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Comparative Genomic Analysis of the Biotechnological Potential of the Novel Species *Pseudomonas wadenswilerensis* CCOS 864^T and *Pseudomonas reidholzensis* CCOS 865^T

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Abstract: In recent years, the use of whole-cell biocatalysts and biocatalytic enzymes in biotechnological applications originating from the genus *Pseudomonas* has greatly increased. In 2014, two new species within the *Pseudomonas putida* group were isolated from Swiss forest soil. In this study, the high quality draft genome sequences of *Pseudomonas wadenswilerensis* CCOS 864^T and *Pseudomonas reidholzensis* CCOS 865^T were used in a comparative genomics approach to identify genomic features that either differed between these two new species or to selected members of the *P. putida* group. The genomes of *P. wadenswilerensis* CCOS 864T and *P. reidholzensis* CCOS 865T were found to share genomic features for the degradation of aromatic compounds or the synthesis of secondary metabolites. In particular, genes encoding for biocatalytic relevant enzymes belonging to the class of oxidoreductases, proteases and isomerases were found, that could yield potential applications in biotechnology. Ecologically relevant features revealed that both species are probably playing an important role in the degradation of soil organic material, the accumulation of phosphate and biocontrol against plant pathogens.

Keywords: biotechnological application; ecology; aromatic degradation; secondary metabolites; secretion systems; biocontrol; zeaxanthin

1. Introduction

The genus *Pseudomonas* taxonomically belongs to the gamma subclass of Proteobacteria [1]. Members of *Pseudomonas* have a saprophytic lifestyle and are ubiquitously found in the environment surviving at most temperatures [2]. They can be isolated from samples of soil, water, air, plants and animal or human related sources [3]. Pseudomonads are highly versatile in relation to genetics, physiology and metabolism [2]. They grow rapidly under simple nutritional conditions and some species are able to use more than 100 different sources of carbon and energy. Members of the genus *Pseudomonas* may catabolize aromatic and aliphatic hydrocarbons (phenols, toluene, n-alkanes, cyclohexane) by using specific pathways [4–6].

For industrial biotechnology application, *Pseudomonas putida* and related species have particularly been scrutinized following the discovery of degradation pathways for natural and xenobiotic compounds [5,7]. Members of the genus *Pseudomonas* are being used as whole-cell biocatalyst or for the production of specific enzymes for bulk- and fine-chemicals manufactory [8]. Another advantage is that they are resistant against environmental stress, making them robust against the presence of toxins or inhibiting solvents and under extreme pH and temperature conditions [7,9]. The use of solvent-tolerant pseudomonads as biocatalysts thus provides a solution in the production of toxic products [10]. Furthermore, numerous extracellular enzymes are being produced by pseudomonads, such as for example lipases and proteases, which find important application in biotechnology [11,12]. Examples for the application are the synthesis of bio-based polymers, small molecular weight chiral compounds, biosurfactants and heterologous proteins [7,12]. In agriculture, pseudomonads are known for their potential as biocontrol agents of soilborne plant pathogens or insects [13–15]. Nevertheless, the biocatalytic potential of some newly found isolates is still unknown. Research in this direction would be of great interest for the biotechnology industry.

In 2014, two isolates belonging to the *P. putida* group were obtained from soil samples of the Reidholz forest in Richterswil, Switzerland, during the search for novel biocatalysts [16]. Genotypic and phenotypic data clarified that the isolates differed from other species of the *P. putida* group. Because of the clear separation of the strains, *Pseudomonas wadenswilerensis* CCOS 864^T and *Pseudomonas reidholzensis* CCOS 865^T were proposed as novel species [16]. Recently, both genomes were published [17,18]. In this study, we used comparative genomics to elucidate the potential of these two species as a source for novel biocatalytic features while investigating also their ecological role.

2. Materials and Methods

2.1. Bacterial Strains

P. wadenswilerensis CCOS 864^T (LMG 29327^T) and *P. reidholzensis* CCOS 865^T (LMG 29328^T) were both isolated from soil from the Reidholz forest (47°13′ N and 8°41′ E), located between the city of Wädenswil and the village of Richterswil, Switzerland in 2014, during research for novel biocatalysts for biotechnological application [16].

2.2. Comparative Genomics

The core genome phylogenetic relationships were obtained using EDGAR v.2.2 [19]. Briefly, the core genome was defined by iterative pairwise comparison of the gene content of each of the selected genomes using the bidirectional best hits (BBH) strategy as orthology criterion. For all calculations, protein BLAST (BLASTp) was used with BLOSUM62 as similarity matrix [20]. Genes were considered orthologous when a reciprocal best BLAST hit was found between two genes, and when both BLAST hits were based on alignments with a score ratio value [21] exceeding 30%. Multiple alignments of each orthologous gene set of the core genome were calculated using the MUSCLE software [22] and non-matching parts of the alignments were removed using GBLOCKS [23]. The resulting alignments were concatenated and used to construct an approximately-maximum-likelihood phylogenetic tree using the FastTree software [24], which computes local support values with the Shimodaira-Hasegawa test. The resulting tree was edited for display with the MEGA7 software [25].

Species differentiation was additionally checked by calculation of ANI, Tetra and GGDC values between the closest related strain in the tree and the draft genome sequences of *P. wadenswilerensis* CCOS 864^T and *P. reidholzensis* CCOS 865^T. For ANI and Tetra, the online service of JSpeciesWS version 3.0.20 with default parameters was used [26], while for GGDC, the online service version 2.1 [27] was used, reporting values obtained with Formula 2 for draft genomes. *Pseudomonas* species names not fitting with the type strains of the designated species were not maintained and renamed as *Pseudomonas* sp. (Table 1).

Table 1. List of bacterial strains of the *Pseudomonas putida* group used for comparative genomics and features of the individual genomes. Strain names labeled with "T" represent the type strains of this species.

Strain Name (NCBI)	Taxonomic Group	Name Used in this Study	Assembly Level	Contigs/Scaffolds	Genome Size [bp]	Genes	G+C [%]	GenBank Accession No.
P. alkylphenolica KL28^T	*P. alkylphenolica*	*P. alkylphenolica* KL28^T	Complete	1	5′764′622	5′454	60.63	CP009048
P. cremoricolorata DSM 17059^T	*P. cremoricolorata*	*P. cremoricolorata* DSM 17059^T	Draft	26	4′655′082	4′072	63.35	AUEA00000000
P. donghuensis P482	*P. donghuensis*	*P. donghuensis* P482	Draft	69	5′623′997	5′259	62.40	JHTS00000000
P. donghuensis HYS^T	*P. donghuensis*	*P. donghuensis* HYS^T	Draft	64	5′646′028	5′239	62.40	AJJP01000000
P. donghuensis SVBP6	*P. donghuensis*	*P. donghuensis* SVBP6	Draft	71	5′701′342	5′355	62.40	NWCB01000000
P. entomophila L48^T	*P. entomophila*	*P. entomophila* L48^T	Complete	1	5′888′780	5′223	64.20	NC_008027
P. fulva NBRC 16637^T = DSM 17717^T	*P. fulva*	*P. fulva* NBRC 16637 ^{T} = DSM 17717^T	Draft	46	4′768′229	4′331	61.80	BBIQ00000000
P. guariconensis LMG 27394^T	*P. guariconensis*	*P. guariconensis* LMG 27394^T	Draft	29	5′079′034	4′703	62.20	FMYX00000000
P. hunanensis P11	*P. hunanensis*	*P. hunanensis* P11	Draft	171	6′644′424	6′469	61.20	PISL00000000
P. japonica NBRC 103040^T = DSM 22348 ^{T}	*P. japonica*	*P. japonica* NBRC 103040^T = DSM 22348 ^{T}	Draft	162	6′663′130	5′845	64.20	BBIR00000000
P. monteilii MO2	OTU06	*Pseudomonas* sp. MO2	Draft	1′589	6′240′608	5′947	62.00	JFBC00000000
P. monteilii SB3078	OTU06	*Pseudomonas* sp. SB3078	Complete	1	6′000′087	5′620	62.50	CP006978
P. monteilii SB3101	OTU06	*Pseudomonas* sp. SB3101	Complete	1	5′945′120	5′546	62.50	CP006979
P. monteilii GTC 10897	OTU08	*Pseudomonas* sp. GTC 10897	Draft	149	5′547′282	5′313	60.40	BCAO00000000
P. monteilii NBRC 103158 = *DSM 14164^T*	*P. monteilii*	*P. monteilii* NBRC 103158 = *DSM 14164^T*	Draft	132	6′299′985	6′005	61.50	BBIS00000000
P. monteilii USDA-ARS-USMARC-56711	OTU13	*Pseudomonas* sp. USDA-ARS-USMARC-56711	Complete	1	4′714′359	4′100	64.40	CP013997
P. mosselii DSM 17497^T	*P. mosselii*	*P. mosselii* DSM 17497^T	Draft	55	6′260′844	5′916	64.00	JHYW00000000
P. parafulva DSM 17004^T	*P. parafulva*	*P. parafulva* DSM 17004^T	Draft	32	4′956′622	4′536	62.50	AUEB00000000
P. parafulva PRS09-11288	*P. fulva*	*P. fulva* PRS09-11288	Complete	1	4′690′783	4′246	61.70	CP019952
P. parafulva CRS01-1	OTU12	*Pseudomonas* sp. CRS01-1	Complete	1	5′087′619	4′457	63.50	CP009747
P. plecoglossicida KCJK7865	OTU05	*Pseudomonas* sp. KCJK7865	Draft	205	5′806′309	5′606	63.00	QANO00000000
P. plecoglossicida NyZ12	OTU06	*Pseudomonas* sp. NyZ12	Complete	1	6′233′254	5′843	62.40	CP010359
P. plecoglossicida NBRC 103162^T	*P. plecoglossicida*	*P. plecoglossicida* NBRC 103162^T	Draft	97	5′341′796	4′946	63.00	BBIV00000000
P. putida DOT-T1E	*P. hunanensis*	*Pseudomonas* sp. DOT-T1E	Complete	1	6′260′702	5′803	61.40	CP003734
P. putida KT2440	*P. hunanensis*	*Pseudomonas* sp. KT2440	Complete	1	6′181′873	5′420	62.30	AE015451
P. putida GB-1	OTU01	*Pseudomonas* sp. GB-1	Complete	1	6′078′430	5′586	61.90	AAXR01000000
P. putida H8234	OTU02	*Pseudomonas* sp. H8234	Complete	1	5′956′110	6′512	61.60	NC_021491
P. putida S13.1.2	OTU03	*Pseudomonas* sp. S13.1.2	Complete	1	6′621′848	5′993	62.30	CP010979
P. putida NBRC 14164^T	*P. putida*	*P. putida* NBRC 14164^T	Complete	1	6′156′701	5′610	62.30	NC_021505
P. putida W15Oct28	*P. putida*	*P. putida* W15Oct28	Draft	119	6′320′510	5′703	62.80	JENB00000000
P. putida S16	OTU06	*Pseudomonas* sp. S16	Complete	1	5′984′790	5′585	62.30	NC_015733
P. putida IEC33019	OTU11	*Pseudomonas* sp. IEC33019	Complete	1	5′847′120	5′445	62.27	CP016634
P. putida CSV86	OTU14	*Pseudomonas* sp.CSV86	Draft	209	6′469′780	5′906	63.10	AMWJ01000000
P. putida W619	OTU09	*Pseudomonas* sp. W619	Complete	1	5′774′330	5′378	61.40	NC_010501
P. reidholzensis CCOS 865^T	*P. reidholzensis*	*P. reidholzensis* CCOS 865^T	Draft	45	6′163′129	5′441	64.09	UIDD01000000
P. soli CCOS 191	*P. soli*	*P. soli* CCOS 191	Complete	1	6′012′947	5′301	64.19	LN847264
P. soli LMG 27941^T	*P. soli*	*P. soli* LMG 27941^T	Draft	34	5′644′910	5′188	64.00	FOEQ00000000
Pseudomonas sp. TJI-51	OTU04	*Pseudomonas* sp. TJI-51	Draft	208	5′805′096	5′508	62.10	AEWE00000000
Pseudomonas sp. GM84	OTU10	*Pseudomonas* sp. GM84	Draft	384	5′818′772	5′299	63.20	AKJC00000000
P. taiwanensis DSM 21245^T	*P. taiwanensis*	*P. taiwanensis* DSM 21245^T	Draft	67	5′415′134	5′056	61.90	AUEC00000000
P. taiwanensis SJ9	OTU06	*Pseudomonas* sp. SJ9	Draft	736	6′253′055	6′075	61.80	AXUP00000000
P. vranovensis DSM 16006^T	*P. vranovensis*	*P. vranovensis* DSM 16006^T	Draft	36	5′697′807	5′295	61.50	AUED00000000
P. wadenswilerensis CCOS 864^T	*P. wadenswilerensis*	*P. wadenswilerensis* CCOS 864^T	Draft	18	5′966′942	5′437	62.39	UNOZ01000000

For investigation of genomic features, both genomes were annotated in GenDB [28]. Based on the obtained annotation data, a comparative pan-genome analysis was performed. Genomic features for potential biotechnological applications or features of ecological interests were selected using EDGAR [19] and examined for their presence in other strains of the P. putida group. Information on transport proteins was obtained from the transporter classification database (TCDB) by using the BLAST search on the website [29], while for aromatic degradation the Kyoto Encyclopaedia of Genes and Genomes (KEGG) [30] and the Biocatalysis / Biodegradation Database from Eawag (EAWAG-BBD) [31] were used. Maps of individual features were created using different subroutines of the LASERGENE package version 11.0 (DNASTAR, Madison, WI, USA).

3. Results

3.1. The Genomes of P. wadenswilerensis CCOS 864^T and P. reidholzensis CCOS 865^T

The genome sizes of 5'966'942 bp for P. wadenswilerensis CCOS 864^T [17] and 6'163'129 bp for P. reidholzensis CCOS 865^T [18] were well within the range of the minimum (4'655'082 bp) and maximum (6'663'130 bp) of other members of the P. putida group. A total number of 5'437 genes for P. wadenswilerensis CCOS 864^T and 5'441 genes for P. reidholzensis CCOS 865^T were annotated. When comparing the number of genes to genomes of the P. putida group, the values of P. wadenswilerensis CCOS 864^T and P. reidholzensis CCOS 865^T were within the average range of 5'382.8 ± 579.9 genes (average ± standard deviation of genome metrics; Table 1) as determined for members of the P. putida group. A comparison of the G+C content values revealed that P. wadenswilerensis CCOS 864^T was, with a value of 62.39%, within the average range of 62.45% ± 0.96% of the P. putida group. P. reidholzensis CCOS 865^T, however, was with a value of 64.09% closer to the highest observed G+C content of 64.4%. Based on the comparison of genome characteristics, we conclude that P. wadenswilerensis CCOS 864^T and P. reidholzensis CCOS 865^T share similar genome characteristics as other members of the P. putida group.

The draft genome sequences were screened for antibiotic resistance genes using ResFinder [32] and CRISPR sequences using CRISPRfinder [33]. Analysis of the draft genome of *P. wadenswilerensis* CCOS 864^T and *P. reidholzensis* CCOS 865^T revealed no similarities to known antibiotic resistance genes. Screening for CRISPR repeat sequences resulted in three questionable CRISPR repeat regions in *P. wadenswilerensis* CCOS 864^T and eight in *P. reidholzensis* CCOS 865^T, but none of them could be confirmed.

3.2. Selection of Genomes and Phylogeny

In order to perform a pan-genome analysis to identify genomic features that differ between the genomes of either *P. wadenswilerensis* CCOS 864^T [17] or *P. reidholzensis* CCOS 865^T [18], a total of 123 genome sequences were selected from GenBank. The selection criteria were: a) allocation to a species known to belong to the *P. putida* group; and b) covering the species with as many as possible genomes to allow statements on species-specific traits. Several reference strains and species, for which the complete 16S rRNA gene was indicating that the species could be closely related to the *P. putida* group, such as *Pseudomonas fuscovaginae*, *Pseudomonas asplenii* or *Pseudomonas oryzihabitans*, were also included in the core phylogeny to understand its phylogenetic position within the genus [3,34]. Strains indicated as *Pseudomonas* sp. in GenBank were not analyzed for their membership of the *P. putida* group. The final selection had a total of 123 genome sequences and included the genomes of 17 type strains belonging to the *P. putida* group (Figure 1).

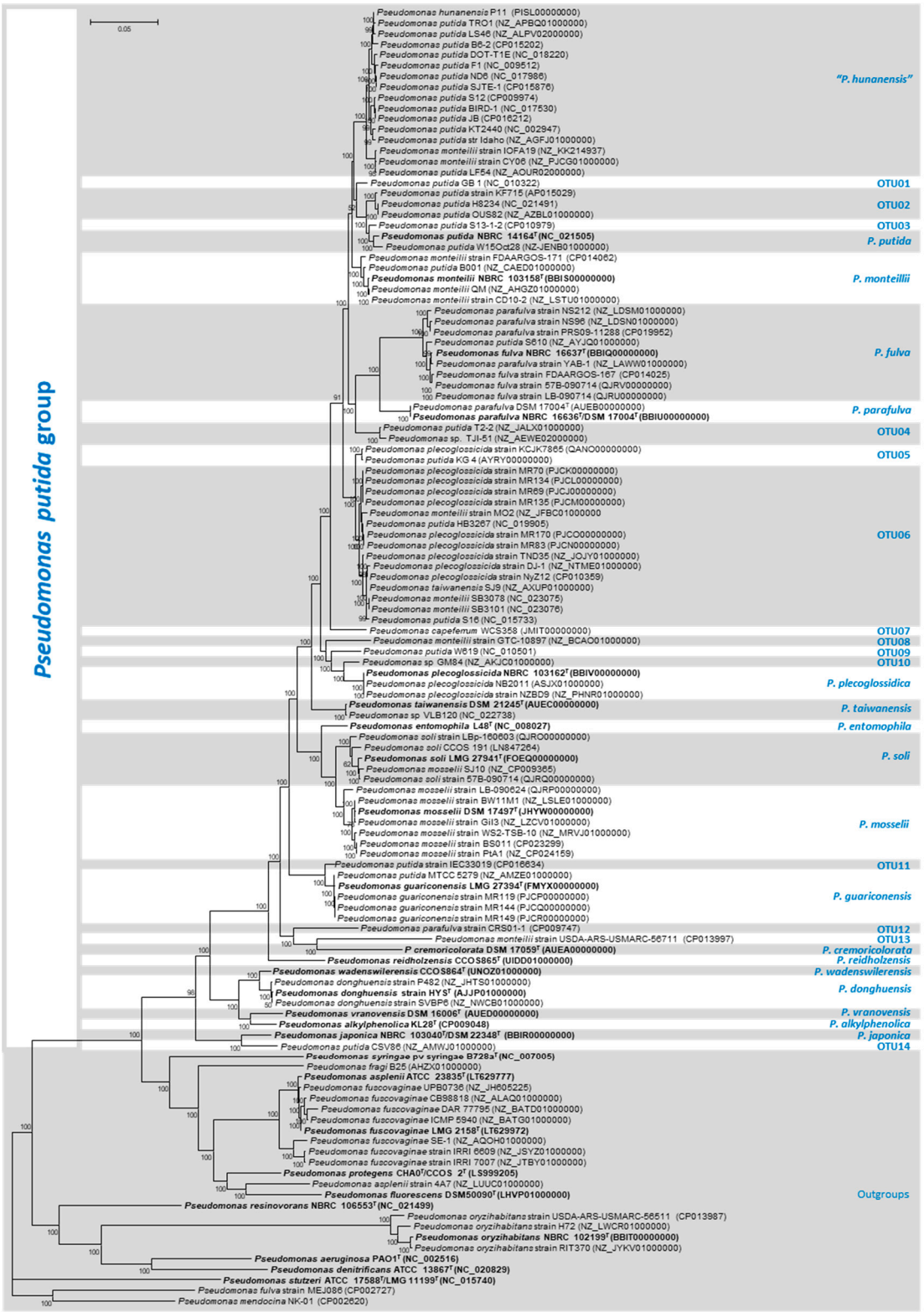

Figure 1. Core genome phylogeny with 123 *Pseudomonas* genomes, build out of a core of 471 genes per genome (131′448 amino acid residues per genome) as calculated by EDGAR version 2.2 [19]. Names in the graphic are directly derived from the NCBI database (with accession numbers). Type strains are indicated in bold. The taxonomic speciation in the *P. putida* group based on ANIb is indicated by the use of different backgrounds.

The core genome tree and comparisons based on average nucleotide identities using BLASTn as performed in EDGAR using the same BLAST parameters as JSpecies [19], identified a further 14 operational taxonomic units (OTUs) each potentially representing a novel species. From this analysis, we selected 43 genomes to cover the phylogenetic groups of the *P. putida* group with at least one representative (Figure 2; Table 1). The table includes 20 of 41 isolates that were renamed to *Pseudomonas* sp. as they were in different taxonomic groups as the type strains of the species to which the sequence was assigned in GenBank [34]. Based on this selection of strains, the comparative genomics platform EDGAR [19] was used to identify some of the most relevant differential genomic features of *P. wadenswilerensis* CCOS 864^T and *P. reidholzensis* CCOS 865^T in order to reveal their yet unknown potential as biocatalyst for biotechnological applications and in ecological processes.

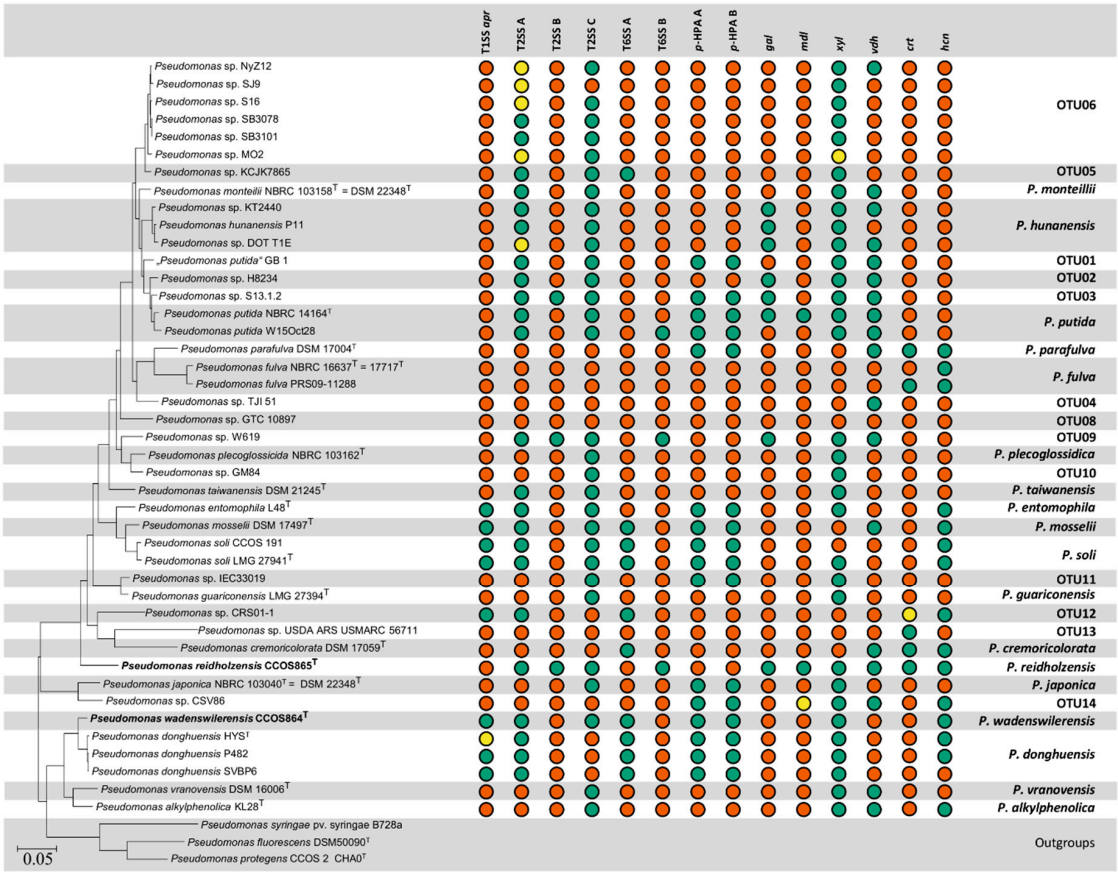

Figure 2. Core genome phylogenetic tree of the *Pseudomonas putida* group genomes analyzed generated using EDGAR [19] based on a total of 1'558 orthologs (573'549 amino acid residues per genome) and presence of shared features to *Pseudomonas wadenswilerensis* CCOS 864^T or *Pseudomonas reidholzensis* CCOS 865^T. Red dot: absence; green dot: presence, yellow dot: partial presence. Species taxonomy is used as given in Table 1. Abbreviations: T1SS *apr*: type 1 secretion system with *apr* genes; T2SS: type 2 secretion system; T6SS: type 6 secretion system; *p*-HPA: gene cluster for degradation of *p*-hydroxyphenylacetic acid; *gal*: gene cluster for gallic acid degradation; *mdl*: gene cluster for mandelate degradation; *xyl*: lower degradation pathway of benzoate degradation; *vdh*: vanillate dehydrogenase; *crt*: carotenoid biosynthesis gene cluster; *hcn*: cyanide biosynthesis cluster.

3.3. Degradation of Aromatic Compounds

Aromatic compounds are widely distributed in nature in form of aromatic amino acids in organisms, plant cell material such as lignin or derivates of gasoline (benzene, toluene, xylene, ethylbenzene) [35]. *Pseudomonas* spp. are reported to be able to degrade aromatic compounds in two

steps, beginning with a ring modification to yield a catechol (upper pathway) followed by a ring fission by either *ortho*- or *meta*-cleavage (lower pathway) [4].

The aromatic compound *p*-hydroxyphenylacetic acid (*p*-HPA) is a product of lignin degradation [36]. Investigation of the pan-genome revealed that *P. wadenswilerensis* CCOS 864^T has a complete gene set for *p*-HPA degradation (Figure 3A), which was absent from the genome of *P. reidholzensis* CCOS 865^T. Positive growth of *P. wadenswilerensis* CCOS 864^T with this compound was obtained before [16], supporting our findings from the genomes. The *hpaBC* genes for degradation of *p*-HPA to homoprotocatechuic acid (HPC) and the *hpaAG1G2EDF-nicT-hpaHIR* gene cluster for the degradation from HPC to succinyl-CoA and acetyl-CoA by *meta*-cleavage [35,37] were found in the genome of *P. wadenswilerensis* CCOS 864^T. While most genes were located in one cluster (CCOS864_03099–03108), the *hpaBC* genes were located at a different location in the genome (CCOS864_02954–02955). The presence of both *p*-HPA degradation gene clusters was confirmed for 15 of 43 members of the *P. putida* group (Figure 2). Comparative genomic results of this feature matched with the phenotypical results obtained before for strains that were tested in their studies [16,38]. In comparison to the cluster in *P. wadenswilerensis* CCOS 864^T, the cluster in *P. entomophila* L48^T differed by the absence of the putative metabolite transport protein *nicT*, while in *Pseudomonas* sp. 13.1.2 (OTU03), different genes were found in the flanking region (Figure 3B).

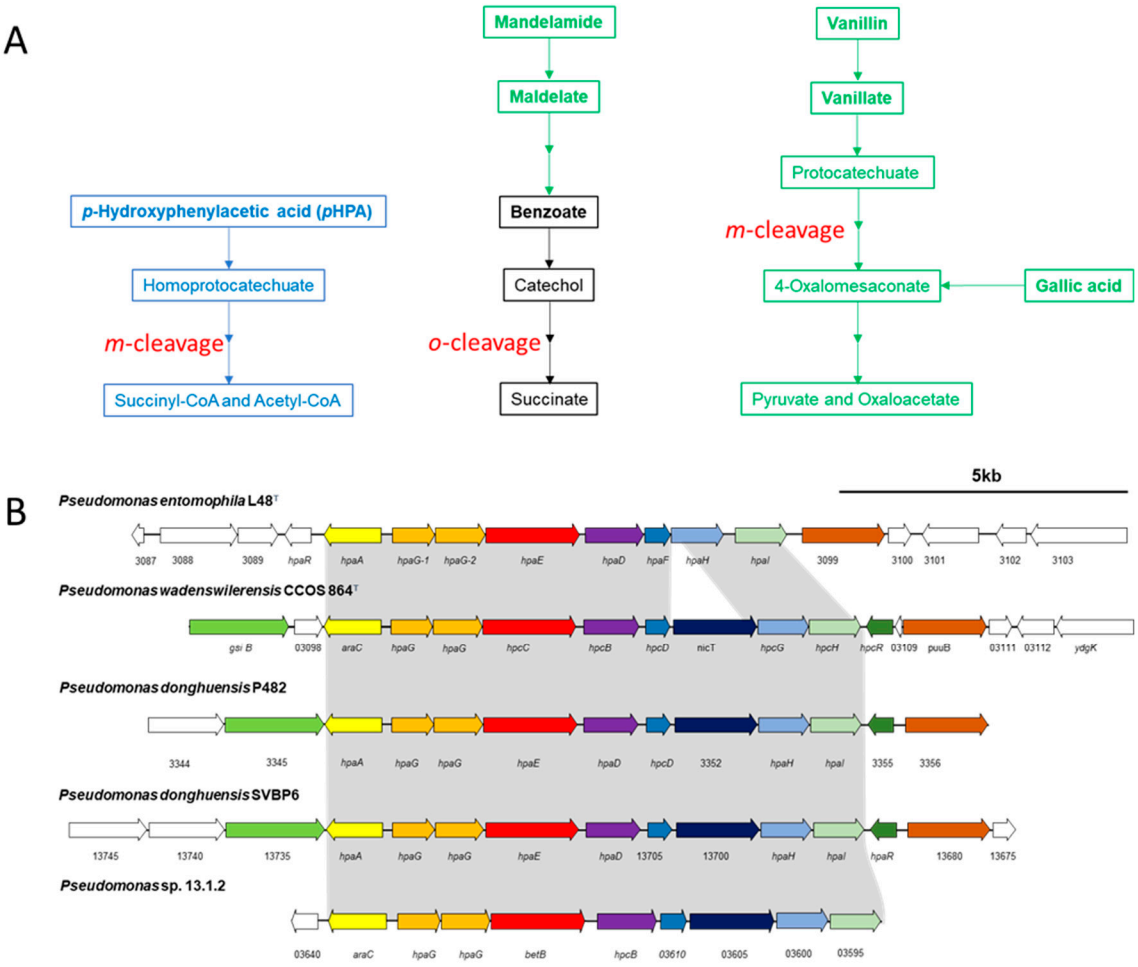

Figure 3. (**A**) Schematic degradation pathways for aromatic compounds in *Pseudomonas wadenswilerensis* CCOS 864^T and *Pseudomonas reidholzensis* CCOS 865^T. Colors represent the organisms in which they are found: blue: only *P. wadenswilerensis* CCOS 864^T; green: only *P. reidholzensis* CCOS 865^T, black: both species. (**B**) Gene cluster for *p*-hydroxyphenylic acid (*p*-HPA) degradation. Orthologous genes are marked in the same color. The grey shading shows identical gene arrangements within the genomes.

Another gene cluster identified in the genome of *P. reidholzensis* CCOS 865^T may allow this organism to degrade mandelic acid. Mandelic acid occurs in its free form in plant tissues of wheat leaves and grapes [39]. Furthermore, glycosides of mandelonitrile can be found in almonds, peach and apricot pits [39]. Apart from this, the repellent mixtures of various arthropods and urine from animals are a potential source of mandelate or mandelonitrile, which can then find its way into the soil [39]. Four genes are known to encode enzymes involved in mandelic acid degradation (*mdlABCD*) [40]. Later studies revealed that also mandelamide dehydrolase (MdlY) is involved in the degradation pathway from mandelamide [41]. The mentioned genes were conserved in *P. reidholzensis* CCOS 865^T (CCOS865_04122–04126) and only occurred as well in *P. putida* NBRC 14164^T (Figure 2).

Additionally, in *P. wadenswilerensis* CCOS 864^T and *P. reidholzensis* CCOS 865^T, a putative gene cluster encoding for enzymes involved in the *ortho*-cleavage of benzaldehyde was found (CCOS864_00141–00148; CCOS865_04272–04279) (Figure 3A). Both species should therefore be able to degrade benzaldehyde via benzoate to catechol. Catechol will then be degraded to muconolactone and in a last step to 3-oxoadipate [30]. Furthermore, 3-oxoadipate enol-lactonases, involved in the last degradation reaction, were found at multiple locations in both genomes. Genes for the oxidation from benzene, toluene ethylbenzene or xylene (BTEX upper pathways) were not identified [42]. However, this pathway could be involved in the further degradation of mandelic acid in *P. reidholzensis* CCOS 865^T, whereas upper pathways in *P. wadenswilerensis* CCOS 864^T could not be identified. This *ortho*-cleavage pathway was also found in 32 other members of the *P. putida* group (Figure 2), while the cluster in *Pseudomonas* sp. MO2 (OTU06) was incomplete due to assembly gaps.

In the *P. reidholzensis* CCOS 865^T genome, we identified a putative gene cluster for the degradation of gallic acid (CCOS865_04015–04020) (Figure 3A). In nature, gallic acid and structurally related compounds have been discovered in many different fruits, plants and in oak wood [43,44]. Furthermore, this compound occurs mainly in black and green tea as phenolic component together with its catechol derivatives [44]. Degradation of gallate, a derivative of gallic acid, was observed in *Pseudomonas* sp. (formerly *P. putida*) KT2440, *Klebsiella pneumoniae* and other Alpha-, Beta- and Gammaproteobacteria. Genes involved in the degradation process of gallic acid are commonly in a single gene cluster (*galABCDPRT*) [45]. However, the cluster in the genome of *P. reidholzensis* CCOS 865^T lacked *galP*, encoding an outer membrane porin, responsible with GalT for the transport of gallic acid into the cell. This gene was located at another location in the genome (CCOS865_00376). The absence of *galP* in some gal-clusters was already reported before [45]. The putative gallic acid degradation cluster was also found in the genome of seven other *P. putida* group members and was not present in *P. wadenswilerensis* CCOS 864^T (Figure 2).

Additionally, the *P. reidholzensis* CCOS 865^T genome also contains genes for vanillin degradation (Figure 3A). Vanillin is a popular aromatic flavor compound and a natural degradation product of lignin or ferulic acid [46]. The *vdh* gene (CCOS865_02494) encodes the enzyme vanillin dehydrogenase, which catalyzes the oxidation of vanillin to vanillic acid [47]. The *vdh* gene occurred in 17 other members of the *P. putida* group (Figure 2). In the next step, the two subunits VanA (CCOS865_03327) and VanB (CCOS865_03328) are needed for the demethylation reaction of vanillic acid to protocatechuic acid [47]. Degradation thereof will occur via the intermediate 4-oxalomesaconate in the gallic acid degradation pathway.

3.4. Secondary Metabolites

In the genomes of *P. wadenswilerensis* CCOS 864^T and *P. reidholzensis* CCOS 865^T, a complete gene cluster for hydrogen cyanide synthesis (*hcnABC*) was found (CCOS864_00134–00136, CCOS865_00148–00150). Hydrogen cyanide (HCN) is a secondary metabolite and functions as cytochrome *c* oxidase and other metalloenzymes inhibitor [48]. The synthesis of HCN was observed in several pseudomonads, like *Pseudomonas aeruginosa*, *Pseudomonas protegens*, and *Pseudomonas soli* [15,48,49]. In Proteobacteria, hydrogen cyanide synthase is a protein involved in the oxidation of glycine to HCN and carbon dioxide on membranes [48]. HCN synthesis is of ecological interest

as it is involved in biological control of plant root diseases and as broad-spectrum antimicrobial compound [50]. Additionally, HCN increases the nutrients availability in the soil through sequestration of metals, resulting in higher phosphate concentrations necessary for plant growth [51]. As an example for biological control, HCN-producing fluorescent pseudomonads can suppress *Thielaviopsis basicola*, a plant pathogenic fungus involved in black root rot of tobacco [15,52]. Nevertheless, cyanide production of bacteria can have different effects on plants depending on the plant species and cyanide tolerance of the plant [53]. The *hcnABC* gene cluster also occurred in 13 other members of the *P. putida* group (Figure 2). Although the clusters in the different pseudomonads were highly similar, the flanking regions differed in most of the strains (Figure 4), which may be an indication that this cluster was introduced by horizontal gene transfer.

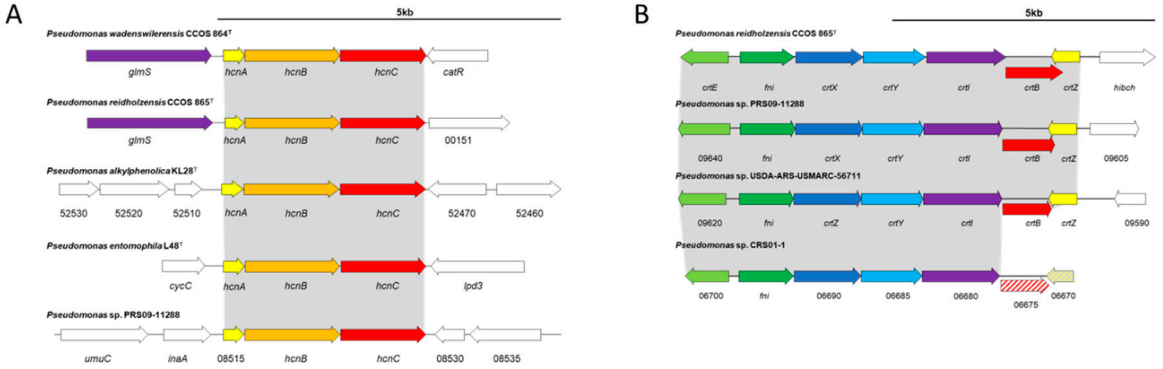

Figure 4. Secondary metabolites. (**A**): Gene cluster for hydrogen cyanide (HCN) biosynthesis. Orthologous genes are marked in the same color. Identical gene arrangements within the genomes are shaded in grey. (**B**): Carotenoid gene cluster for the synthesis of zeaxanthin. The grey shading shows identical arrangements within the genomes, while orthologous genes are marked in the same color. Dashed color indicates weak levels of sequence identity.

Furthermore, a putative carotenoid gene cluster for zeaxanthin biosynthesis in *P. reidholzensis* CCOS 865^T (CCOS865_02931–02936) was identified. Synthesis of carotenoids is widely present in Proteobacteria and has been demonstrated among others in *Erwinia*, *Pantoea*, or *Pseudomonas* sp. strain Akiakane [54,55]. Zeaxanthin, phenotypically seen as a yellow color of the colony, plays likely a role in photo-oxidative damage protection [54]. In *P. reidholzensis* CCOS 865^T, the cluster consisted of *crtE-fni-crtXIYBZ*, which corresponds to the cluster setup as in *Cronobacter* or *Erwinia* spp. [55,56]. When comparing with other members of the *P. putida* group (Figure 2), only four other strains had orthologous genes forming a complete cluster, while *Pseudomonas* sp. CRS01-1 (OTU12) displayed an incomplete cluster. However, closer inspection of the region downstream of *crtI* showed the presence of alternative *crtBZ* orthologs with lower sequence identity at this location (Figure 4). Although the presence of a complete gene cluster, a yellow color, indicating the synthesis of this carotenoid, was not observed phenotypically for *P. reidholzensis* CCOS 865^T [16]. Therefore, it might be possible that it is only weak or not at all expressed in *P. reidholzensis* CCOS 865^T under the tested conditions.

Bacteria from the genus *Pseudomonas* are known for possessing several protein secretion mechanisms, which are important for commensal, mutualistic or pathogenic interactions between them and their environment [57]. We identified one type I secretion (T1SS) cluster in *P. wadenswilerensis* CCOS 864^T, while three and two type II secretion system (T2SS) gene clusters were present in *P. reidholzensis* CCOS 865^T and *P. wadenswilerensis* CCOS 864^T, respectively. Further observations showed also the presence of two different type VI secretion system (T6SS) gene clusters.

3.5. Secretion Systems

3.5.1. Type I Secretion Systems

The alkaline protease AprA is secreted by a type I secretion (T1SS) [58,59]. The T1SS complex is built by three proteins; a specific outer membrane protein (OMP), an ATP-binding cassette (ABC) and an adapter or membrane fusion protein (MFP) [60]. In *P. aeruginosa*-associated infections, alkaline protease inhibits the connection between natural killer cells and target cells and plays therefore an important role in pathogenesis [61]. It was also proven that it occurs in other bacterial species such as *Dickeya dadantii*, *Escherichia coli* and several *Bacillus* or fungal species [61,62]. A T1SS gene cluster for alkaline protease was found in the genome of *P. wadenswilerensis* CCOS 864^T (CCOS864_01636–01640), but was not present in *P. reidholzensis* CCOS 865^T. The *apr* gene cluster in *P. wadenswilerensis* CCOS 864^T consists of five genes (*aprDEFAI*) as already observed before in other strains [60,63]. Orthologous *apr* gene clusters were only found in eight further strains within the *P. putida* group (Figure 2) although it was only partially identified in *Pseudomonas donghuensis* HYS^T (Figure 5). The sequence of the region pointed towards an assembly problem within the *aprD* gene due to the presence of unidentified bases (Ns).

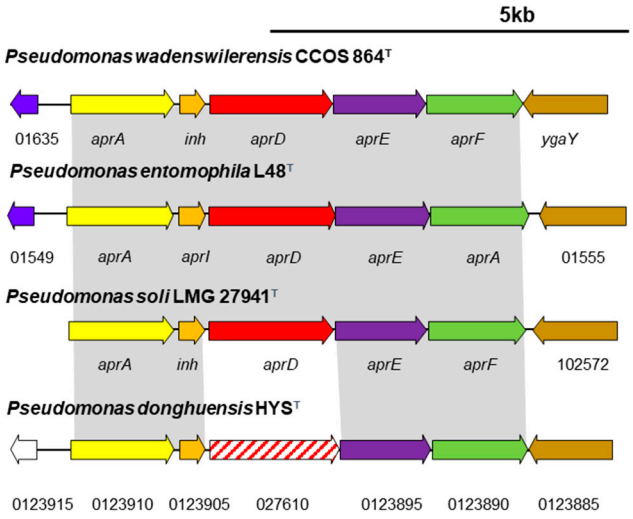

Figure 5. Type I secretion cluster (T1SS) for the secretion of alkaline protease. The same gene arrangement of the genomes is shaded in grey. Orthologous genes are marked in the same color. Dashed color indicates weak levels of sequence identity.

3.5.2. Type II Secretion Systems

Analysis of the pan-genome revealed several type II secretion system gene clusters (T2SS) in numerous strains. Two T2SS clusters were identified in *P. wadenswilerensis* CCOS 864^T, while three were present in *P. reidholzensis* CCOS 865^T. The main function of the T2SS being used by Gram-negative bacteria is the secretion of proteins (lipases, pectinases, phospholipases, cellulases and toxins), which are associated with tissue destruction and leading to cell damage and diseases [64]. At least 12 genes (*xcp* genes) are encoding for the T2SS and in some species it is known that they are regulated by quorum-sensing signals [64]. The two gene clusters present in *P. wadenswilerensis* CCOS 864^T were identified in a broad range in the *P. putida* group but are not omnipresent, while the third cluster in *P. reidholzensis* CCOS 865^T only was detected in *Pseudomonas* sp. KCJK7865 (OTU05) and *Pseudomonas* sp. W619 (OTU09) (Figure 2). Differences were only observed in the flanking regions of the genes, not within the clusters, indicating a foreign origin of these systems.

3.5.3. Type VI Secretion Systems

Type VI secretion systems (T6SSs) gained a lot of attention because of their potential involvement in pathogenic bacterial-host interactions [65]. Nevertheless, this is rarely the case and T6SSs are more often used to mediate in cooperative and competitive bacterial interactions, or to encourage commensal or mutualistic relationships between bacteria and eukaryotes [65–67]. In co-infections with a T6SS-positive *P. putida* strain and a plant pathogen (*Xanthomonas campestris*), a reduced plant leaf necrosis was seen [68]. Based on these investigations, the T6SS may also play an important role in plant protection and biocontrol of plant pathogens [66,68,69]. Gram-negative bacteria are able to possess up to five structurally different T6SS clusters in a genome [70]. In our study, we observed a single T6SS in *P. reidholzensis* CCOS 865^T, which was only present in two other members of the *P. putida* group (Figure 2). This T6SS cluster shares all conserved genes with *Pseudomonas* sp. W619 (OTU09) and *P. putida* W15Oct28 and therefore belongs to the phylogenetic group 2 as described before [68]. However, a group of hypothetical proteins, located in the center of the gene cluster, is only shared but not absolutely conserved between *P. reidholzensis* CCOS 865^T and *P. putida* W15Oct28 (Figure 6A). The T6SS group 2 is related to the T6SS-3 of *Erwinia* or *Pantoea* spp. [66,68,69].

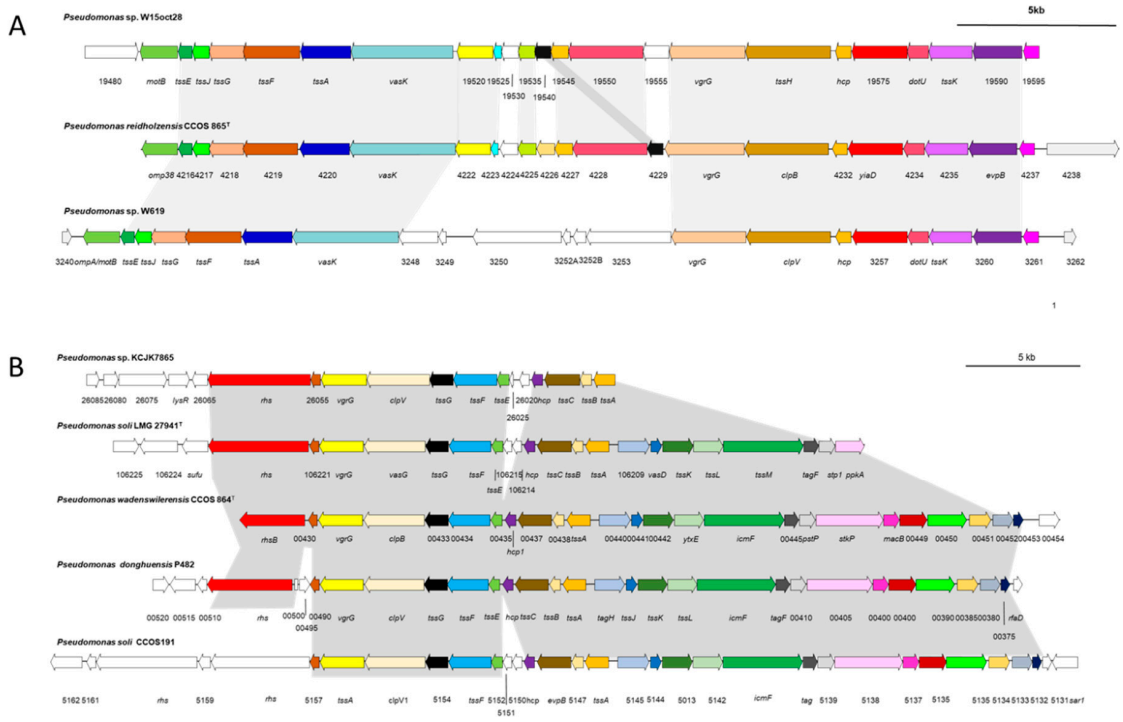

Figure 6. Type VI secretion systems. (**A**): The type VI secretion cluster (T6SS) of *Pseudomonas reidholzensis* CCOS 865^T and related sequences in other strains. Genes aligning to each other are marked in the same color, while the grey background marks identical alignments of the genes within the cluster. (**B**): The type VI secretion cluster (T6SS) of *Pseudomonas wadenswilerensis* CCOS 864^T and related systems in other *Pseudomonas* strains. Identical genes are marked in the same color, while the grey shading indicates the same gene arrangement.

Additionally, we discovered a different T6SS in the genome of *P. wadenswilerensis* CCOS 864^T (Figure 2), which was also existent in nine other members of the *P. putida* group, but was absent in *P. reidholzensis* CCOS 865^T. The T6SS cluster from *P. wadenswilerensis* CCOS 864^T showed structural differences due to insertions of genes encoding hypothetical proteins in the other strains (Figure 6B). However, this T6SS could not be categorized into a phylogenetic group as defined by Barnal et al. because similar clusters only occurred in species not investigated in the previous study [68]. This T6SS cluster thus represents an additional phylogenetic group of T6SS.

4. Discussion

4.1. Potential Ecological Role of P. wadenswilerensis CCOS 864^T and P. reidholzensis CCOS 865^T

The aim of our study was to highlight some of the most important genomic features in the genome of P. wadenswilerensis CCOS 864T and P. reidholzensis CCOS 865T. As both species were found in forest soil, we can now, after studying their genome sequences, use the identified features to hypothesize about their potential ecological role. In this study, we found some gene clusters encoding enzymes for the degradation of aromatic compounds such as p-HPA, mandelic acid, gallic acid and accompanying ortho- or meta-cleavage pathways (Figure 3A). We thus assume that P. wadenswilerensis CCOS 864^T and P. reidholzensis CCOS 865^T may play a role as degraders of organic material from forest litter. The presence of the T1SS in P. wadenswilerensis CCOS 864^T, several T6SSs and the HCN biosynthesis gene cluster in both species indicate that P. wadenswilerensis CCOS 864^T and P. reidholzensis CCOS 865^T are most probably able to competitively act in their environment. Additionally, they could be involved in biocontrol of plant pathogens and therefore in plant protection [50,66,68,69]. Furthermore, their ability for HCN biosynthesis may lead to a better phosphate availability in the soil, which allows for better plant-growth performance [51]. In conclusion, we assume that P. wadenswilerensis CCOS 864^T and P. reidholzensis CCOS 865^T represent ecologically relevant microorganisms based on their potential for degradation of organic material, nutrient accumulation for plants and protection against plant pathogens.

4.2. Potential Role of P. wadenswilerensis CCOS 864^T and P. reidholzensis CCOS 865^T as a Biocatalyst

Screening for biocatalytic enzymes has gained great importance due to the rapid increase of their usage in industrial applications [71,72]. At the same time, sequencing of genomes has become simpler, cheaper and faster [73], which has made genomics highly valuable in the search for biocatalysts and in the understanding of the whole systems biology [74–76]. In this study, we used the large amount of genomic information available for the *P. putida* group to determine the potential features of *P. wadenswilerensis* CCOS 864^T and *P. reidholzensis* CCOS 865^T that could qualify them as potential biocatalysts in biotechnological applications.

The discovery of several aromatic compound degradative pathways can yield novel biocatalysts with alternative substrate spectrum or catalytic specifications. However, as we only identified an *ortho*-cleavage pathway as required for BTEX compounds, it would be possible the genomes still contain upper pathways, including several mono- and dioxygenases that would act on aromatic compounds [42]. A first inspection of the genomes of *P. wadenswilerensis* CCOS 864^T and *P. reidholzensis* CCOS 865^T revealed several enzymes of this type, but it was impossible to assign a clear substrate from the annotation of the genes. The low sequence identity to known systems could thus yield enzymes with different catalytic properties.

The mandelic acid pathway as discovered in the genome of *P. reidholzensis* CCOS 865^T and *P. putida* NBRC 14164^T are highly similar to that of other species [39]. The mandelate racemase from *P. putida* ATCC 12633 is commonly used in biocatalysis for a broad range of conversions in which stereochemistry is relevant [77]. Here, we have shown that related enzymes are present in specific *Pseudomonas* strains that can be used to test stereo- and regiospecificity [77,78].

The potential for production of carotenoids by *P. reidholzensis* CCOS 865^T might be of interest for further studies. In particular, zeaxanthin showed promising effects against human cancer cells [79,80]. However, due to the missing phenotypical manifestation [16], the activity of the carotenoid biosynthesis genes for potential biocatalysis must be studied in more details.

5. Conclusions

This study has examined the potential role of *P. wadenswilerensis* CCOS 864^T and *P. reidholzensis* CCOS 865^T as biocatalysts. This revealed many interesting gene clusters encoding for enzymes for

potential use in biotechnological applications, such as proteases, isomerases, oxidoreductases and enzymes of the carotenoid biosynthesis pathway. Apart from biotechnological applications, we can assume that *P. wadenswilerensis* CCOS 864^T and *P. reidholzensis* CCOS 865^T are playing a role in the degradation of aromatic compounds commonly found in organic material in nature. Furthermore, we discovered many ecological relevant genomic features and we expect that, based on the presence of several secretion systems (T2SS, T6SS) and the ability to synthesize HCN, both species may be involved in biocontrol against plant pathogens and solubilization of phosphate in the soil, which may improve plant growth. Nevertheless, additional research will be necessary to confirm the expression of these enzyme systems in vivo and determine their activity. Altogether, we can conclude that both species have the potential as an alternative source for novel biocatalysts.

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