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ORIGINAL ARTICLE

Degradation of phenylacetate by *Acinetobacter* spp.: evidence for the phenylacetyl-coenzyme A pathway

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Abstract The aerobic degradation of phenylacetate (PA) by many bacteria has recently been shown to proceed via an unprecedented catabolic route. A typical feature of this pathway is the transformation of PA to phenylacetyl-coenzyme A (PACoA). However, the aerobic degradation of PA by *Acinetobacter* spp. is not sufficiently understood. To gain insight into the catabolism of PA by *Acinetobacter* spp., we isolated several PA-degrading *Acinetobacter* spp. from a wastewater treatment plant in Germany using enrichment cultures with PA as a sole carbon source. We also conducted in vitro PA transformation assays based on the detection of PACoA. The identification of the isolated bacteria was based on partial 16S rDNA sequences. Phylogenetic analysis revealed that the isolated strains are members of the *Acinetobacter* group and could be regarded as strains of *Acinetobacter* spp. The soluble protein fraction obtained from cells cultured on PA-containing medium transformed PA to several intermediates, as detected by thin layer chromatography and autoradiography. The formation of one intermediate was CoA dependent and comigrated with a sample of PACoA, the earliest characteristic intermediate of the PA catabolic pathway, suggesting that the isolated PA-degrading *Acinetobacter* spp. utilize the recently elucidated PA catabolic pathway. A database search revealed that many *Acinetobacter* spp. harbor PA catabolic genes analogous to the *paa* gene cluster of *Escherichia coli* K-12.

Keywords *Acinetobacter* · Phenylacetate · Phenylacetyl-coenzyme A · Aerobic catabolism

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Introduction

The bacterial aerobic degradation pathway for phenylacetate (PA) remained an unsolved problem for more than 50 years (Kunita 1955). Under oxic conditions, the microbial degradation of aromatic compounds depends on oxygen as a co-substrate in the well-known β-ketoadipate pathway (Hayaishi 1994; Harwood and Parales 1996). In the initial step, oxygenases introduce hydroxyl groups into the aromatic nucleus, resulting in the formation of a few key intermediates, such as catechol, protocatechuate, and gentisate. These are substrates for subsequent oxygenolytic ring opening catalyzed by ring cleavage dioxygenases.

The fungal degradation of PA follows this strategy (Mingot et al. 1999). It was long believed that the bacterial aerobic degradation of PA proceeds via a monohydroxylated derivative of PA. Two different pathways were proposed with either homogentisate (2,5-dihydroxyphenylacetate) (Chapmann and Dagley 1961) or homoprotocatechuate

(3,4-dihydroxyphenylacetate) (Agrawal et al. 1996) as intermediates. However, none of the proposed aerobic catabolic pathways could be proven experimentally in PA-degrading bacteria.

It has recently been shown that the bacterial aerobic degradation of PA proceeds via an unorthodox catabolic strategy (Fig. 1) (Ismail et al. 2003; Teufel et al. 2010; Teufel et al. 2011; Ismail and Gescher 2012). Interestingly, the recently characterized pathway involves reactions from both the aerobic and anaerobic pathways. The pathway starts with the transformation of PA to phenylacetyl-coenzyme A (PACoA). Transformation of the aromatic acids to CoA thioesters is a typical step in the anaerobic degradation pathways of aromatic compounds (Fuchs et al. 2011). The ring of PACoA becomes activated to a ring-1,2-epoxide in a reaction catalyzed by a novel multicomponent oxygenase, the PACoA epoxidase PaaABC(D)E. The reactive non-aromatic epoxide is then isomerized to an unprecedented seven-membered *O*-heterocyclic enol-ether, an oxepin. The latter is the substrate of hydrolytic ring cleavage that produces a semialdehyde which in turn is oxidized to the corresponding carboxylic acid. Hydrolytic cleavage of the rings of aromatic substrates is another characteristic reaction of the anaerobic degradation pathways (Fuchs et al. 2011).

The enzymes of this pathway are encoded by the *paa* (*p*henyl*a*cetic *a*cid) gene cluster which has been studied in *Escherichia coli*, *Pseudomonas* spp., *Azoarcus evansii*, and *Rhodococcus* sp. (Fig. 1) (Blakley et al. 1967; Agrawal et al. 1996; Ferrández et al. 1998; Olivera et al. 1998; Mohamed et al. 2002; Bartolome-Martin et al. 2004; Navarro-Liorens et al. 2005; Ferrandez et al. 2006; Di Gennaro et al. 2007). The crystal structure of the novel PaaAC oxygenase subcomplex was recently reported (Grishin et al. 2010; Grishin et al. 2011). To date, this is the only proven pathway for aerobic bacterial PA degradation. A similar pathway for aerobic benzoate degradation which also involves CoA-bound intermediates was recently elucidated (Rather et al. 2010).

Although, there are several reports on the degradation of hydroxylated derivatives of PA by *Acinetobacter* spp. (Sucharitakul et al. 2005), the degradation of PA by these bacteria is not sufficiently understood (O'Connor et al. 2005). Here we report the isolation of some *Acinetobacter* strains capable of aerobic PA degradation. We also present biochemical and in silico evidence for the presence of the recently elucidated PA catabolic pathway in several *Acinetobacter* strains isolated from wastewater.

Materials and methods

Chemicals and reagents

Chemicals were obtained from Fluka (Neu-Ulm, Germany), Sigma-Aldrich (Deisenhofen, Germany), Merck (Darmstadt,

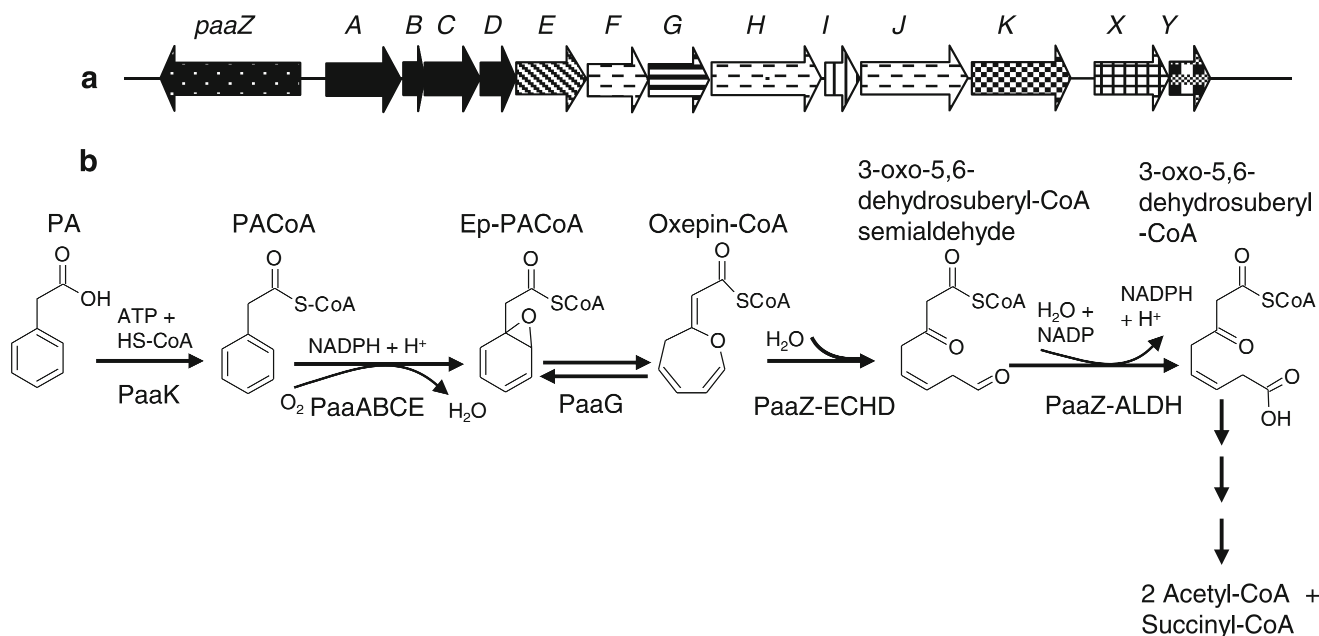

Fig. 1 **a** The phenylacetate (*PA*) catabolic gene cluster of *Escherichia coli* K-12. **b** The aerobic PA catabolic pathway. *HS-CoA* Coenzyme A, *PACoA* phenylacetyl-CoA, *Ep-CoA* ring-1,2-epoxy-PACoA, *oxepin-CoA* 2-oxepin-2 (3H)-ylideneacetyl-CoA, *PaaK* PA-CoA ligase, *PaaABCE* ring-1,2-PACoA epoxidase, *PaaG* ring-1,2-epoxy-PACoA isomerase, *PaaZ* oxepin-CoA hydrolase/3-oxo-5,6-dehydrosuberyl-CoA semialdehyde dehydrogenase, *PaaZ-ECH* enoyl-CoA hydratase domain of the ring-cleavage enzyme PaaZ, *PaaZ-ALDH* aldehyde dehydrogenase domain of the ring-cleavage enzyme PaaZ, *PaaFGHJ* enzymes catalyzing β-oxidation-like reactions, *PaaI*, *PaaY* thioesterases (Ferrández et al. 1998; Teufel et al. 2010)

Germany), or Roth (Karlsruhe, Germany). Biochemicals were obtained from Genaxxon Bioscience (Biberach, Germany), MBI Fermentas (St. Leon-Rot, Germany), Applichem (Darmstadt, Germany), Roth or Gerbu Biochemicals (Gaiberg, Germany). C1-[^{14}C]PA was obtained from Amersham (Braunschweig, Germany). Materials for PCR, DNA purification, cloning, and sequencing were purchased from Promega (Madison, WI), Takara (Otsu, Japan), MBI Fermentas, Biomers (Ulm, Germany), and QIAGEN (Hilden, Germany).

Enrichment and isolation of PA-degrading bacteria

Samples from a wastewater treatment plant (Freiburg, Germany) were collected to isolate bacteria capable of PA aerobic degradation. The enrichment was carried out in minimal medium containing 5 mM of PA as a sole carbon source (Mohamed et al. 2002). The various samples (5 mL) were inoculated into 200 mL of the relevant medium in Erlenmeyer flasks and incubated at 30 °C with shaking (200 rpm). After 1 week of incubation, samples were taken from enrichments showing bacterial growth, inoculated into the same medium, and incubated under the same conditions. This step was repeated three times. Isolation and purification of bacteria were carried out on minimal medium agar plates containing PA at 30 °C. Cultures were periodically examined by the microscope throughout the whole enrichment and isolation process.

Preparation of cell extract

Frozen or fresh cells were suspended in an equal volume of 10 mM Tris/HCl (pH 7.8) containing 0.1 mg DNase I mL^{-1}. Cells were ruptured by passing the cell suspension through a French pressure cell (American Instruments, Silver Spring, MD) at 137 MPa. The soluble protein fraction was obtained by centrifugation at 100,000×g for 1 h. Small cell amounts (<0.5 g wet weight) were extracted with a Mixer Mill (MM 200; Retsch, Haan, Germany). Thus, 0.6 mL of ice-cold cell suspension was mixed with 1.2 g of glass beads (0.1–0.25 mm) and subjected to shaking for 7 min at 30 Hz. The cell lysate was then centrifuged for 15 min at 20,000×g and 4 °C (centrifuge 5420; Eppendorf, Hamburg, Germany), and the supernatant was used immediately or stored at −20 °C. The protein content of the cell extracts was determined by the Bradford method using bovine serum albumin as a standard (Bradford 1976).

In vitro assays for the transformation of PA

Consumption of C1-[^{14}C]PA and the formation of metabolic intermediates thereof was followed by in vitro assays using the soluble protein fraction. Some of the isolated bacteria were grown with PA in minimal medium, and cells were then harvested and extracted as described above. Assays (500 μL) contained 1 mM CoA, 2 mM ATP, 10 mM $MgCl_2$, 1 mM NADH, 1 mM NADPH, 0.2 mM PA, and C1-[^{14}C]PA (200 kBq μmol^{-1}) in 100 mM Tris/HCl buffer (pH 7.8). An ATP-regenerating system consisting of phosphoenolpyruvate (2 mM), 8 nkat myokinase, and 8 nkat pyruvate kinase was included in all assays. The assays were started by adding 100 μL of the soluble protein fraction (approx. 3–4 mg protein). The assays were then incubated at 30 °C, and samples (80 μL) were retrieved at different time intervals. The reaction was stopped by the addition of 5 μL of 20 % (v/v) formic acid, and the samples were centrifuged (20,000×g, at 4 °C for 15 min). The supernatants were analyzed by thin-layer chromatography (TLC) followed by autoradiography. C1-[^{14}C]PACoA was synthesized in similar assays with C1-[^{14}C]PA as a precursor using the soluble protein fraction.

Thin-Layer chromatography

Labeled products originating from [^{14}C]PA or [^{14}C]PACoA in cell extracts were separated on silica gel TLC plates (0.2 mm, 20×20 cm silica gel; 60 F 254; Merck), using the following solvent system: *n*-butanol/acetic acid/water (12:3:5, v/v). Radioactive spots were localized by phospho-imaging (Fuji Photo Film, Tokyo, Japan).

Genomic DNA isolation and amplification of 16S rDNA

Genomic DNA was extracted from the various isolates according to published procedures (Sambrook et al. 1989). PCR assays were performed to amplify bacterial 16S rDNA using the 16S-1f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16S-1500r (5′-ACGGCTACCTTGTTACGACT-3′) universal primers (Arturo et al. 1995). The amplification reaction mixture contained 1 μL of 10× amplification buffer, 0.2 μL each of the forward and reverse primers (12.5 μM), 1 μL dNTPs, 1 μL template DNA (100 μg mL^{-1}), 4 μL *Taq* DNA polymerase (5 U μL^{-1}), and autoclaved MilliQ water up to a final volume of 10 μL. After denaturation at 94 °C for 5 min, samples were subjected to 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min, followed by an additional extension step of 10 min at 72 °C. The different amplicons were gel purified using the GenElute Minus EtBr Spin columns (Sigma-Aldrich).

Cloning and sequencing of 16S rDNA

Purified 16S rDNA PCR products were ligated into the pGEM-T Easy Vector System (Promega) and used to transform *E. coli* JM109 (Takara) according to the manufacturer's instructions. Recombinant plasmids were isolated from transformed cells using the standard miniprep method for plasmid DNA isolation (Promega). Sequencing of selected clones of

16S rDNA was performed by the automated fluorescent dye terminator sequencing method (Sanger et al. 1977) using the DYEynamic ET Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech, Arlington Heights, IL) with a model ABI 310 genetic sequence analyzer (Applied Biosystems, Foster City, CA). Dye terminator-based sequencing was performed on a PCR-amplified segment covering about 500 bp. Unidirectional sequencing of the 16S rDNA was performed using the standard vector primer (M13).

In silico analysis

The 16S rDNA sequences were analyzed by Genetyx-Win MFC application software ver, 4.0. The reference 16S rDNA gene sequences of the species were retrieved from the GenBank database (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD). The partial 16S rDNA gene sequences were compared with sequences in GenBank using nucleotide–nucleotide BLAST to obtain the nearest phylogenetic neighbors (www.ncbi.nlm.nih.gov/BLAST/). Multisequence alignments were performed by ClustalX, and phylogenetic trees were constructed with MEGA 3.1 (The Biodesign Institute, Tempe, AZ) (Kumar et al. 2004) using evolutionary distance and the neighbor-joining method (Saitou and Nei 1987). Sequences showing more than 97 % similarity were considered to belong to the same generic level (Altschul et al. 1997).

The search for the PA catabolic gene products in the finished genomes of *Acinetobacter* spp. was conducted using the blastp program against the non-redundant protein sequences database (http://blast.ncbi.nlm.nih.gov/Blast). The query sequences were retrieved from the complete genome sequence of *E. coli* K-12 MG1655 (accession number: NC_000913.2). The genome of *Acinetobacter baumannii* AYE (accession number: NC_010410.1) was searched for the complete PA catabolic gene cluster.

Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study were submitted to the GenBank database and assigned accession numbers (AB695279 to AB695285).

Results and discussion

Isolation and phylogeny of PA-degrading bacteria

By using enrichment cultures in minimal medium, we successfully isolated seven bacterial strains capable of the aerobic degradation of PA from a wastewater treatment plant. All isolated strains were able to grow on PA as a sole carbon source, indicating the presence of a pathway for PA catabolism in these bacteria.

The isolates were identified based on their 16S rDNA partial sequence (400 nucleotides) and closest matches of the isolates were searched for in the GenBank database. All seven PA-degrading strains were affiliated with the genus *Acinetobacter* (Table 1, Fig. 2). Three strains, designated PAD1, PAD2, and PAE1, formed one cluster with *Acinetobacter* sp. QN6, *A. baumannii* MMG5b, and *A. baumannii* BDHIBO5, respectively. The remaining four strains were distributed into different *Acinetobacter* clades. PAE2 was clustered with *A. baumannii* 10-6-1-9, while PAW1 was clustered with *A. calcoaceticus* AIMST 4. Strains PAC1 and PAC2 were classified as members of *A. seohaensis and Acinetobacter* sp., respectively. However, they were localized in different phylogenetic branches from the enclosed reference *Acinetobacter* strains. The isolated strains showed some degree of variation in terms of sequence similarity. Low sequence similarities were found in strain PAD2 which was regarded as a strain of *A. baumannii*.

PA catabolism by the isolated bacteria

Cell extracts of most of the isolated strains transformed C1-[^{14}C]PA into several labeled products (Fig. 3). Taking into account that PACoA is a typical intermediate of the PA catabolic pathway (Fig. 1), we looked for it among the labeled products that were detected. Only one polar radiolabeled product was detected by TLC analysis, and the formation of this product was dependent on the presence of CoA, Mg^{+2}, and ATP. This product co-migrated with a sample of PACoA and was therefore identified as PACoA. These observations confirm the transformation of PA to PACoA by cell extracts (soluble protein fraction) of the

Table 1 Identification of isolates based on partial 16S rDNA sequence

Strain	Growth substrate	Closest match	Identity %	Accession no.
PAC1	Phenylacetate	*Acinetobacter seohaensis* SA-A5-61	98	EU420936
PAC2	Phenylacetate	*Acinetobacter* sp. PD12	98	AY673994
PAD1	Phenylacetate	*Acinetobacter* sp. QN6	100	DQ640274
PAD2	Phenylacetate	*Acinetobacter baumannii* MMG 5b	97	JN162444
PAE1	Phenylacetate	*Acinetobacter baumannii* BDHIBO5	98	FJ550344
PAE2	Phenylacetate	*Acinetobacter baumannii* 10-6-1-9	98	JF919847
PAW1	Phenylacetate	*Acinetobacter calcoaceticus* AIMST 4	99	JF939018

Acinetobacter sp. QN6 (DQ640274)
PAD2 (AB695282)
PAE1 (AB695283)
Acinetobacter baumannii MMG 5b (JN162444)
Acinetobacter sp. TW (FJ753401)
PAD1 (AB695281)
Acinetobacter baylyi NBRAJG74 (EU734817)
PAE2 (AB695284)
Acinetobacter calcoaceticus AIMST (HM104473)
PAW1 (AB695285)
Acinetobacter baumannii IISR WP 35 (JF907698)
Acinetobacter johnsonii a153 (EU434432)
Acinetobacter beijerinckii (JN644620)
Acinetobacter lwoffii EQH10 (FJ999939)
Acinetobacter berezinae (HQ396906)
Acinetobacter radioresistens LEH7 (JN644591)
Acinetobacter sp. PD12 (AY673994)
Acinetobacter towneri (HQ180191)
Acinetobacter towneri DSM14962 (EF611416)
Acinetobacter sp. G16 (EF204279)
Acinetobacter seohaensis SA-A5-61 (EU420936)
PAC1 (AB695279)
PAC2 (AB695280)
85
54
80
76
50
86
99
65
54
86
92
99
81
0.005

Fig. 2 Neighbor-joining tree of 16S rDNA sequences showing the relationship of PA-degrading isolates with closest matches from reference GenBank sequences. Sequences determined in this study are given in *bold*. Reference sequences from GenBank include the accession numbers. Bootstrap values are shown near the nodes (based on 1000 replicate trees)

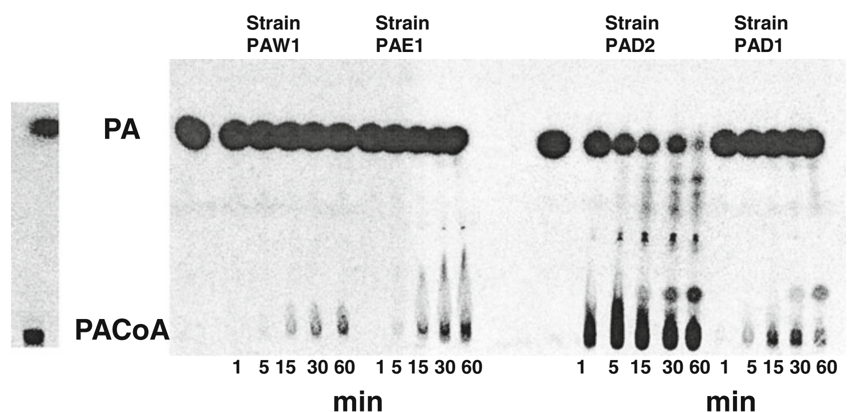

Fig. 3 In vitro transformation of C1-[^{14}C]PA by cell extracts from the isolated strains

Table 2 Occurrence of the key phenylacetate (*paa*) catabolic genes in *Acinetobacter* spp.

Query sequence (accession no.)	Function in the PACoA pathway	Number of closest matches in *Acinetobacter* spp.	Identity (%)	Examples of *Acinetobacter* homologues (accession no.)
PaaK (AAC74480)	PACoA ligase	12	66–69	*Acinetobacter lwoffii* SH145 (ZP_06068877), *Acinetobacter baumannii* AYE (YP_001714198)
PaaA (NP_415906)	Oxygenase subunit (epoxidation of the aromatic ring)	16	71–78	*Acinetobacter baumannii* ACICU (YP_001845995), *Acinetobacter calcoaceticus* PHEA-2 (ADY81268)
PaaB (NP_415907)	Oxygenase subunit (epoxidation of the aromatic ring)	6	72–74	*Acinetobacter calcoaceticus* RUH2202 (ZP_06056312), *Acinetobacter baumannii* ATCC 17978 (YP_001084367)
PaaC (NP_415908)	Oxygenase subunit (epoxidation of the aromatic ring)	13	52–55	*Acinetobacter baumanni*I ATCC 19606 (ZP_05827700), *Acinetobacter* sp. P8-3-8 (ZP_09141787)
PaaD (NP_415909)	Oxygenase subunit (epoxidation of the aromatic ring)	11	46–56	*Acinetobacter calcoaceticus* RUH2202 (ZP_06056314), *Acinetobacter baumannii* ATCC 17978 (YP_001084369)
PaaE (NP_415910)	Oxygenase reductase (electron transfer protein)	11	44–52	*Acinetobacter baumannii* ATCC 17978 (ABO11768), *Acinetobacter* sp. RUH2624 (ZP_05823859)
PaaZ (AAC74469)	Ring cleavage and aldehyde oxidation	15	58–59	*Acinetobacter baumannii* ATCC 19606 (ZP_05827703), *Acinetobacter radioresistens* SK82 (ZP_05359935)

PACoA, Phenylacetyl-coenzyme A

PA-grown bacteria and are in line with the detection of PACoA as the first common intermediate of aerobic bacterial PA catabolism (Fuchs et al. 2011). It is worth noting that the tested strains did not exhibit an identical pattern of labeled products derived from [^{14}C]PA transformation, possibly reflecting the different levels of enzyme activities in the various strains. Other strain-specific factors, such as regulation, gene duplication, and thioesterases, could also play a role. Interestingly, the cell extract from strain PAW1 transformed [^{14}C]PA into a single radio-labeled product, which was shown to be PACoA, as mentioned above. The inability to detect metabolic intermediates other than PACoA by incubating cell extracts of some strains with PA could be attributed to several reasons. First, one can envisage the dissociation of an enzyme complex acting on PACoA upon cell disruption. Secondly, this enzyme complex may be sensitive to high oxygen levels, particularly if it contains oxygen-labile components such as iron-sulfur clusters (Teufel et al. 2011). The inability to demonstrate the hydroxylation of PA by cell extracts of different bacteria in the presence of various cofactors has been reported by several research groups (Sparnins et al. 1974; Lee and Desmazeud 1986). The data suggest that PA degradation by the isolated strains follows the recently elucidated pathway (Teufel et al. 2010). However, further confirmation is still needed through the identification of other characteristic intermediates of the pathway.

The PA catabolic genes in *Acinetobacter* spp.

Phylogenetic analysis revealed the presence of key genes of the PA catabolic pathway in the genomes of many

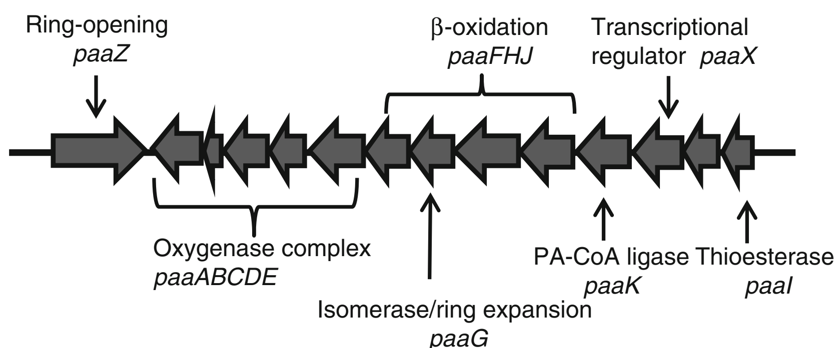

Fig. 4 The PA catabolic gene cluster of *Acinetobacter baumannii* AYE

Acinetobacter spp. (Table 2). For instance, we found the *paaK* gene encoding the PA-CoA ligase which catalyzes the first step in the PA catabolic pathway and is one of the most important characteristics of this pathway (Ferrández et al. 1998; Olivera et al. 1998; Mohamed 2000; Mohamed et al. 2002; Bartolome-Martin et al. 2004; Navarro-Liorens et al. 2005; Ferrandez et al. 2006; Di Gennaro et al. 2007; Nogales et al. 2007). Moreover, we found genes encoding the PACoA multicomponent oxygenase paaABC(D)E which catalyzes the second characteristic step of the pathway (epoxide formation). Genes coding for the bifunctional fusion protein PaaZ, which catalyzes the hydrolytic ring cleavage and subsequent aldehyde oxidation, were also found. The presence of these genes in the genome of a bacterium shows that the bacterium possesses the PACoA degradation pathway. To check for the presence of the other PA catabolic genes in the genomes of *Acinetobacter* spp., we searched the genome of *A. baumannii* AYE as a representative and found that it harbors the complete PA catabolic gene cluster (Fig. 4).

Conclusion

Our data demonstrate the presence of a functional PACoA degradation pathway in several *Acinetobacter* spp., thereby further confirming the significance and the wide occurrence of the PACoA degradation pathway.

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