheless, biotin biosynthesis encompasses myriads of biochemical reactions requiring other associated enzymes such as 8-amino-7-oxononanoate synthase, adenosylmethionine-8-amino-7-oxononanoate aminotransferase and dethiobiotin synthase which were not detected in the annotated genomes of strains S1 and PBC [38].

Protocatechuate and 4-sulfocatechol catabolism among helper strains

Ralstonia sp. PBA lacked a substantial portion of integral enzymes associated with the catabolism of protocatechuate and/or 4-sulfocatechol (Fig. 1A) corroborating with previous biochemical results [5, 40]. Unlike *Ralstonia* sp. PBA, *A. radiobacter* S2 could grow on 4-sulfocatechol secreted by *H. intermedia* S1 during 4-ABS catabolism as its sole carbon source [1]. Two types of protocatechuate dioxygenases were identified in the annotated genome of *A. radiobacter* S2 (Fig. 1B) which is consistent with previous molecular work showing that both variants could degrade protocatechuate with high efficiency but only one of them (protocatechuate 3,4-dioxygenase type II),

showed activity towards 4-sulfocatechol [41].

Taxonomic revision of *Agrobacterium radiobacter* S2 and *Ralstonia* sp. PBA

Phylogenomic analysis (Fig. 2) showed that strains currently assigned as *A. tumefaciens* (identical to *A. radiobacter* [36]) shared a common ancestor with *A. fabrum* and *A. sp.* ATCC31974. Among them, *A. tumefaciens* F2 was sister taxa to strain S2 with maximal SH-like bootstrap support. Surprisingly, with a suggested ANI parameter of ~95-96% for species delineation [20], 5 distinct genospecies could be identified for members within this clade. Two genomic clusters represented by two genome pairs e.g. (i) *A. tumefaciens* NCPPB3001-*A. tumefaciens* B6 and (ii) *A. fabrum* C58-*A. sp.* ATCC31749 could be designated with a valid *Agrobacterium* species name [20]. For strain S2 however, given the lack of >95% ANIm similarity to known *Agrobacterium* species, its current taxonomic assignment as *A. radiobacter* is not valid and we propose a more conservative classification of strain S2 as *Agrobacterium* sp. pending future taxonomic description.

The basal position of *Ralstonia* sp. PBA within the *Cupriavidus* clade indicates that it is more closely related to the genus *Cupriavidus* than *Ralstonia* (Fig. 3A). Pairwise comparison of the whole proteome of strain PBA against members of genus *Cupriavidus* generated median POCP value of 51% (Fig. 1D), which slightly surpassed the genus demarcation threshold of 50% proposed by Qin et al., 2014 [22] (Fig. 3B). Taken together, the data seemed to consolidate the proposed phylogenetic affinity of strain PBA to the genus *Cupriavidus* instead of *Ralstonia* but considering the intertwined biography of the genus *Cupriavidus* with several other β -proteobacteria (*Ralstonia*, *Wautersia*, *Alcaligenes*), genus classification for this group of bacteria could be fairly misleading [42]. In fact, strains that retain several basonyms, such as *Ralstonia eutropha* - previously known as *Cupriavidus metallidurans* or *Alcaligenes eutrophus*, well represent the history of intermingled nomenclature and taxonomy of the genus *Ralstonia* [42]. With the availability of more genomic resources, a more comprehensive genomic analysis of members of these related genera will be beneficial which is beyond the scope of this study.

Data Availability

The assembled draft genomes of strains S1 and S2 are 5 MB (124 contigs) and 5.7 MB (60 contigs), respectively, and have been deposited into the NCBI database under the accession numbers CCAE01000000 and CCAN01000000, respectively.

Whole genome sequencing for strains PBA and PBC has been previously reported [11, 12].

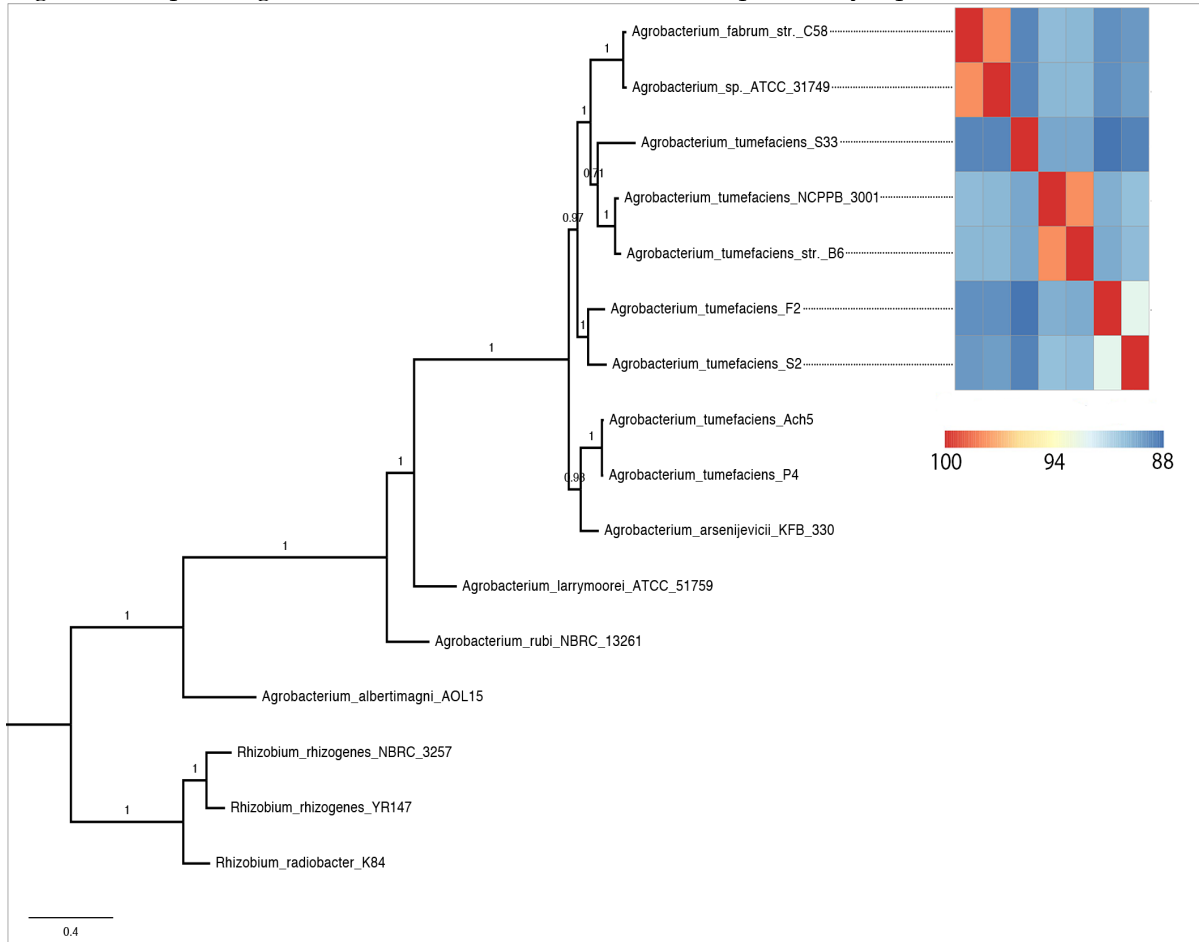


Figure 2. Maximum likelihood tree of the genus *Agrobacterium* rooted with members of the genus *Rhizobium* as the outgroup. Heatplot next to the tree is ANIm matrix generated from the 7 selected *Agrobacterium* strains.

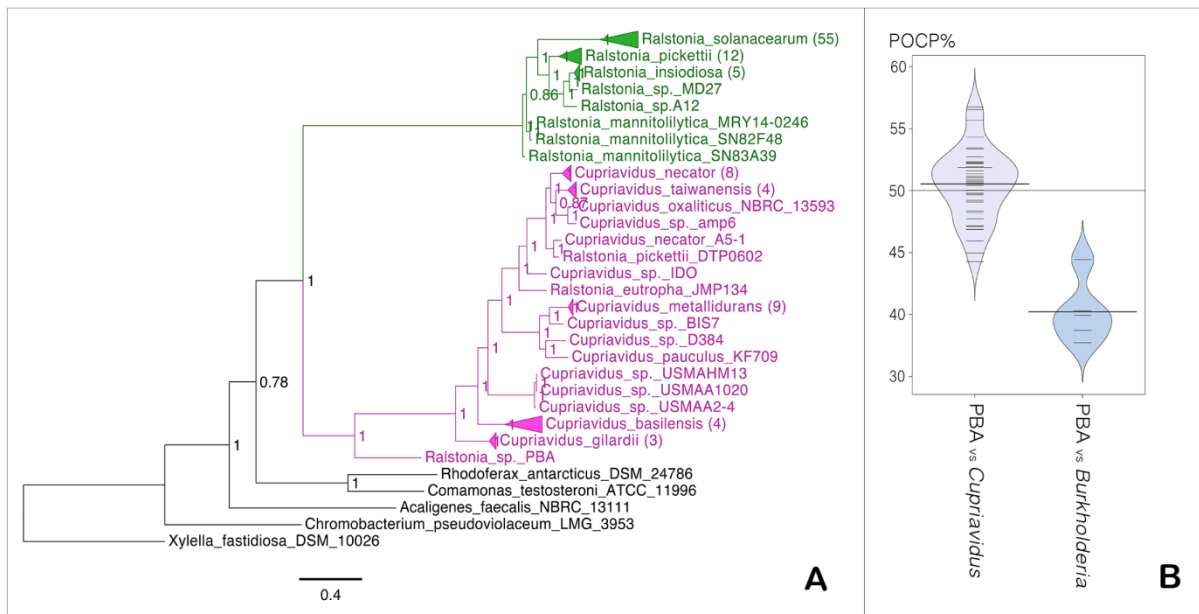


Figure 3. (A) Maximum likelihood tree of the genera *Cupriavidus* and *Ralstonia*. Values in nodes indicate SH-like support values, numbers in brackets indicate number of branches collapsed and scale bar indicates number of amino acid substitutions per site (B) Bean plot of POCP data calculated between PBA vs members of the genus *Cupriavidus* and *Burkholderia*.

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Competing Interests

The authors have declared that no competing interests exist.

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