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ORIGINAL PAPER

Genome sequence analysis of *Pseudomonas extremaustralis* provides new insights into environmental adaptability and extreme conditions resistance

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Abstract The genome of the Antarctic bacterium *Pseudomonas extremaustralis* was analyzed searching for genes involved in environmental adaptability focusing on anaerobic metabolism, osmoregulation, cold adaptation, exopolysaccharide production and degradation of complex compounds. Experimental evidences demonstrated the functionality of several of these pathways, including arginine and pyruvate fermentation, alginate production and growth under cold conditions. Phylogenetic analysis along with genomic island prediction allowed the detection of genes with probable foreign origin such as those coding for acetate kinase, osmotic resistance and colanic acid biosynthesis. These findings suggest that in *P. extremaustralis* the horizontal transfer events and/or gene redundancy could play a key role in the survival under unfavorable conditions. Comparative genome analysis of these traits in other representative *Pseudomonas* species highlighted several similarities and differences with this extremophile bacterium.

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Introduction

Pseudomonas extremaustralis is a non-pathogenic species isolated from a temporary pond located on the west side of the Antarctic Peninsula, at 64°09′S, 60°57′W (López et al. 2009). This species shows high thermal and oxidative stress resistance associated with high polyhydroxybutyrate (PHB) production and it is also able to tolerate and degrade hydrocarbons (Ayub et al. 2004; Tribelli et al. 2012a). PHB biosynthesis genes are located within an adaptive genomic island and were probably acquired by horizontal transfer events (Ayub et al. 2007, 2009).

In extreme environments, metabolic plasticity could be a key factor to cope with unfavorable conditions. For example, variation in temperature, salt concentration, nutrient and oxygen availability are frequently found in natural environments, and then the presence and diversity of different metabolic pathways could be essential in colonization and survival (Su and Hassett 2012; Lawrence et al. 2004). *Pseudomonas* species were traditionally described as aerobic bacteria; however, a few years ago different metabolisms were described regarding energy production under low oxygen tension in some species of this genus (Arai 2011). Most of these studies were made in the model human opportunistic pathogen *P. aeruginosa* in which three main microaerobic metabolisms for energy production were described: denitrification and arginine and pyruvate fermentation (Arai et al. 1999; Eschbach et al. 2004; Vander Wauven et al. 1984). However, less information is available about other

environmental *Pseudomonas* strains with biotechnological and ecological importance (Mascher et al. 2003; Sohn et al. 2010).

While microaerobic metabolism is important for energy production under low oxygen tension, other mechanisms that enable the microorganisms to survive and adapt to stressful conditions are important for bacterial persistence in the environment. Cold habitats are widely distributed and present challenges at different cellular levels. Low temperature affects membrane fluidity, efficiency of RNA transcription and translation due to the stabilization of nucleic acid secondary structures, the folding of several proteins and also produces an increase in the reactive oxygen species (ROS) (D'Amico et al. 2006). Additionally, in subzero conditions ice presence leads to a decrement in water availability with a consequent osmotic shock (D'Amico et al. 2006). Therefore, growth and survival depend on the ability of organisms to cope with these unfavorable conditions more successfully than competing species (Rodriguez Frigi and Tiedje 2008). Among the mechanisms involved in stress resistance the presence of cold-shock proteins, the production of compatible solutes to counteract ice crystal presence, the production of exopolysaccharides to protect cells from ROS and to enhance biofilm formation and the ability to degrade complex compounds are relevant (Ferenci and Spira 2007). Ecological adaptation is essential to survive in extreme environments and also constitutes the most important driving force for bacterial genome rearrangement processes such as gene loss and acquisition and the movement of whole genetic islands (Wiedenbeck and Cohan 2011).

We hypothesized that bacterial adaptability to extreme environments involves the presence of genes related to different metabolism as energy production, stress tolerance and utilization of alternative compounds as carbon source but in addition, the acquisition of foreign genes through horizontal transfer and/or gene redundancy play a key role in survival under unfavorable conditions.

In this work, we analyzed *P. extremaustralis* genome focusing on the study of genes related to several metabolic processes important for environmental adaptability, such as anaerobic metabolism, cold growth, osmoregulation, exopolysaccharide production and degradation of complex compounds. We also assessed experimentally the functionality of some of these pathways in *P. extremaustralis.* The contribution of horizontal transfer events and gene redundancy in these processes was also analyzed. The comparison of these traits in other representative *Pseudomonas* species was used to recognize similarities and differences with this extremophile bacterium.

Materials and methods

Bacterial strains

P. extremaustralis 14-3b, a PHB producing organism with high stress resistance, was isolated from a temporary water body in Antarctica and its genome was sequenced (López et al. 2009; Tribelli et al. 2012b). Model *Pseudomonas* strains *P. putida* KT2440 (Ferenci and Spira 2007) *P. aeruginosa* PAO1 (Holloway 1955) and *P. protegens* Pf-5 (Paulsen et al. 2005) were also used in experimental assays.

Selection of strains

Seven representatives previously sequenced and well-studied *Pseudomonas* strains were selected for genomic analysis based upon their characterized and distinctive properties and their association with different habitats (Table 1).

Accession numbers

The complete genome sequence of *P. extremaustralis* has been deposited at GenBank/EMBL/DDBJ under accession AHIP01000001–AHIP01000135 (Tribelli et al. 2012b). Accession numbers for the complete genome sequences are shown in Table 1. Locus tags of all individual analyzed sequences of *P. extremaustralis* were included in Table S1.

Bioinformatic and phylogenetic analyses

Sequences were analyzed using the following programs available on line: BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), ClustalW (http://www.ebi.ac.uk/Tools/clustalw/) and bioinformatic tools included in the RAST server (http://rast.nmpdr.org/). The *Pseudomonas* genome database (Winsor et al. 2011) was also used to obtain comparative information. Phylogenetic analyses of proteins were performed using MEGA 5 (Takamura et al. 2011). Protein sequences were aligned using the ClustalW program. Phylogenetic trees were constructed using the neighbor-joining (NJ) method with genetic distances computed using Poisson correction model and maximum parsimony method, bootstrap analysis of 500 replicates and root on midpoint. Analysis of genomic islands was performed using the Island Viewer software (Langille and Brinkman 2009). This software integrates different methods for genomic island prediction including measures of codon usage, abnormal sequence composition or the presence of genes functionally related to mobile elements. In silico genome-to-genome distance calculations were performed using the service available in http://ggdc.dsmz.de/. The genome-to-genome distance calculator (GGDC) uses

Table 1 Characteristics of the analyzed bacterial strains

Strain	Relevant features	Isolation place	Genome accession number	Reference
P. extremaustralis 14-3b	Extremophile	Temporary pond in Antarctica	AHIP01000001–AHIP01000135	Tribelli et al. (2012b)
P. aeruginosa PAO1	Human opportunistic pathogen	Infected burn/wound of a patient in Melbourne, Australia	AE004091.2	Stover et al. (2000)
P. stutzeri A1501	Diazotroph	Rhizosphere of a *Sorghum nutans* cultivar	CP000304.1	Yan et al. (2008)
P. syringae pv *syringae* B728a	Plant pathogen	Snap bean leaflet in Wisconsin	CP000075.1	Feil et al. (2005)
P. fluorescens SBW25	Biocontrol agent	Field-grown sugar beet	AM181176.4	Silby et al. (2009)
P. protegens Pf-5	Plant growth promoting bacteria	Rhizosphere of cotton seedlings	CP000076.1	Paulsen et al. (2005)
P. putida KT2440	Organic pollutant degrader	Strain derived from a toluene-degrading isolate, mt-2	AE015451.1	Nelson et al. (2002)

tools such as BLAST to identify high-scoring segment pairs (HSPs) or maximally unique matches (MUMs) in each combination of query and reference genome for inferring intergenomic distances for species delimitation. The final result of this calculation is the conversion of these distances in percent-wise similarities analogous to DNA–DNA hybridization (DDH). The rationale of the distance calculation and its relation to DDH values is described in Auch et al. (2010).

Phenotypes associated with gene functions

Growth under low temperature

P. extremaustralis, P. aeruginosa PAO1, *P. putida* KT2440 and *P. protegens* Pf-5 were used in growth experiments. Overnight pre-cultures were performed in Luria-Broth medium (LB) at 30 °C and 200 rpm. These pre-cultures were used to inoculate cultures in LB supplemented with 0.25 % octanoate or 2 % glucose to allow PHA accumulation that enhances cold resistance in *P. extremaustralis* and *P. putida* KT2440 (Ayub et al. 2009) at an initial OD_{600nm} of 0.05. Cultures were incubated at 8 or 30 °C (as a control) at 200 rpm and OD_{600nm} was monitored through time.

Arginine fermentation

Arginine fermentation was measured by determination of arginine deiminase activity. *P. extremaustralis* was cultured in 100-ml completely filled hermetically sealed bottles under low oxygen tension in 0.5NE2 medium at 50 rpm supplemented with 50 mM of arginine at 30 °C for 72 h (Huisman et al. 1992). Cell extracts were obtained by sonication followed by centrifugation at 5,000×*g* for 30 min at 4 °C. The arginine deiminase activity was measured in the culture supernatant by quantifying citruline production colorimetrically as was described before (Archibald 1944), using a standard curve of citruline (Sigma). Protein content was determined by Bradford method using BSA as standard. The enzymatic activity was calculated as μmol of citruline per mg of protein.

Pyruvate fermentation

Presence of acid compounds derived of pyruvate fermentation was analyzed by high-performance liquid chromatography (HPLC). Microaerobic cultures of *P. extremaustralis* were carried out in 100-ml hermetically sealed bottles containing 50 ml of 0.5NE2 medium with pyruvate as sole carbon source (0.1 and 0.2 % w/v) and were incubated at 30 °C for 7 days. Cultures were centrifuged at 3,000×*g* for 20 min and the supernatant was diluted and filtrated. Aliquots from the supernatants were analyzed by HPLC, using an Aminex column HPX-87-H (Cat no. 125-0140) at 50 °C and an UV detector set to 215 nm (for organic acids) and standards of the different organic acids (Sigma).

Alginate production

P. extremaustralis cultures were performed in 0.5NE2 medium supplemented with 0.25 % octanoate sodium or 3 % glucose, grown at 30 °C and 200 rpm for 24 h. Alginate was obtained by precipitation of the culture supernatant by addition of 2 volumes of isopropanol and quantified spectrophotometrycally as previously described (May and Chakrabarty 1994). Calibration curve was performed using different concentrations of commercial alginate (Sigma). Values were normalized by cellular dry weight.

Statistical analysis

The significance of each treatment was evaluated by Student's *t* test with confidence levels at 95 % (i.e. $P < 0.05$

was considered as significant). For growth experiments comparing different *Pseudomonas* species one-way ANOVA test was used and $P < 0.05$ was considered as significant.

Results and discussion

Genomic relationship of *P. extremaustralis*

In order to estimate the overall similarity between the genome of *P. extremaustralis* and each genome of the model strains selected for comparison, an in silico analysis was performed to mimic DDH lab technique, in which the definition of species is based. All the selected strains showed in silico DDH values lower than 70 %, further demonstrating that they are different species. The highest genome similarity was found with *P. fluorescens* strain SBW25 that showed 39.50 ± 2.51 %, followed by *P. protegens* Pf-5 with 30.80 ± 2.45 %. Both species are found in terrestrial habitats associated with the surfaces of plant roots and leaves (Silby et al. 2009). Values of 23.60 ± 2.38 and 26.20 ± 2.42 % were obtained for the human opportunistic pathogen *P. aeruginosa* PAO1 and the soil bacteria *P. putida* KT2440, respectively. The genome of the plant pathogen *P. syringae pv syringae* B728a shared 26.70 ± 2.42 % similarity with *P. extremaustralis* genome. *P. stutzeri* A1501, which can survive in the soil, fix nitrogen, colonize the root surface and was also reported as endophyte in the superficial layers of the root cortex (Yan et al. 2008), showed only a 24.40 ± 2.39 % of DDH estimated. However, *P. stutzeri* A1501 shares a genomic island containing PHB genes with similarity higher than 90 % with the genomic island of *P. extremaustralis.* PHB genes are essential for survival under cold condition in *P. extremaustralis* (Ayub et al. 2009).

Microaerobic metabolism in *P. extremaustralis*

Denitrification is the process by which nitrate is successively reduced to gaseous nitrogen species, being the final product N_2. Four enzymatic complexes are required for this pathway: nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS) (Arai 2011). Several genes involved in the denitrification pathway were found in *P. extremaustralis* genome. In particular, all genes needed for nitrate reduction were observed sharing a similar organization with *P. aeruginosa* PAO1 (Fig. 1). *P. stutzeri* A1501 also showed the complete set of genes, except for the nitrate transporter *narK2;* however, in this strain another gene encoding a protein with a similar function was found. Genes involved in nitrate reduction were not found in *P. putida* KT2440, *P. protegens* Pf-5, P. *syringae* pv *syringae* B728a and P. *fluorescens* SBW25 (Fig. 1).

P. extremaustralis only presented one gene involved in nitrite reduction, *nirM* in contrast with *P. aeruginosa* PAO1 (Fig. 1). These results were in line with experimental data regarding the impairment of *P. extremaustralis* to carry out nitrite reduction (Tribelli et al. 2010). Interestingly, all genes related with nitric oxide reduction were present in *P. extremaustralis* (Fig. 1). This region showed a high degree of synteny in comparison with *P. aeruginosa* PAO1. *P. stuzeri* A1501 showed some of the genes involved in the nitric oxide conversion with the exception of *norE* and *norF*. The NOR cluster of genes was not present in *P. putida* KT2440, *P. protegens* Pf-5*, P. syringae* pv *syringae* B728a and *P. fluorescens* SBW25. Additionally, *P. extremaustralis* presented the complete nitrous oxide reduction pathway. Genes encoding nitrous oxide reductase *nosRZDYFL* were present in all the analyzed species, with the exception of *P. putida* KT2440, regardless the presence of the genes encoding NAR, NIR and NOR enzymes (Fig. 1). N_2O is emitted from different environments such as soils, ocean, estuaries and

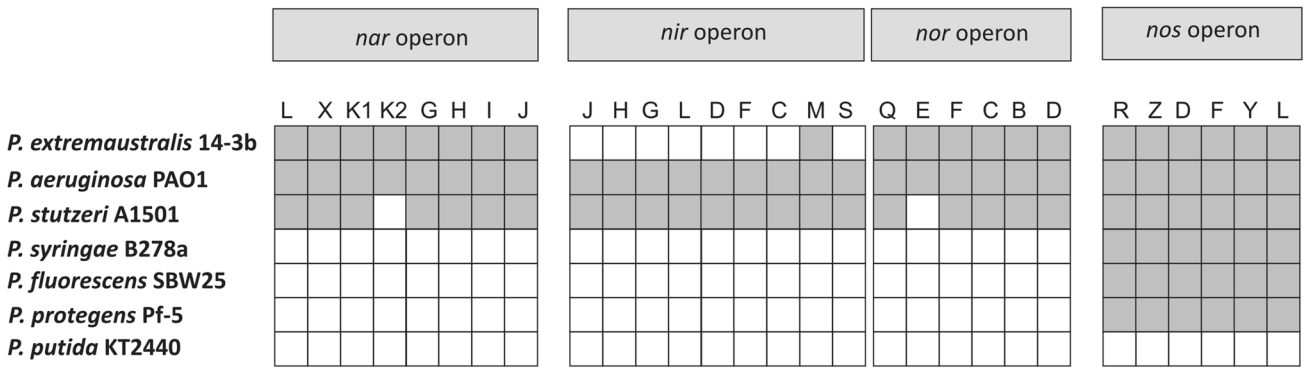

Fig. 1 Comparison of genes belonging to the denitrification pathway. Genes belonging to *nar, nir,nor and nos* operons are *narLXKGHJI*, *nirJHGLDFCMS*, *norQEFCBD* and *nosRZDFYL*. Gray boxes represent the presence of a gene within a genome, while absence of a gene is represented by white boxes; numbers inside a box represent the number of copies of a gene within a genome

freshwater habitats including wastewater treatment plants (Schreiber et al. 2012). Soils and aquatic habitats exposed to intense agricultural activities are the largest sources of nitrous oxide due to high N-input through fertilization, increasing N_2O levels considered actually a powerful greenhouse gas (Henry et al. 2006). Therefore, the presence of NOS genes in non-denitrifying bacteria could be related to the detoxification of this compound in environments derived from both natural and anthropogenic sources.

Arginine deiminase pathway was described in *P. aeruginosa* as an alternative metabolism to denitrification, with ATP production at substrate level phosphorylation (Vander Wauven et al. 1984). Proteins related with this metabolism are encoded by the *arcDABC* operon in *P. aeruginosa*. The product of *arcA* is the arginine deiminase, a key enzyme in the pathway; the catabolic ornithine carbamoyltransferase is coding by *arcB,* while *arcC* encodes a carbamate kinase and *arcD* is an ornithine–arginine antiporter (Vander Wauven et al. 1984). This pathway was present in *P. extremaustralis* and was conserved across different *Pseudomonas* species. In all the analyzed *Pseudomonas*, with the exception of *P. syringae* pv *syringae* B728a and *P. aeruginosa* PAO1, a duplication of the *arcD* gene was observed, but in *P. extremaustralis* four copies of this sequence were found. Among them, one (*arcD*1) is part of the *arcDABC* operon and other (*arcD*2) is divergent from this operon. The deduced aminoacid sequences presented around 75 % of identity to each other and with the rest of analyzed *Pseudomonas* species. A third copy (*arcD*3) was located in a genomic island and the deduced protein sequence presented 77 % of identity and 95 % of similarity with the *arcD*1 gene. Additionally, a fourth copy (*arcD4*) was located in a genome region that contains genes that showed high similarity with *Burkholderiales.* Similarity and identity at protein level of *arcD* copies in *P.extremaustralis* were around 55 and 80 %, respectively; these values were similar to other analyzed *Pseudomonas* species.

In order to analyze the functionality of arginine fermentation pathway in *P. extremaustralis*, the activity of arginine deiminase was determined. *P. extremaustralis* presented a positive activity for this enzyme, with values of 0.027 ± 0.014 μmol of citruline/mg of protein in cultures of 72 h under low oxygen conditions in medium supplemented with arginine, while without arginine, the basal activity was significantly lower (0.003 ± 0.002 μmol of citruline/mg, $P < 0.05$). A similar arginine deiminase activity was found in *P. putida* KT2240 (0.035 ± 0.021 μmol of citruline/mg of protein) while in *P. aeruginosa* the activity was higher with values of 0.139 ± 0.01 μmol of citruline/mg of protein.

Until now, pyruvate fermentation in *Pseudomonas* was described only in *P. aeruginosa* in which the pyruvate is converted to acetyl-CoA by the pyruvate dehydrogenase (encoded by *pdh*) followed by the conversion to acetyl-phosphate by the phosphotransacetylase (encoded by *pta*) and finally converted to acetate by the acetate kinase (encoding by *ack*) with generation of energy. This pathway also includes a lactate dehydrogenase (encoded by *ldh*) which catalyzes the conversion of pyruvate to lactate and an alcohol dehydrogenase (encoded by *adh*) that is involved in ethanol production from acetyl-CoA (Eschbach et al. 2004). In all analyzed *Pseudomonas* species *pta, ldh* and *adhA* were found. However, the presence of the gene coding for Ack was only found in *P. aeruginosa* PAO1, *P. stutzeri* A1501 and *P. extremaustralis.* Two copies of this gene were present in *P. extremaustralis* and *P. stutzeri* A1501 (*ack1* and *ack2*). The *ack1* sequence was similar in both species and was located in a genomic island found in both strains. Phylogenetic analysis of the deduced protein sequences showed that they were related to those found in α- and β- *Proteobacteria* (Fig. 2). Interestingly, the phylogenetic tree showed that, while the Ack2 of *P. stutzeri* A1501 clustered with similar proteins belonging to other *Pseudomonas* species, the *P. extremaustralis* Ack2 was not related to *Pseudomonas* species, clustering with β-*Proteobacteria* (Fig. 2). To test the functionality of this pathway in *P. extremaustralis* the presence of organic acid compounds was determined in supernatants of microaerobic cultures. Lactate, formate and acetate (product of the Ack enzyme) were detected in preliminary HPLC experiments and also a low amount of residual pyruvate, showing that *P. extremaustralis* was able to perform pyruvate fermentation (data not shown).

The extent of alternative pathways to aerobic respiration in *Pseudomonas* species highlights its importance to thrive under the different oxygen tensions found in their habitats. In the case of *P. aeruginosa* nitrite reduction contributes to virulence by the generation of NO, a signal molecule in the pathogenic context (Van Alst et al. 2009) and pyruvate fermentation seems to be important for biofilm formation, a key process in chronic infections (Petrova et al. 2012). Waterlogged soils or those with high humidity content present low oxygen relative pressure (Mirleau et al. 2001) and, as a consequence, the capability of bacteria to grow under microaerobic conditions could result in an advantage for their survival. Moreover, the presence of nitrate reductase is an advantage for bulk and rhizosphere soil bacteria (Mirleau et al. 2001). *P. extremaustralis*, isolated from an extreme environment, presented functional pathways as well as redundant genetic information regarding some important genes relevant for energy production in low oxygen environments.

Cold growth in *P. extremaustralis*

Although almost all microorganisms are exposed to temperature changes, not all of them are capable to grow under

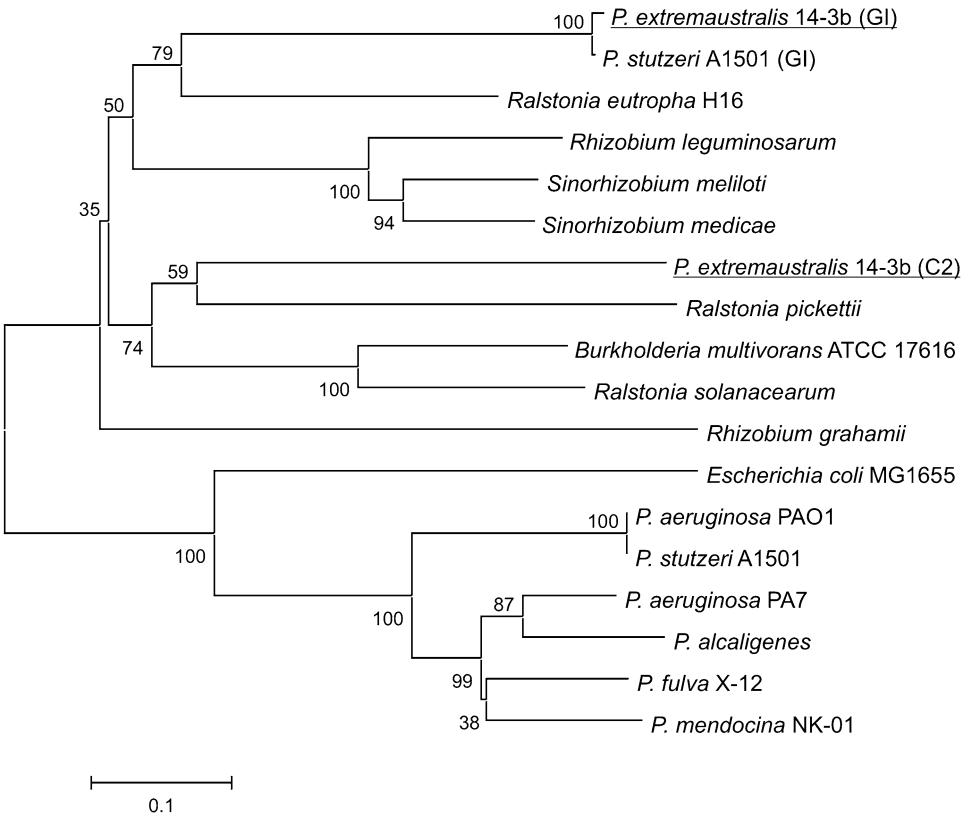

Fig. 2 Neighbor-joining phylogeny inferred from aligned amino acid sequences of Ack proteins from different *Proteobacteria*. Two copies of Ack were found in *P. extremaustralis* 14-3b and *P. stutzeri* A1501. The Ack1 copy located within a genomic island is indicated as GI. Ack was also present in *P. aeruginosa* PAO1, among the other analyzed strains. Bootstrap values equal to or greater than 50 % are shown, and the scale bar represents the number of substitutions per site. GI denotes that this copy was found in a genomic island. Tree topology was similar using maximum parsimony method (data not shown)

cold conditions (Feller 2013). *P. extremaustralis*, an Antarctic isolate, was able to grow under low temperature with either glucose or octanoate as carbon source more efficiently than the other tested *Pseudomonas* (*P. protegens* Pf-5, *P. putida* KT2440, *P. aeruginosa* PAO1) (Fig. 3). *P. protegens* Pf-5 reaches a similar OD_{600nm} as *P. extremaustralis* after 72 h, but the lag phase was longer in Pf-5 strain (Fig. 3). Moreover, in *P. extremaustralis* the OD_{600nm} at the end of the experiment was significantly different in comparison with *P. aeruginosa* PAO1 ($P < 0.05$), an opportunistic human pathogen, and *P. putida* KT2440, a soil isolate. At 28° C the growth was similar for all strains in both carbon sources (Fig. 3). These experiments showed that *P. extremaustralis* is able to grow under low temperatures reaching an OD_{600nm} of 6.4 ± 0.6 in culture medium supplemented with sodium octanoate after 72 h of incubation. In order to identify possible adaptations to cold conditions we analyzed the presence of cold-shock proteins in the genomes of the selected *Pseudomonas* strains. These small proteins, called Csps, constitute a conserved family of nucleic acid-binding proteins related to cold-shock and are usually induced under cold conditions (Horn et al. 2007). The Csp proteins are synthesized in psychrophilic, mesophilic and thermophilic microorganisms to cope with temperature downshift. In addition to Csp proteins, only psycrotolerant and psycrophile bacteria synthesize cold acclimation proteins (Caps) in response to continuous growth at low temperatures (Ermolenko and Makhatadze, 2002). Among the selected species, *P. syringae* pv. *syringae* B278a and *P. aeruginosa* PAO1, which were not able to grow under cold conditions, presented the lowest number of cold shock proteins. Interestingly, *P. extremaustralis* presented a complete cold shock genetic battery composed by all the cold-shock proteins described before, including Csp family proteins and Cap proteins coding genes and three additional ORFs with cold-shock domains (Fig. 4). These results suggest that these proteins contribute to cold adaptation of *P. extremaustralis*.

Osmotic stress resistance in *P. extremaustralis*

In order to adapt to a reduction in external water activity, cells accumulate low-molecular-weight solutes to maintaining the proper intracellular osmotic balance (Thusitha et al. 2008). Particularly, it has been reported that in Antarctica, freezing tolerance of psychrotrophic bacteria is often accompanied by tolerance to osmotic stress caused by a lack of free water (Song et al. 2013); thus *P. extremaustralis,* proceeding from an Antarctic environment, could have a machinery to cope with osmotic stress.

The ability to respond to an osmotic up-shock by accumulating compatible solutes can be found in different domains of life and includes the production of sugar,

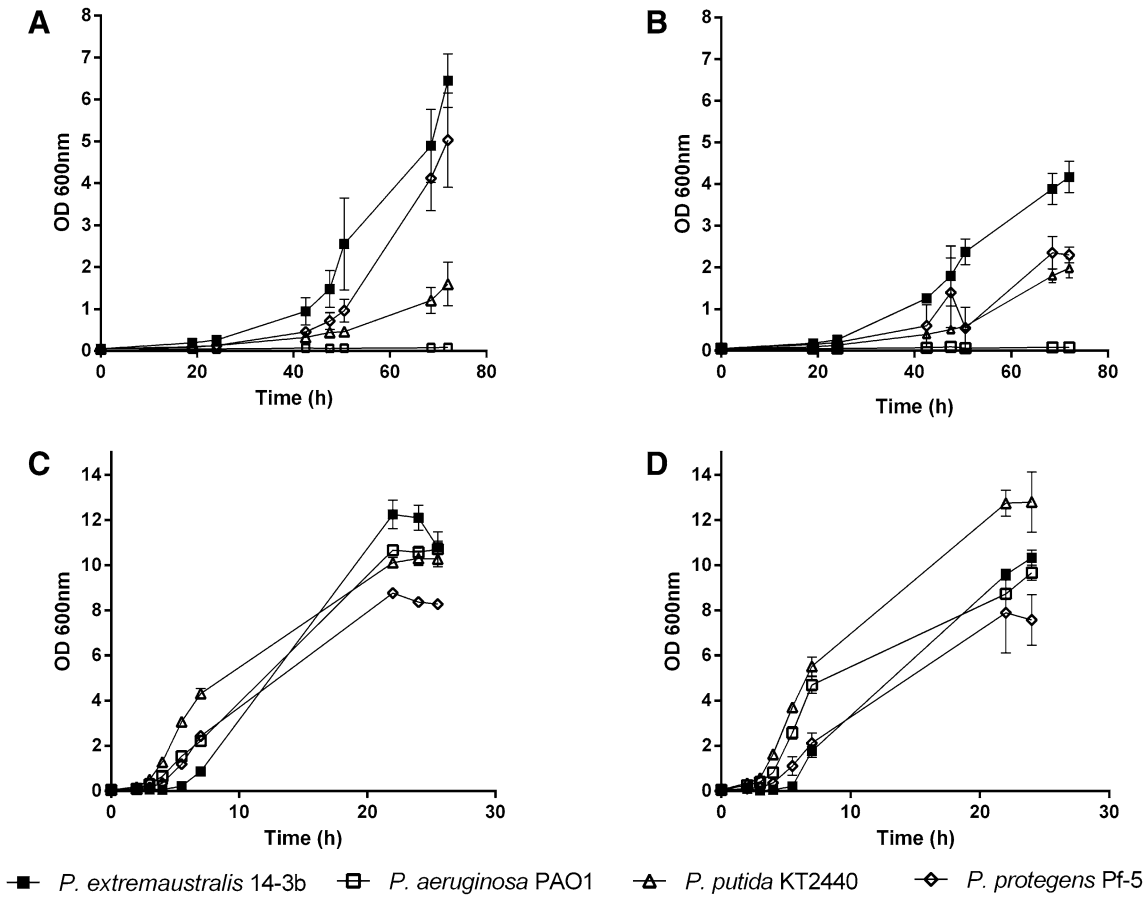

Fig. 3 Growth of *P. extremaustralis* 14-3b, *P. fluorescens* SBW25, *P. protegens* Pf-5, *P. aeruginosa* PAO1, *P. putida* KT2440, *P. stutzeri* A1501 and *P. syringae* pv *syringae* B728a. Growth at 10 °C (**a** sodium octanoate, **b** glucose). Growth at 28 °C (**c** sodium octanoate, **d** glucose)

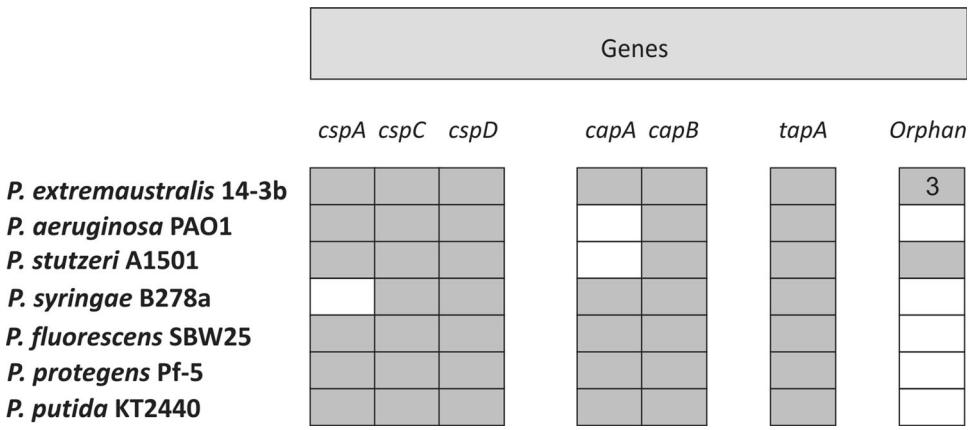

Fig. 4 Comparison of genes encoding cold shock proteins among *Pseudomonas* species. Gray boxes represent the presence of a gene within a genome, while absence of a gene is represented by white boxes. Orphan genes codify uncharacterized proteins containing cold shock domains. Numbers inside a box represent the number of copies of a gene within a genome

polyols and aminoacids and derivatives (Roeûler and Mueller 2001). Among them, glycine betaine (GB) is one of the most widespread and effective compatible solutes (Wargo 2013). The genes related to the biosynthesis of GB from choline were found in *P. extremaustralis*. Although this pathway was conserved across the analyzed *Pseudomonas* species, *P. extremaustralis* presented a different number of copies of these genes (Fig. 5). Moreover, the *gsmT* gene, coding a key protein (glycine-sarcosine methyltransferase) for the biosynthesis of GB from glycine, not usually present in bacteria, was found in *P. extremaustralis* genome (Fig. 5). The biosynthesis of betaine from glycine was described in some halotolerant Archaea like *Aphanothece halophytica* and *Methanohalophilus portucalensis* (Waditee et al. 2003; Lai and Lai 2011). The deduced sequence of the GsmT protein in *P. extremaustralis* (256

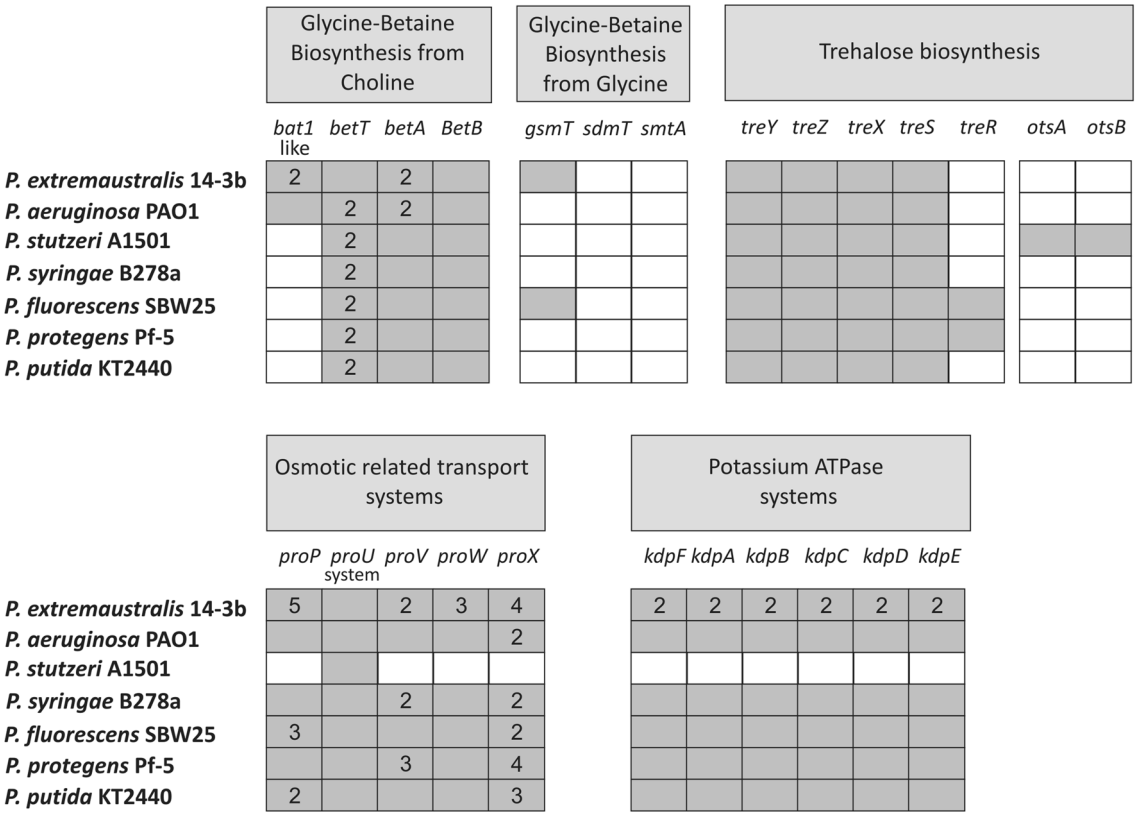

Fig. 5 Genes related to osmotic resistance pathways. Gray boxes represent the presence of a gene within a genome, while absence of a gene is represented by white boxes. Numbers inside a box represent the number of copies of a gene within a genome

aminoacids) was compared to those described in *A. halophytica* (265 aminoacids) and in *M. portucalensis* (263 aminoacids). The analysis showed that GmsT of *P. extremaustralis* shared a 38 % of identity and 75.4 % of similarity with the GmsT found in *A. halophytica* and 37 % of identity and 72.5 % of similarity with the GmsT belonging to *M. portucalensis*. The average percent G+C content for *gmsT* gene was 54.6 %, lower than 60.66 %, the average for the *P. extremaustralis* genome (Tribelli et al. 2012b). Among the analyzed *Pseudomonas,* only *P. fluorescens* SBW25 presented the *gsmT* gene and the deduced protein (255 aminoacids) showed 83 and 94 % of identity and similarity, respectively, compared to *P. extremaustralis*. The presence of *gsmT* gene allows sarcosine accumulation, a compound which can be found in antibiotics and in lactones molecules but more importantly, is considered an osmoprotector itself suggesting a function in osmotic balance in *P. extremaustralis* (Jackson et al. 2004; Simon et al. 2009).

All genes for trehalose biosynthesis from maltose and glycogen (*treYZXS*) were present in all the analyzed *Pseudomonas* species, including *P. extremaustralis* (Fig. 5).

Other important mechanism in osmotic resistance is the high-affinity potassium uptake mediated by the ATP-driven KdpFABC complex and regulated by KdpD and KdpE described in *Escherichia coli* (Irzik et al. 2011). In *P. extremaustralis* two clusters related to potassium ATP-dependent transport were found (Fig. 5). One of these clusters was similar to those present in *P. fluorescens* SWB25, *P. protegens* Pf-5, *P. putida* KT2440, *P. syringae* pv. *syringae* B278a and *P. aeruginosa* PAO1. However, in *P. extremaustralis* another cluster of the *kdpFACBDE* genes, arranged in different order, was found in a genomic island detected using the IslandViewer software. A phylogenetic analysis was performed to analyze the origin of each Kdp gene cluster using the deduced aminoacid sequence of *kdpC*, encoding the potassium-transporting ATPase C chain, as representative protein. This analysis showed that KdpC (C1) of *P. extremaustralis* was related to *P. protegens* Pf-5 and *Burkholderia* species but not to other *Pseudomonas* (Fig. 6), probably suggesting its acquisition by horizontal transfer events in line with its location in a putative genomic island. By contrast, the second copy of KdpC (C2) seems to belong to the core genome as was associated with *Pseudomonas* species (Fig. 6).

The presence of osmotic balance-related genes and the existence of several copies of some of them could be important for stress resistance in the Antarctic environment due to the role of betaine, proline and trehalose in the

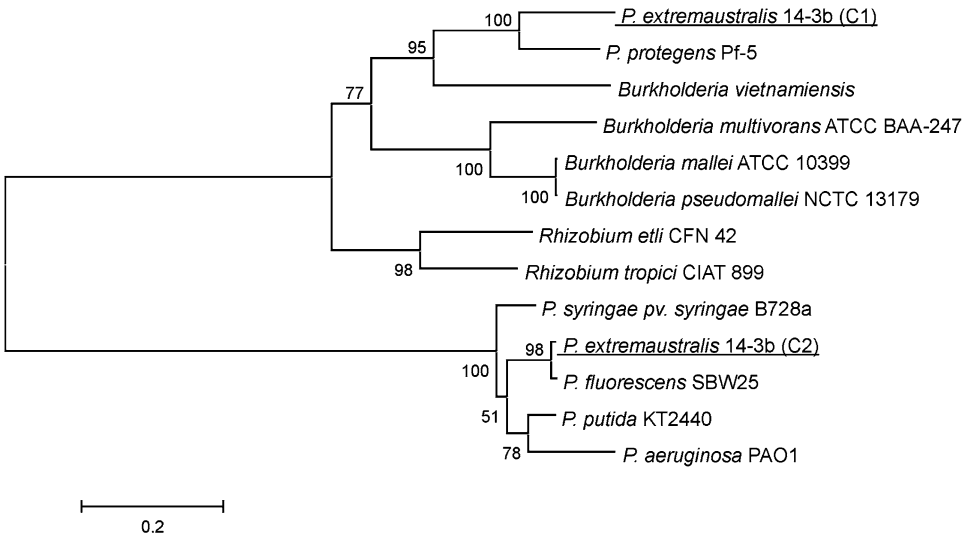

Fig. 6 Neighbor-joining phylogeny inferred from aligned amino acid sequences of KpdC proteins from different *Proteobacteria*. In *P. extremaustralis* 14-3b two copies of KpdC were found. Bootstrap values equal to or greater than 50 % are shown, and the scale bar represents the number of substitutions per site. C1 and C2 correspond to the two copies of this gene found in *P. extremaustralis*. Tree topology was similar using maximum parsimony method (data not shown)

stabilization of proteins in repeated freeze–thaw cycles (Carpenter et al. 1993). Additionally, the presence of the *gsmT* gene found in Archaea, but rarely in Bacteria, could allow sarcosine accumulation, another compatible osmolyte, from glycine. The redundancy of the entire *kdpFACBDE* operon in *P. extremaustralis* could result important for adaptability since it has been proposed that the increased concentration of cytoplasmic K^+ is the central regulatory signal that turns on other responses to cope with osmotic stress (Sutherland et al. 1986). The relevance of osmotic balance in extreme environments is exemplified in the wide gene battery related to this function present in *P. extremaustralis* genome.

Exopolysaccharides production

Mechanisms related to water stress tolerance, including freezing, involve the production of exopolymeric substances that protect bacterial cells but also enhance tolerance to desiccation, oxidizing agents and host defensive processes (Chang et al. 2007; Friedman and Kolter 2004; Jackson et al. 2004). *P. extremaustralis* presented several groups of genes related to polysaccharide production. Genes involved in Pel (a glucose-rich, cellulose-like exopolysaccharide), poly-β-1,6-*N*-acetylglucosamine (Pga), alginate and other capsular exopolysaccharides, such as colanic acid, were detected.

Genes implicated in the biosynthesis of two exopolysaccharides, Pga and Pel, described as a matrix biofilm component, were found in *P. extremaustralis*. Interestingly, only *P. extremaustralis* and *P. protegens* Pf-5 presented both clusters of genes, while in *P. aeruginosa* PAO1 and in *P. fluorescens* SBW25 only the Pel cluster and the Pga cluster, respectively, were found (Fig. 7).

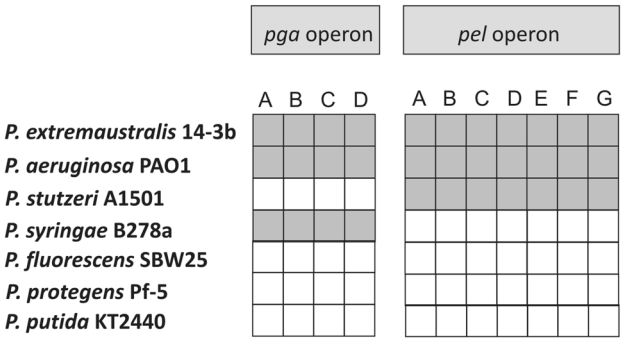

Fig. 7 Exopolysaccharide genes related to biofilm formation. Gray boxes represent the presence of a gene within a genome, while absence of a gene is represented by white boxes

Besides its role in enhancing water stress tolerance, alginate is an important exopolysaccharide with biotechnological interest (Hay et al. 2014). The biosynthesis of alginate has been demonstrated in *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. syringae*, *P. mendocina* and *A. vinelandii* (Govan et al. 1981; Fett et al. 1986). The alginate production pathway has been deeply studied in *A. vinelandii* and *P. aeruginosa* (Ramsey and Wozniak 2005; Trujillo-Roldan et al. 2003). The sequences of the genes involved in the alginate metabolism in these bacteria were used for comparative analysis of *P. extremaustralis* genome. In *P. aeruginosa* structural genes related to alginate biosynthesis are arranged in an operon containing 12 genes (a*lgD, alg8, alg44, algK, algE, algG, algX, algL, algI, algJ, algF* and *algA*) (Chitnis and Ohman 1993). Regulator genes are organized in two groups, the first composed by *algU*, *mucA*, *mucB*, *mucC* and *mucD* (Ramsey and Wozniak 2005) and the second one represented by *algR*, *algQ*, *algP, algZ* and *algB*. All structural and regulatory genes,

except *mucC,* were present in *P. extremaustralis* genome. The entire genomic region encoding structural alginate genes showed high degree of synteny with other species belonging to *Pseudomonas* genus. The deduced aminoacid sequences showed more than 95 % similarity with homologous proteins belonging to *P. fluorescens* SBW25. In addition, *algC*, encoding a phosphomannomutase also involved in rhamnolipid and lipopolysaccharide biosynthesis (Olvera et al. 1999), was observed in *P. extremaustralis a*s well as in the other analyzed *Pseudomonas*. Bioinformatics analysis showed that *P. extremaustralis* has all structural genes related to the alginate production and most of the regulatory genes, excluding *mucC*. Comparative analysis of the region containing this gene in the selected *Pseudomonas* strains showed that *mucC* was only present in *P. aeruginosa* PAO1 and was absent in *P. fluorescens* SWB25, *P. protegens* Pf-5, *P. putida* KT2440, *P. syringae* pv. *syringae* B278a and *P. stutzeri* A150, in concordance with that observed in *P. extremaustralis.*

In *P. aeruginosa*, insertional inactivation of *mucC* does not cause any overt effects on alginate synthesis; however, it affected growth under conditions of combined elevated temperature and increased ionic strength or osmolarity (Boucher et al. 1997). The absence of *mucC* in environmental *Pseudomonas* and its presence in the pathogenic species *P. aeruginosa* could represent another evidence of the genetic flexibility of the *Pseudomonas* species, showing particular phenotypes adapted to many different ecological niches.

The functionality of alginate biosynthesis genes in *P. extremaustralis* was demonstrated by quantitative analysis. Alginate production was 3.27 ± 0.44 mg alginate/g dry weight when the carbon source was glucose. When the fatty acid sodium octanoate was used as carbon source, alginate was also produced (1.08 ± 0.26 mg alginate/g dry weight). These results show that the absence of *mucC* could be not essential for alginate production.

The production of the capsular polysaccharide colanic acid has been described as an important element against desiccation, oxidative and osmotic stress and not related to virulence, in *Escherichia coli* (Ophir and Gutnick 1994; Chen et al. 2004). Several genes involved in colanic acid biosynthesis were found in *P. extremaustralis* genome (Table S1) among them *wcaF, wcaA,gmd wcaB, wcaC, wcaG, wcaI, wcaL* and *amm*. We also detected *wza, wzb* and *wzc* genes necessaries in *E. coli* for the late stages of colanic acid production (Stevenson et al. 1996). Additionally, two copies of *gmd, wcaG and amm* genes were found. Interestingly, colanic acid biosynthesis genes were not observed in the other analyzed *Pseudomonas* strains with the exception of *P. fluorescens* SBW25. The analysis of the *P. extremaustralis* genomic region around these genes showed high similarity with *Enterobacteria* like *Salmonella* sp., *E. coli, Citrobacter* sp. and *Yersinia* sp. while the *gmd, wcaG and amm* extra copies were located in a genomic island identified by Island viewer program, with an average of 51.6 % of G+C lower than 60.66 % corresponding to the whole genome G+C average (Tribelli et al. 2012b). These results could indicate the acquisition of this metabolic pathway by horizontal transfer.

Capsular polysaccharides (CPS) and exopolysaccharides (EPS) possess important functions under unfavorable conditions. Alginate and colanic acid have been reported as relevant for desiccation and osmotic resistance (Freeman et al. 2013). Since many EPSs are hygroscopic, their presence presumably creates a more hydrated microenvironment in the immediate vicinity of the cells, thereby contributing to desiccation tolerance. The capability of *P. extremaustralis* to produce alginate from different carbon sources could be relevant for tolerating water stress deriving from freezing conditions. On the other hand, Pel and Pga EPS are relevant for biofilm formation, especially Pel is responsible for the pellicle air–liquid observed in *P. aeruginosa.* Moreover, it has been demonstrated that it also enhanced cell-to-cell interactions as well as increased biomass of biofilms (Ghafoor et al. 2011), while Pga was found in *E. coli* biofilms as an important matrix component (Branda et al. 2005). Previous studies have demonstrated the capability of *P. extremaustralis* to produce well-developed biofilms under different conditions, including low temperatures or stressful conditions such as the presence of hydrocarbon compounds (Tribelli and López 2011, Tribelli et al. 2012a). The genetic information related to the production of the four exopolysaccharides observed in *P. extremaustralis,* not found together in other *Pseudomonas* strains so far, suggests the importance of these compounds to cope with unfavorable conditions prevailing in extreme environments.

Degradation of complex compounds: ferulic acid metabolism

In poor nutrient environments the ability to catabolize complex aromatic compounds could confer advantages to cope with famine. Ferulic acid is one of the most common hydroxycinnamic acids present in lignin. All genes necessary for ferulic acid catabolism were present in *P. extremaustralis.* The *P. extremaustralis* ferulic acid degradation gene cluster and the presence of these genes in other studied *Pseudomonas* are shown in Fig. 8a. Hydroxyccinamate-specific porin (*orpD*) and hydroxycinnamate transporter (*mhpT*), key for the cinamic acid derivative uptake, were only observed in *P. extremaustralis, P. fluorescens* SBW25 *and P. syringae* pv *syringae* B782a. Interestingly, a second copy of *orpD* was only found in *P. extremaustralis* (Fig. 8a).

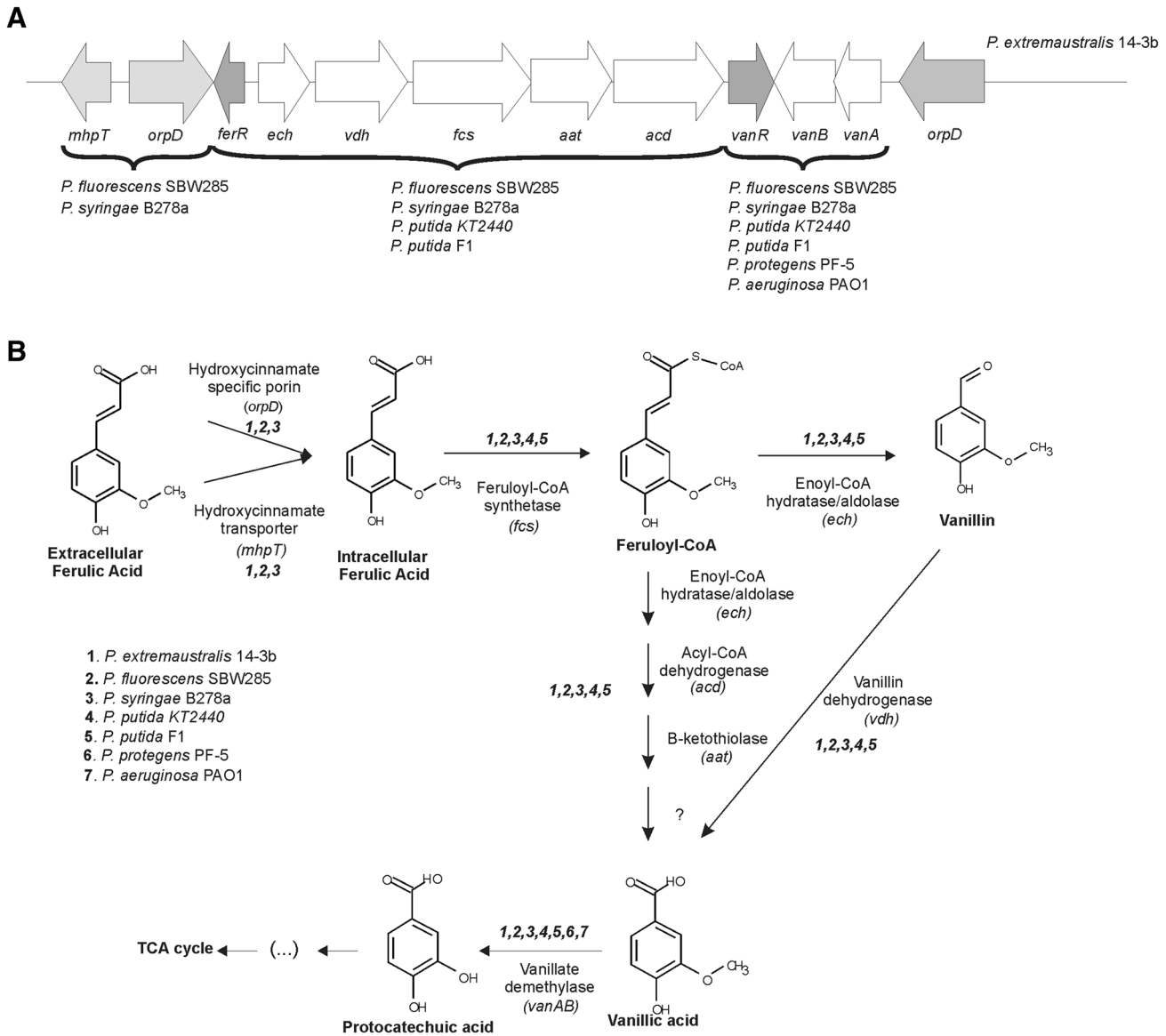

Fig. 8 Ferulic acid metabolism. **a** Genes involved in ferulic acid degradation and its presence in the analyzed *Pseudomonas*. **b** Ferulic acid degradation pathway

Two pathways involved in the conversion of ferulic acid into vanillic acid have been described in *Pseudomonas* (Overhage et al. 1999). One of them transforms feruloyl-CoA into vanillic acid by an acyl-CoA-dehydrogenase and a β-ketothiolase enzymes, and the second one has vanillin as intermediate driving to vanillic acid by the enzyme vanillin dehydrogenase (Fig. 8b). Both pathways were found in all the analyzed *Pseudomonas* species with the exception of *P. aeruginosa* PAO1 and *P. protegens* Pf-5 (Fig. 8b). The conversion of vanillate to protocatechuate was observed in the seven analyzed strains.

Besides the advantage that represents the usage of complex compounds to survive in poor nutrient environments, the ability to tolerate and degrade lignocellulosic material constitutes an important characteristic for biotechnological applications. Lignocellulosic material from agricultural residues represents a wide, inexpensive and renewable carbon source. Studies were conducted to analyze the tolerance of bacteria to several inhibitor compounds derived from the degradation of lignocellulosic residues to achieve polyhydroxyalkanoates production from this material (Dietrich et al. 2013). *P. extremaustralis* could be a good candidate for this purpose due to the presence of the machinery to use some of these compounds and the capability to synthesize polyhydroxyalkanoates. In addition, vanillin is an important flavoring agent. In a recent work, genetic manipulation of *P. putida* KT2440 aimed to improve the biotechnological production of this

compound from ferulic acid was performed (Graf and Altenbuchner 2014).

Concluding remarks

P. extremaustralis genome showed several particular features regarding environmental adaptability that could enable it to survive under unfavorable conditions. *P. extremaustralis* was able to grow under cold condition more efficiently than other *Pseudomonas* species. Functionality of arginine deiminase pathway, pyruvate fermentation and alginate production has been experimentally tested in this bacterium. In spite of some genes involved in the analyzed pathways were proven to belong to *Pseudomonas* core genome, several genes responsible for survival under unfavorable conditions, such as acetate kinase (pyruvic acid fermentation), genes encoding potassium transporter and colanic acid biosynthesis, which are relevant for osmotic resistance, seem to be acquired through horizontal transfer events. Gene duplication was also found in *P. extremaustralis* genome, as is the case of those encoding specific cinnamic acid porins, arginine/ornitine antiporter *arcD*, osmotic-related transport systems genes, genes involved in potassium ATPase system and glycine-betaine synthesis. These findings suggest that the acquisition of genes by horizontal transfer events and/or the increasing of fitness by gene redundancy could contribute to survival of *P. extremaustralis* in extreme environments.

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