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ABSTRACT

This work aimed to establish the taxonomic status of six strains (A212-S19-A16 ; A127-S21-F16 ; A105-S21-F16 ; A104-S21-F16 ; A101-S19-F16 and A35-S23-M15) isolated from three different waterways in 2015 and 2016 in the south east of France. Amplification and sequencing of the *gap*A housekeeping gene clustered these six strains together inside the *Pectobacterium* genus outside of already described or proposed *Pectobacterium* species and supspecies. Phenotypic analysis, using GENIII biolog plates performed with strains A212-S19-A16, A105-S21-F16, A101-S19-F16 and the closely related *P. polaris* (CFBP 1403), *P. carotovorum* subsp *odoriferum* (CFBP 1878^T), "*P carotovorum* subsp *actinidiae"* (CFBP 7370), *P. carotovorum* subsp *carotovorum* (CFBP 2046^T), "*P. carotovorum* subsp *brasiliense"* (CFBP 6617) or the most distantly related *P. aroidearum* (CFBP 8168^T) failed to identify specific compounds metabolized by these three strains but a weak activity was specifically observed at pH5 with these three strains. Illumina sequencing was used to sequence these six strains. Based on phylogenetic data, ANI, and in silico DNA-DNA hybridization, strains A212-S19-A16 ; A127-S21-F16 ; A105-S21-F16 ; A101-S19-F16 ; A35-S23-M15 ; A104-S21-F16 are suggested to represent a novel species of the genus *Pectobacterium*, for which the name *Pectobacterium aquaticum* sp. nov. is proposed. The type strain is A212-S19-A16 ^{T} (=CFBP 8637^T =NCPPB 4640^T).

The genus *Pectobacterium* gathers seven recognized species, *Pectobacterium carotovorum*, *P. atrosepticum*, *P. betavasculorum*, *P. wasabiae, P. aroidearum, P. polaris*, *P. parmentieri,* and the recently proposed "*P. peruviense"*(1-6). The *P. carotovorum* species is itself highly heterogeneous and several recognized and proposed subspecies have been ascribed to *P. carotovorum* to account for this heterogeneity: *P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *odoriferum*, "*P. carotovorum* subsp. *brasiliense"* and "*P. carotovorum* subsp. *actinidiae"* (7-12).

Altogether, bacteria belonging to the *Pectobacterium* genus have a broad host range and cause soft rot and blackleg disease on a wide variety of plants, including important crops

and ornamentals (13). As these bacteria collectively cause big losses on agricultural field, *Pectobacterium* species have been mainly described so far following bacterial sampling and isolation from diseased host plants. For example, *P. wasabiae* type strain has been isolated from the diseased rhizomes and fibrous roots of Japanese horseradish (14) and *P. parmentieri* type strain from symptomatic potato plants (4).

In contrast, the diversity of *Pectobacterium* sp. outside the plant context remains largely unexplored. Early studies showed that *Pectobacterium* sp. could be recovered from various non-host environments, such as water, soil or air (15-18). However, the taxonomic status of most of the isolated strains in these early works was not clearly assigned since most *Pectobacterium* species were described later on. Therefore, is it still unclear which particular *Pectobacterium* species could be recovered from various non-host environments. Furthermore, it remains to be determined if sampling outside diseased plants will allow the discovery of new *Pectobacterium* species.

Isolation and Ecology

This study aimed to establish the taxonomic status of six strains isolated from three different fresh water river streams in 2015 and 2016 in the south east of France (Table1). Following sampling, 500 ml of fresh water was filtered through 0.2 µm pore filters (Sartorius cellulose acetate filters), the bacteria present on the filters were suspended in 1 ml sterile distilled water and 100 µl of the suspension were poured onto semiselective modified single-layers CVP_{AG366} plates (same medium as described in (19) except that tryptone was not added to the medium, hereafter described as CVP). After 2 days of growth at 28°C, strains forming pits on CVP medium were further isolated, named A35-S23-M15, A101-S19-F16, A104-S21-F16, A105-S21-F16, A127-S21-F16 and A212-S19-A16 (table 1) and stored in 40% /60% glycerol/ LB liquid medium (10 g tryptone, 5 g yeast extract, 10 g NaCl per one liter of medium) at -80°C.

16S RNA and *gap*A phylogeny

The 16S rRNA rrs barcode was amplified and sequenced using the universal PCR primers 8F (AGAGTTTGATCCTGGCTCAG) and 1542R (AAGGAGGTGATCCAGCCGCA). The sequenced DNA fragments were identical between strains A35-S23-M15, A101-S19-F16, A127-S21-F16 and A212-S19-A16 and displayed 5 nucleotide changes with the sequences of the strains A104-S21-F16, A105-S21-F16 (Fig. S1). Blast search with complete genome of *Pectobacterium* sp. identified 16S rRNA sequences of related *Pectobacterium* sp. that were used to build up a 16S phylogenetic tree (supplemental S1). There was only 33 informative sites along the sequences and the generated rRNA tree is not informative at the specie level as already stated by Aldeolu *et al.* (2016) (20). However, amplification and sequencing of the *gap*A housekeeping gene was recently described to rapidly characterize the different *Pectobacterium* species (21). Therefore, to delineate the taxonomic position of the six isolated strains, amplification and sequencing of the *gap*A housekeeping gene was performed (sequences provided in supplemental S2). This highlighted that these six strains clustered together and formed a new clade outside of the already described *Pectobacterium* species and subspecies (Fig. 1 (a)).

Genome Features

To further gain insight into the taxonomic status of these six strains, draft illumina sequences were obtained. For preparation of genomic DNA, the strains were first grown overnight at 28°C on solid LB medium. A single colony was then picked up and grown overnight in 2ml of liquid LB medium at 28°C with 120 rpm shaking. Bacterial cells were harvested by centrifugation (5min at 12,000 rpm) and DNA was extracted with the wizard® genomic DNA extraction kit (Promega) following the supplier's specification. DNA was suspended in 100µl of sterile distilled water and the quantity and quality of DNA was assessed by nano-drop measurement, spectrophotometry analysis and agarose gel electrophoresis at 1%.

Genome sequencing was performed at the next generation sequencing core facilities of the Institute for Integrative Biology of the Cell, Avenue de la Terrasse 91190 Gif-sur-Yvette France. Nextera DNA libraries were prepared from 50ng of high quality genomic DNA. Paired end 2x75pb sequencing was performed on an Illumina NextSeq500 instrument, with a High Output 150 cycle kit. CLC Genomics Workbench (Version 9.5.2, Qiagen Bioinformatics) was used to assemble reads. Final sequencing coverages were between 60 and 180 (table 1). Coding sequences were predicted using the RAST server (22) with the Glimmer 3 prediction tool (23). Statistics of the six draft genomes are presented in table 1. A phylogenetic tree, constructed from concatenated sequences of 523 homologous genes, confirmed that these six strains clustered together outside of the previously described species or subspecies (Fig. 1 (b)).

Pairwise comparison of the six genomes and other *Pectobacterium* genomes representative of known and proposed species and subspecies was performed using the average nucleotide identity (ANI) calculator (Table 2 and supplemental S3 for more distantly related *Pectobacterium* sp.) (24). Pairwise ANI values between the six studied genomes were ≥ 98%. In contrast, pairwise ANI values of these six genomes with those of known *Pectobacterium* species and subspecies dropped below 94,5%, below the suggested cut-off value of 95–96% to delineate bacterial species. Digital DNA-DNA hybridisation (dDDH) is an *in silico* method to approach the wet-lab DDH method as closely as possible (25). dDDH were calculated between the six genomes and *Pectobacterium* genomes representative of known species and subspecies (Table 2 and Table S1 for more distantly related *Pectobacterium* sp.). dDDH pairwise values between the six studied genomes were above 86%, well above the 70% species boundary. When pairwise calculations were performed between these six genomes with those of known *Pectobacterium* species and subspecies the estimated dDDH values dropped below 57%, well below the species boundary.

Physiology and Chemotaxonomy

Tests on potato showed that the six studied strains were able to macerate potato tuber slices (supplemental S4). Biochemical tests were performed using BIOLOG GENIII plates with strains A212-S19-A16, A105-S21-F16, A101-S19-F16 and the closely related *P. polaris* (CFBP 1403), *P. carotovorum* subsp *odoriferum* (CFBP 1878^T), "*P carotovorum* subsp *actinidiae*" (CFBP 7370), *P. carotovorum* subsp *carotovorum* (CFBP 2046^T), "*P. carotovorum* subsp

brasiliense" (CFBP 6617) or the most distantly related *P. aroidearum* (CFBP 8168^T). The plates were treated according to the manufacturer's instructions using inoculation fluid A except for strains CFBP 1878^T and CFBP 2046^T for which inoculation fluid B was used to better adjust the metabolism rate of these two strains and read with an Omnilog^{TM} apparatus at 48h. With most of the tested compounds (68 out of 93), all the *Pectobacterium* sp. tested showed similar reactions. For 25 compounds or conditions tested, variable reactions were observed depending on the *Pectobacterium* species tested (Table 3). The three tested *Pectobacterium* strains, A212-S19-A16 , A101-S19-F16 and A105-S21-F16 always reacted similarly. However, strain A212-S19-A16 and strain A101-S19-F16 showed a weaker activity than strain A105-S21-F16 when tested for lithium chloride and L-Glutamic acid (Table 3). We could not identify specific compounds specifically metabolized by these three strains. With the three studied strains, contrary to the other tested strains, a weak activity was observed at pH5 and the three strains were also specifically unable to use D-Trehalose and D-Cellobiose as carbon source (Table 3). However, strains of *P. betavasculorum* not tested in our study, were also described by Gardan *et al* (2) as unable to use D-Cellobiose, therefore this feature is not specific to the three tested strains.

In conclusion, DNA-DNA relatedness analysis, whole genome sequences, phylogenetic data, phenotypic characterisation, indicates that this cluster of six strains represent a novel species, included in the *P. carotovorum* complex but distinct from the already described and proposed species and subspecies, for which the name *Pectobacterium aquaticum* sp. nov. is proposed.

Protologue

Pectobacterium aquaticum (*aquaticum* pertaining to the fresh water river stream from which the type strain was isolated).

Gram-negative, motile bacterium. Grows optimally at 28°C in LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar per one liter of medium) forming colonies within 24h. Causes maceration of potato tuber slices and, when on grown at 28°C on CVP medium (19), forms pits within 48h. Using GENIII biolog® plates, positive for D-Raffinose, α-D-Glucose, Pectin, α-D-Lactose, D-Mannose, D-Mannitol, D-Galacturonic Acid, Methyl pyruvate, D-Melbiose, D-Fructose, β-Methyl-D-Glucoside, D-Galactose, myo-Inositol, D-Gluconic Acid, D-Salicin, Glycerol, L-Aspartic Acid, Gentiobiose, N-Acetyl-D-Glucosamine, D-Glucose-6-PO4, Sucrose, D-Fructose-6-PO4, Mucic Acid, L-Rhamnose, D-Aspartic Acid, L-Malic Acid, Acetic Acid, L-Serine, D-Saccharic Acid, Bromo-Succinic Acid, 1% and 4% NaCl, 1% Sodium Lactate, Troleandomycin, Vancomycin, pH6, Rifamycin SV, Tetrazolium violet, Niaproof 4, Tetrazolium blue, L-Galactonic Acid Lactone, Citric Acid, L-Glutamic Acid, Lincomycin, Fusidic Acid, Guanidine HCl, Lithium Chloride, Sodium Butyrate and weakly positive at pH5.

P. aquaticum strains clusters separately in phylogenetic analyses based on the *gap*A housekeeping gene and whole genome alignment of *Pectobacterium* described or proposed species strains.

The type strain is A212-S19-A16^T (=CFBP 8637^T =NCPPB4640^T) and was isolated from fresh water river Verdon, close to the confluence with the river Durance in France in 2016. The DNA G+C content of the DNA of the type strain is 51,2%. A35-S23-M15 (CFBP8632), A101-S19-F16 (CFBP8633), A104-S21-F16 (CFBP8634), A105-S21-F16 (CFBP8635) and A127-S21-F16 (CFBP8636) are additional strains of the specie.

The whole genome sequence of strains A212-S19-A16^T, A35-S23-M15, A101-S19-F16, A104-S21-F16, A105-S21-F16, and A127-S21-F16 have been deposited at DDBJ/ENA/GenBank under the accession QHJR00000000, QHJW00000000, QHJV00000000, QHJU00000000, QHJT00000000, QHJS00000000. The versions described in this paper are versions QHJR02000000, QHJW02000000, QHJV02000000, QHJU02000000, QHJT02000000, QHJS02000000. The bioproject number is PRJNA473080 and the assembly accession numbers are: GCA_003382565.2 for A212-S19-A16^T, GCA_003382645.2 for A35-S23-M15, GCA_003382625.2 for A101-S19-F16, GCA_003382595.2 for A104-S21-F16, GCA_003382585.2 for A105-S21-F16, and, GCA_003382655.2 for A127-S21-F16. The rRNA 16S sequences are available with the following accession numbers : MK035744 for A212-S19-A16^T, MK035739 for A35-S23-M15, MK035740 for A101-S19-F16, MK035741 for A104-S21-F16, MK035742 for A105-S21-F16 and, MK035743 for A127-S21-F16.

AUTHOR STATEMENTS

Funding information

This work was supported by the Agence Nationale de la Recherche (ANR-15-CE21-0003 and ANR-17-CE32-0004-04) and the CNRS Ec2Co program (EC2COBiohefect/Ecodyn/Dril/MicrobiEenCARTOBACTER)

Acknowledgements

We thank Odile Berge and Frédérique Van Gijsegem for their help during river samplings and Ariane Toussaint and Antoine Pourbaix for hosting us during the field samplings. We also thank Cécile Dutrieux and Audrey Lathus for strains conservation. This work has benefited from the expertise of the High-Throughput Sequencing Platform of I2BC, Gif sur Yvette, France.

Ethical statement

not applicable

Conflicts of interest

The authors declare that they have no conflicts of interest

ABBREVIATIONS

ANI: Average Nucleotide Identity
dDDH: digital DNA-DNA hybridization
gapA: glyceraldehyde-3-phosphate dehydrogenase A
MLSA: Multi Locus Sequence Analysis
Pcb: *Pectobacterium carotovorum* subsp. *brasiliense*
Pco: *Pectobacterium carotovorum* subsp. *odoriferum*
Pcc: *Pectobacterium carotovorum* subsp. *carotovorum*

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FIGURES AND TABLES

Figure 1

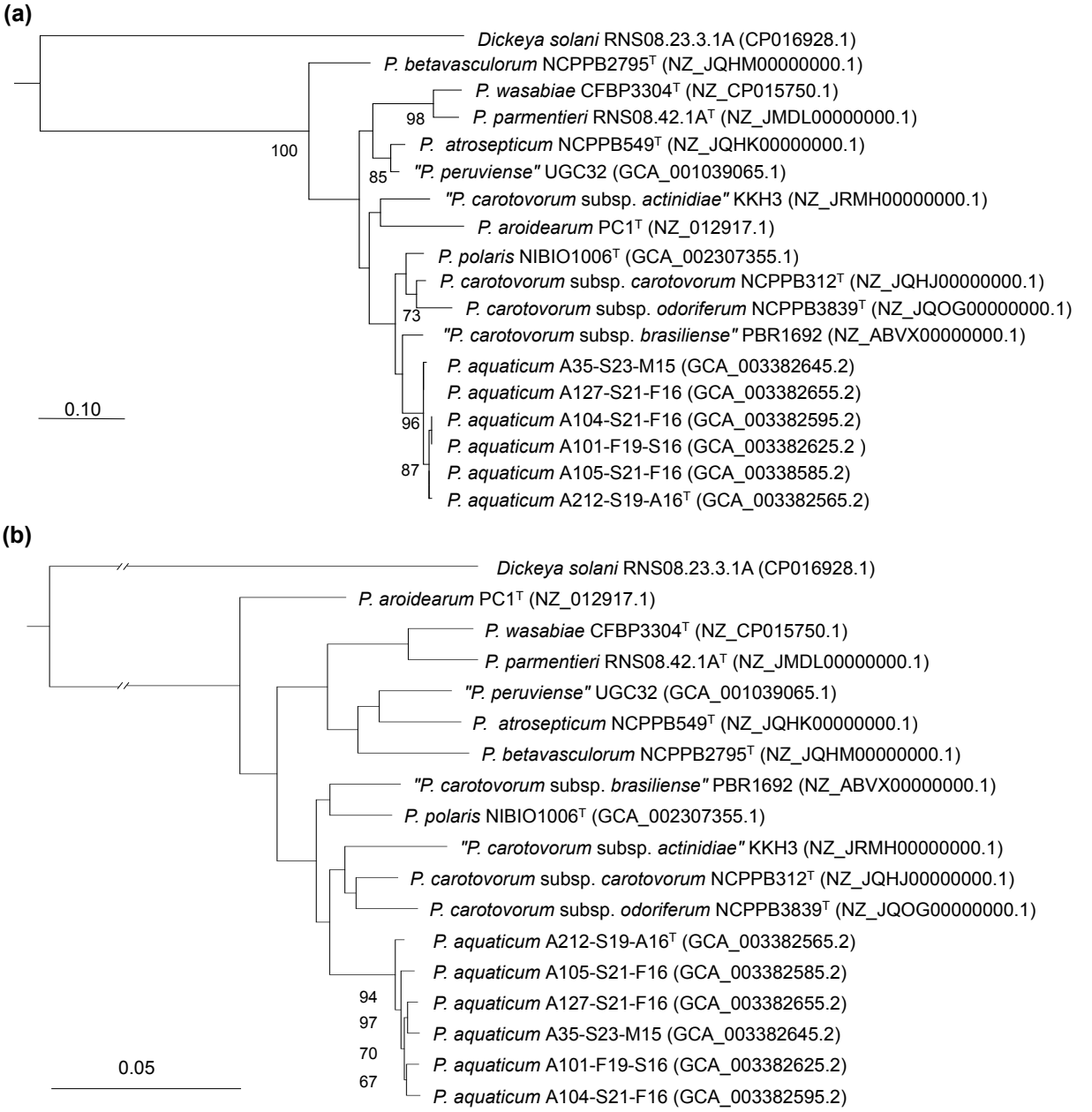
Phylogenetic trees of environmental *P. aquaticum* strains *and* other strains representatives of all the currently described or "proposed" species and subspecies of the *Pectobacterium* genus. (a): Phylogenetic tree constructed from the *gap*A nucleotide sequences. Sequences were aligned using the MUSCLE software (26) and the alignments were filtered by using the program GBLOCKS (27). Tree was computed using PHYML (28). One hundred bootstrap replicates were performed to assess the statistical support of each node. Bootstrap support values (percentages) are indicated if superior to 70 %. *gap*A sequences were retrieved from full genome of type strains (accession numbers are indicated in figure 1 B) or obtained from the sequenced *gap*A amplicon for strains A35-S23-M15 (CFBP8632), A101-S19-F16 (CFBP 8633), A104-S21-F16 (CFBP 8634), A105-S21-F16 (CFBP 8635), A127-S21-F16 (CFBP 8636), A212-S19-A16 (CFBP 8637^{T}). All the gapA sequences used to construct the phylogenetic tree

are provided. (b) : Phylogenetic tree constructed from concatenated sequences of 523 homologous gene sequences. Complete procedure is described in the supplemental S5. All scripts and data are available on GitHub (github.com/jacques-pedron/MLSA_scripts). Briefly, clustering of homologous nucleotide sequences was performed with the SiLix software with 80% identity threshold (29). Homologous sequences of each gene were aligned using the MUSCLE software (26) then concatenated. SeaView (version 4.6.5), a multiplatform program for molecular phylogeny, was used to implement specified tools and options (30). Alignments were filtered by using the GBLOCK tool (27) resulting 568,204 sites dataset. Tree was computed using PHYML (28). One hundred bootstrap replicates were performed to assess the statistical support of each node. Bootstrap support values (percentages) are shown if less than 100%. The accession number for each genome is indicated inside brackets after the strain name. *Dickeya solani* RNS08.23.3.1.A was used as outgroup. Type strains are marked with T after the strain name. The bars indicated the number of changes per nucleotide position.

Table 1
Isolation sites and genome informations for the six *P. aquaticum* strains

Table 2
Pairwise ANI (below diagonal) and dDDH (above diagonal) between the genomes of novel *P. aquaticum* strains and the genomes of closely related *Pectobacterium* described and "proposed" species and subspecies.

Table 3. Distinctive metabolic traits between *P. aquaticum* (A101-S19-F16, A105-S21-F16, A212-S19-A16) and *P. polaris (*CFBP 1403), "*P. carotovorum* subsp. *brasiliense"* (CFBP 6617), "*P. carotovorum* subsp. *actinidiae"* (CFBP 7370), *P. aroidearum* (CFBP 8168^{T}), *P. carotovorum* subsp. *odoriferum* (CFBP 1878^{T}) and *P. carotovorum* subsp. *carotovorum* (CFBP 2046^{T}), using GENIII biolog® plates. All plates read were performed on an Omnilog™ apparatus at 48h. +: positive reaction; -: negative reaction; w: weak reaction.
All strains showed positive reactions for D-Raffinose, α-D-Glucose, Pectin, α-D-Lactose, D-Mannose, D-Mannitol, D-Galacturonic Acid, Methyl pyruvate, D-Melbiose, D-Fructose, β-Methyl-D-Glucoside, D-Galactose, myo-Inositol, D-Gluconic Acid, D-Salicin, Glycerol, L-Aspartic Acid, Gentiobiose, N-Acetyl-D-Glucosamine, D-Glucose-6-PO4, Sucrose, D-Fructose-6-PO4, Mucic Acid, L-Rhamnose, D-Aspartic Acid, L-Malic Acid, Acetic Acid, L-Serine, D-Saccharic Acid, Bromo-Succinic Acid, 1% NaCl, 1% Sodium Lactate, Troleandomycin, Vancomycin, pH6, 4% NaCl, Rifamycin SV, Tetrazolium violet, Niaproof 4 and Tetrazolium blue. All strains showed negative reaction for Gelatin, β-Hydroxy-Phenylacetic Acid, Tween 40, γ-Amino-Butryric-Acid, D-Lactic Acid Methyl Ester, α-Hydroxy-Butyric Acid, L-Arginine, β-Hydroxy-D-L-Butyric Acid, 3-Methyl Glucose, α-Keto-Butyric Acid, Glucuronamide, Acetoacetic Acid, N-Acetyl-β-D-Mannosamine, L-Fucose, L-Histidine, D-Malic Acid, Propionic Acid, N-Acetyl-D-Galactosamine, L-Pyroglutamic Acid, Quinic Acid, N-Acetyl NeuraminicAcid D-Serine, Nalidixic Acid, Aztreonam, 8% NaCl, Minocycline, Potassium tellurite and Sodium Bromat.


(a)
Dickeya solani RNS08.23.3.1A (CP016928.1)
P. betavasculorum NCPPB2795T (NZ_JQHM00000000.1)
P. wasabiae CFBP3304T (NZ_CP015750.1)
P. parmentieri RNS08.42.1AT (NZ_JMDL00000000.1)
P. atrosepticum NCPPB549T (NZ_JQHK00000000.1)
"P. peruviense" UGC32 (GCA_001039065.1)
"P. carotovorum subsp. actinidiae" KKH3 (NZ_JRMH00000000.1)
P. aroidearum PC1T (NZ_012917.1)
P. polaris NIBIO1006T (GCA_002307355.1)
P. carotovorum subsp. carotovorum NCPPB312T (NZ_JQHJ00000000.1)
P. carotovorum subsp. odoriferum NCPPB3839T (NZ_JQOG00000000.1)
"P. carotovorum subsp. brasiliense" PBR1692 (NZ_ABVX00000000.1)
P. aquaticum A35-S23-M15 (GCA_003382645.2)
P. aquaticum A127-S21-F16 (GCA_003382655.2)
P. aquaticum A104-S21-F16 (GCA_003382595.2)
P. aquaticum A101-F19-S16 (GCA_003382625.2)
P. aquaticum A105-S21-F16 (GCA_00338585.2)
P. aquaticum A212-S19-A16T (GCA_003382565.2)
100
98
85
73
96
87
0.10
(b)
Dickeya solani RNS08.23.3.1A (CP016928.1)
P. aroidearum PC1T (NZ_012917.1)
P. wasabiae CFBP3304T (NZ_CP015750.1)
P. parmentieri RNS08.42.1AT (NZ_JMDL00000000.1)
"P. peruviense" UGC32 (GCA_001039065.1)
P. atrosepticum NCPPB549T (NZ_JQHK00000000.1)
P. betavasculorum NCPPB2795T (NZ_JQHM00000000.1)
"P. carotovorum subsp. brasiliense" PBR1692 (NZ_ABVX00000000.1)
P. polaris NIBIO1006T (GCA_002307355.1)
"P. carotovorum subsp. actinidiae" KKH3 (NZ_JRMH00000000.1)
P. carotovorum subsp. carotovorum NCPPB312T (NZ_JQHJ00000000.1)
P. carotovorum subsp. odoriferum NCPPB3839T (NZ_JQOG00000000.1)
P. aquaticum A212-S19-A16T (GCA_003382565.2)
P. aquaticum A105-S21-F16 (GCA_003382585.2)
P. aquaticum A127-S21-F16 (GCA_003382655.2)
P. aquaticum A35-S23-M15 (GCA_003382645.2)
P. aquaticum A101-F19-S16 (GCA_003382625.2)
P. aquaticum A104-S21-F16 (GCA_003382595.2)
94
97
70
67
0.05

Strains	CFBP names	Isolation date	Isolation site	Genome coverage	number of scaffolds	Genome Size (pb)	G+C mol%	Protein coding genes	RNA genes	genebank accession No
A35-S23-M15	CFBP8632	30/05/15	1	102	152	4467243	51.3	4335	50	QHJW01000000
A101-S19-F16	CFBP8633	04/02/16	2	175	104	4274151	51.5	4114	49	QHJV01000000
A104-S21-F16	CFBP8634	17/02/16	3	180	133	4395676	51.4	4297	46	QHJU01000000
A105-S21-F16	CFBP8635	17/02/16	3	167	135	4310812	51.4	4162	50	QHJT01000000
A127-S21-F16	CFBP8636	17/02/16	3	60	209	4465293	51.2	4364	46	QHJS01000000
A212-S19-A16^{T}	CFBP8637^{T}	25/08/16	2	101	119	4348076	51.2	4266	45	QHJR01000000

1 : Canal de Vaucluse ; 2 : Verdon ; 3 Grand anguillon

	P.aquaticum A101-S19-F16	*P.aquaticum* A104-S21-F16	*P.aquaticum* A35-S23-M15	*P.aquaticum* A105-S21-F16	*P.aquaticum* A127-S21-F16	*P.aquaticum* A212-S19-A16^{T}	*Pcc* NCPPB312^{T}	*Pco* NCPPB3839^{T}	*P. polaris* NIBIO1006^{T}	*"Pcb"* PBR1692
P.aquaticum A101-S19-F16	---	88.80	88.80	88.90	88.70	88.10	55.70	53.10	52.90	51.50
P.aquaticum A104-S21-F16	98.76	---	88.50	87.30	89.40	87.70	56.00	53.00	53.00	51.30
P.aquaticum A35-S23-M15	98.72	98.7	---	87.60	90.50	86.80	55.70	52.90	52.90	51.40
P.aquaticum A105-S21-F16	98.73	98.63	98.63	---	87.90	87.80	55.90	53.00	52.90	51.40
P.aquaticum A127-S21-F16	98.70	98.70	98.90	98.63	---	86.20	55.70	52.80	52.80	51.50
P.aquaticum A212-S19-A16^{T}	98.61	98.65	98.53	98.64	98.50	---	55.60	52.70	53.30	51.90
Pcc NCPPB312^{T}	94.23	94.26	94.23	94.30	94.23	94.24	---	61.50	52.40	51.00
Pco NCPPB3839^{T}	93.69	93.68	93.68	93.73	93.66	93.64	95.18	---	49.50	47.40
P. polaris NIBIO1006^{T}	93.66	93.68	93.66	93.67	93.67	93.73	93.52	92.90	---	54.50
"Pcb" PBR1692	93.36	93.34	93.35	93.34	93.37	93.45	93.26	92.40	94.02	---

Test	*P. aquaticum* A101-S19-F16	*P. aquaticum* A105-S21-F16	*P. aquaticum* A212-S19-A16^{T}	*P. polaris* CFBP 1403	*"P. c. brasiliense"* CFBP 6617	*"P. c. actinidiae"* CFBP 7370	*P. aroidearum* CFBP 8168^{T}	*P. c. odoriferum* CFBP 1878^{T}	*P. c. carotovorum* CFBP 2046^{T}
D-Trehalose	-	-	-	+	+	+	+	+	+
D-Cellobiose	-	-	-	+	+	+	+	+	+
Formic Acid	-	-	-	+	+	-	-	+	+
pH5	w	w	w	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	w	-	+	-
Dextrin	-	-	-	-	+	w	-	-	-
Glycyl-L-Proline	-	-	-	-	w	-	-	-	-
D-Maltose	-	-	-	-	+	w	-	+	-
D-Arabitol	-	-	-	-	+	w	-	+	-
L-Alanine	-	-	-	-	+	-	-	-	-
L-Galactonic Acid	+	+	+	+	+	+	+	+	-
L-Lactic Acid	-	-	-	-	+	+	+	-	-
D-Glucuronic Acid	-	-	-	+	+	-	+	+	+
Citric Acid	+	+	+	-	+	+	+	+	+
D-Fucose	-	-	-	-	w	-	-	-	-
L-Glutamic Acid	+	w	w	+	+	+	+	+	+
α-Keto-Glutaric Acid	-	-	-	-	-	-	+	-	-
D-Turanose	-	-	-	-	w	-	-	+	-
Stachyose	-	-	-	-	+	+	+	+	+
Inosine	-	-	-	+	+	-	-	+	-
Guanidine HCl	+	+	+	+	+	+	-	+	-
Lithium Chloride	+	w	w	+	+	+	+	-	+
Sodium Butyrate	+	+	+	-	+	+	+	-	+
Fusidic Acid	+	+	+	+	-	+	+	+	+
Lincomycin	+	+	+	+	+	+	+	-	+

Pectobacterium aquaticum sp. nov, isolated from waterways

Pédron Jacques[1], Bertrand Claire[1], Taghouti Géraldine[2], Portier Perrine[2$] and Barny Marie-Anne [1$]

[1]Sorbonne Université, INRA, Institute of Ecology and Environmental sciences-Paris, 4 place Jussieu, F-75 252 Paris, France
[2]IRHS, INRA, Université d'Angers, Agrocampus-Ouest, SFR 4207 QuaSaV, CIRM-CFBP, 49071, Beaucouzé, France

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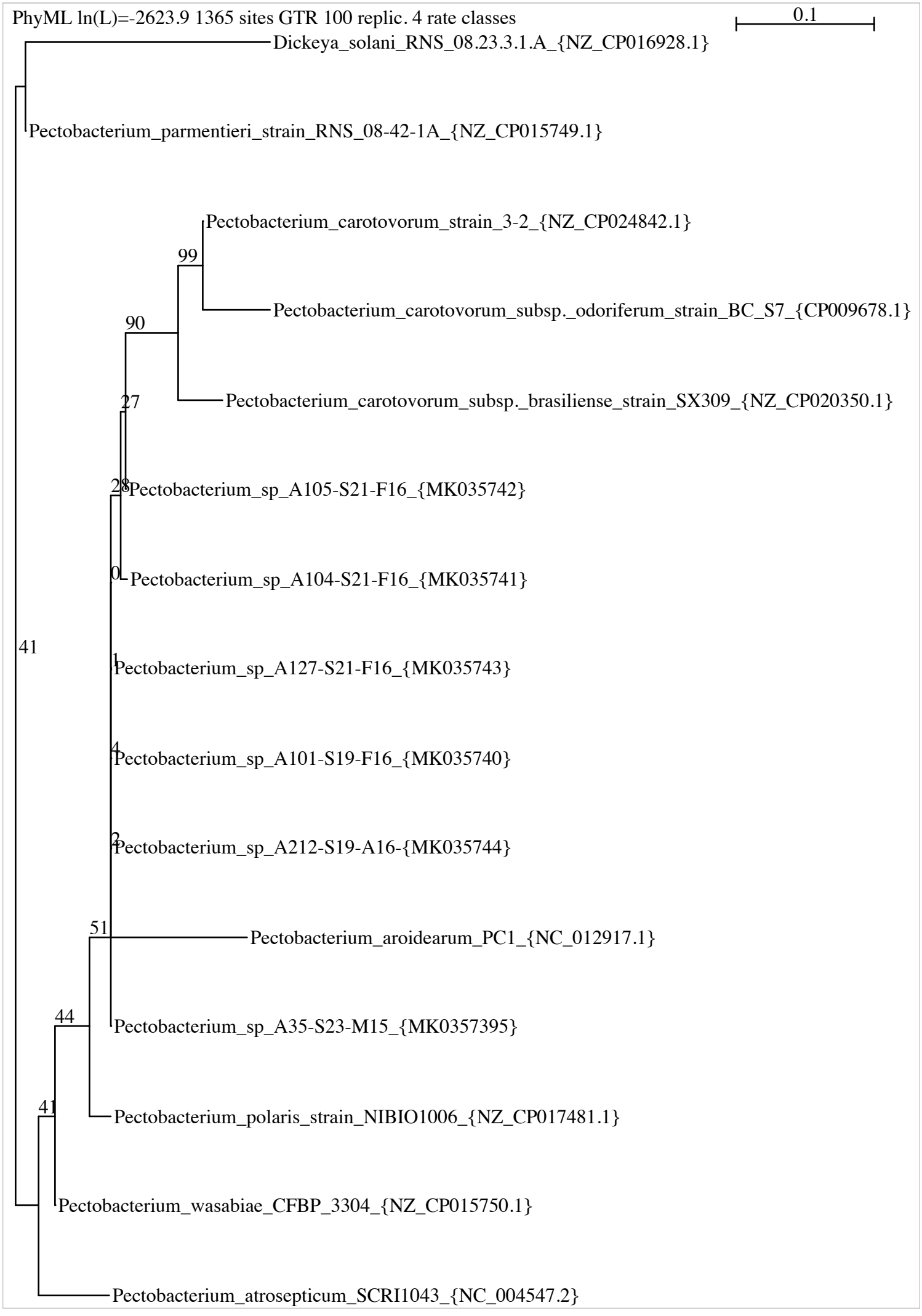
for correspondance: marie-anne.barny@sorbonne-universite.fr

Journal name : International Journal of Systematic and Evolutionnary Microbiology

Supplemental data

Supplemental S1 : 16S phylogenetic tree and associated sequences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Pectobacterium_atrosepticum_SCRI1043_{NC_004547.2}

Legend S1 : Phylogenetic tree constructed from the 16S nucleotide sequences. Sequences were aligned using the MUSCLE software (1) and the alignments were filtered by using the program GBLOCKS (2). Tree was computed using PHYML (3). One hundred bootstrap replicates were performed to assess the statistical support of each node. Bootstrap support values (percentages) are indicated.

1. **Edgar RC.** MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 2004;5:113
2. **Castresana J.** Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* 2000;17:540–52
3. **Guindon S, Gascuel O.** A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol.* 2003;52:696–704

>Pectobacterium_sp_A35-S23-M15_{MK0357395} 16S ribosomal RNA_ partial sequence
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Supplemental S2 : GapA sequences used to construct the phylogenetic tree presented in fig. 1 (a)

Supplemental S2: GapA sequences used to construct the phylogenetic tree presented in fig. 1 (a)
This includes
- Partial gapA sequences of A35-S23-M15, A101-S19-F16, A104-S21-F16, A105-S21-F16, A127-S21-F16 and A212-S19-A16 obtained following amplification and sequencing of the gapA housekeeping gene as described in Cigna et al 2017
- homologous gapA sequences of Pectobacterium sp. type strains retrieved from NCBI genomes through BLAST analysis

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Supplemental S3 : ANI and dDDH values between Pectobacterium sp.

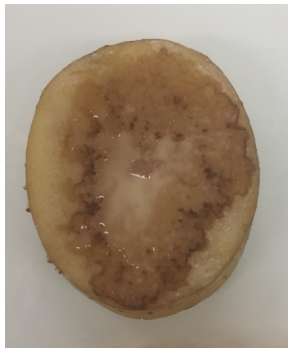
	P.aquaria A101-S19-F16	*P.aquaria* A104-S21-F16	*P.aquaria* A35-S23-M15	*P.aquaria* A105-S21-F16	*P.aquaria* A127-S21-F16	*P.aquaria* A212-S19-A16^T	*P. carotovorum* subsp. *carotovorum* NCPPB312^T	*P. carotovorum* subsp. *odoriferum* NCPPB3839^T	*P. polaris* NIBIO1006^T	*"P. carotovorum* subsp. *brasiliense "* PBR1692	*"P. carotovorum* subsp. *actinidiae"* KKH3	*P. aroidearum* PC1^T	*P. atrosepticum* NCPPB549^T	*"P. peruviense "* UGC32	*P. betavasculorum* NCPPB2795^T	*P. wasabiae* CFBP3304^T	*P. parmentieri* RNS08.42.1A^T
P.aquaria A101-S19-F16	---	88.80	88.80	88.90	88.70	88.10	55.70	53.10	52.90	51.50	46.40	40.00	38.00	38.10	37.70	36.60	36.30
P.aquaria A104-S21-F16	98.76	---	88.50	87.30	89.40	87.70	56.00	53.00	53.00	51.30	46.60	40.00	38.40	38.20	37.60	36.60	36.40
P.aquaria A35-S23-M15	98.72	98.7	---	87.60	90.50	86.80	55.70	52.90	52.90	51.40	46.40	40.00	38.00	37.90	37.60	36.70	36.30
P.aquaria A105-S21-F16	98.73	98.63	98.63	---	87.90	87.80	55.90	53.00	52.90	51.40	46.40	40.00	38.30	38.10	37.60	36.60	36.30
P.aquaria A127-S21-F16	98.70	98.70	98.90	98.63	---	86.20	55.70	52.80	52.80	51.50	46.40	39.80	38.20	38.00	37.60	36.70	36.40
P.aquaria A212-S19-A16^T	98.61	98.65	98.53	98.64	98.50	---	55.60	52.70	53.30	51.90	46.30	40.20	38.20	38.30	37.70	36.60	36.40
P. carotovorum subsp. *carotovorum* NCPPB312^T	94.23	94.26	94.23	94.30	94.23	94.24	---	61.50	52.40	51.00	51.00	40.30	38.80	38.50	38.10	36.80	36.70
P. carotovorum subsp. *odoriferum* NCPPB3839^T	93.69	93.68	93.68	93.73	93.66	93.64	95.18	---	49.50	47.40	50.10	39.50	38.30	38.20	37.70	36.70	36.50
P. polaris NIBIO1006^T	93.66	93.68	93.66	93.67	93.67	93.73	93.52	92.90	---	54.50	45.20	40.40	38.50	38.30	38.30	36.80	36.60
"*P. carotovorum* subsp. *brasiliense*" PBR1692	93.36	93.34	93.35	93.34	93.37	93.45	93.26	92.40	94.02	---	43.90	41.30	38.10	37.90	38.10	36.40	36.30
"*P. carotovorum* subsp. *actinidiae*" KKH3	92.28	92.32	92.28	92.29	92.27	92.25	93.37	93.15	92.02	91.57	---	38.60	37.40	37.00	36.80	35.80	35.60
P. aroidearum PC1^T	90.40	90.43	90.42	90.39	90.37	90.42	90.49	90.22	90.56	90.75	89.96	---	36.60	36.60	36.80	35.60	35.70
P. atrosepticum NCPPB549^T	89.81	89.89	89.86	89.9	89.85	89.87	89.97	89.83	89.91	89.74	89.51	89.30	---	53.90	46.80	40.00	39.40
"*P. peruviense*" UGC32	89.72	89.74	89.72	89.79	89.73	89.77	89.89	89.77	89.81	89.70	89.38	89.20	93.76	---	47.30	39.90	39.60
P. betavasculorum NCPPB2795^T	89.64	89.66	89.67	89.65	89.64	89.67	89.83	89.68	89.81	89.83	89.34	89.28	92.29	92.46	---	38.50	37.80
P. wasabiae CFBP3304^T	89.23	89.32	89.30	89.26	89.29	89.26	89.42	89.27	89.49	89.24	89.16	88.94	90.42	90.42	89.96	---	54.70
P. parmentieri RNS08.42.1A^T	89.20	89.25	89.23	89.20	89.26	89.24	89.36	89.28	89.35	89.20	88.99	88.94	90.24	90.21	89.76	94.14	---

Table S1: Pairwise ANI (below diagonal) and dDDH (above diagonal) between the genomes of the six *P. aquaria* strains and the genomes of all described and "proposed" *Pectobacterium* species and subspecies.

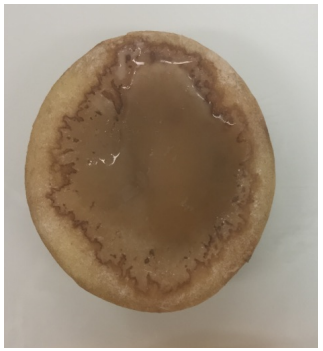
Supplemental S4 : Phenotypic characterization of *P. aquaticum* strains on potato tuber slices

A35-S23-M15

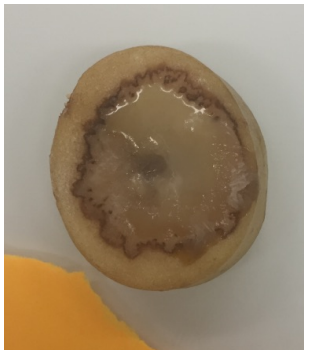
A101-S19-F16



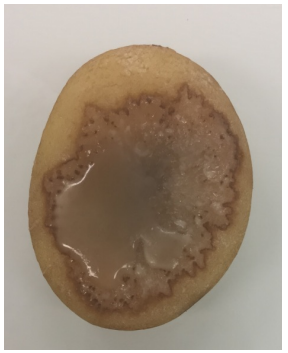
A104-S21-F16



A105-S21-F16

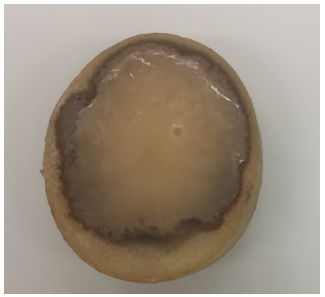


A127-S21-F16



A212-S19-A16^{T}

buffer



CFBP2046^{T}
Pectobacterium carotovorum subsp. *carotovorum*

Legend S4: Phenotypic characterization of *P. aquaticum* strains on potato tuber slices
Bacterial strains A35-S23-M15, A101-S19-F16, A104-S21-F16, A105-S21-F16, A127-S21-F16, A212-S19-A16 were plated on LB medium and cultured for 15h at 28°C. Bacteria were then scrapped off the Petri dish, suspended in 50mM phosphate buffer pH 6,8 and adjusted to 1.0 at OD_{600nm}. Tubers of *S. tuberosum* var. charlotte were cut in slices and 10µl of the cell suspension were deposit in the centre of seven slices. The slices were placed at 28°C on wet paper towel in a plastic box. Typical obtained symptoms are shown.
Positive control; *P. carotovorum* subsp. *carotovorum* $CFBP2046_T$, negative control: 50mM phosphate buffer pH 6,8.

Supplemental S5: Procedure to generate the phylogenetic tree presented in Fig 1 (b)

Dependancies:

Python version 3.7.1
Blast version 2.6.0. To install:

```
conda install blast
```

SiLix version 1.2.9. Dowloading: http://lbbe.univ-lyon1.fr/Download.html
To install:

```
tar zxvf silix-1.x.x.tar.gz
cd silix-1.x.x
./configure
make
make check
make install
```

Muscle version 3.8.1551
To install:

```
conda install muscle
```

Predicted nucleotide protein sequences (.fasta) of all genomes located in the sequences/ directory are first concatenated in a single fasta file (sequences.fasta):

```
cat sequences/* | awk '/^>/ {print "\n"$0} /^[a-z]/ {printf $0} /^[A-Z]/ {print $0}' > sequences.fasta
```

The next command generates a names.txt file containing names of genomes:

```
ls sequences/ | sed 's/.fasta//g' > names.txt
```

The sequences.fasta file is used to create a Blastp database:

```
makeblastdb -in sequences.fasta -dbtype nucl -out dbblastall
```

A Blastn all vs all is then performed:

```
blastn -db dbblastall -query sequences.fasta -outfmt 6 -out blastall.blastn -num_threads 8 -evalue 0.00001
```

The blastp outut (blastall.blastn file) is then processed by the SiLix software that clusters homologous genes in families with a 80% threshold. SiLix generates the seq.80.fnodes file.

```
silix sequences.fasta blastall.blastn -f FAM -i 0.80 > seq.80.fnodes
```

The seq.80.fnodes is processed with the python script core.py that generates a matrix (output fam.80.txt file) with species in columns and number of homologous genes in rows. The script needs the seq.80.fnodes and names.txt files as arguments:

```
python3 core.py seq.80.fnodes names.txt
```

The homologous.py script then searches for homologous genes present in all genomes (core genome). The core.80.txt output file is a list of homologous families.

```
python3 homologous.py fam.80.txt
```

The align.py script extracts homologous sequences, aligns with Muscle and concatenates aligned sequences. The output file is align.fasta.

```
python3 align.py core.80.txt seq.80.fnodes sequences.fasta names.txt
```

Molecular phylogeny is performed by using the multiplatform GUI Seaview software version 4.6.5. Input file is the align.fasta alignment. First, alignment is curated by Gbloks tool with default parameters to remove all gaps or X positions. Phylogeny is performed with PhyML algorithm with the following settings: substitution model GTR, 100 bootstraps, nucleotide equilibrium frequencies, invariable sites optimized, tree searching NNI, starting tree BioNJ.