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Phthaloyl-coenzyme A decarboxylase from *Thauera chlorobenzoica*: the prenylated flavin-, K⁺- and Fe²⁺-dependent key enzyme of anaerobic phthalate degradation

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Running title: UbiD-like phthaloyl-CoA decarboxylase

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Originality-Significance Statement

Phthalates represent major xenobiotic pollutants that are industrially produced at the annual million ton scale, and bioremediation is considered the main process of their elimination from the environment. We report on the first isolation and characterization of the key enzyme of oxygen-independent phthalate degradation, phthaloyl-CoA decarboxylase (PCD) from a denitrifying bacterium. The enzyme is an unusual member of the UbiD-family of (de)carboxylases and contains a prenylated FMN/Fe²⁺/K⁺-complex at the active site. The facile isolation established makes PCD a model for studying UbiD-like (de)carboxylases involved in the degradation of aromatic compounds including phenols, benzenes and polycyclic aromatic hydrocarbons.

Summary

The degradation of the industrially produced and environmentally relevant phthalate esters by microorganisms is initiated by the hydrolysis to alcohols and phthalate (1,2-dicarboxybenzene). In the absence of oxygen the further degradation of phthalate proceeds via activation to phthaloyl-CoA followed by decarboxylation to benzoyl-CoA. Here we report on the first purification and characterization of a phthaloyl-CoA decarboxylase (PCD) from the denitrifying *Thauera chlorobenzoica*. Hexameric PCD belongs to the UbiD-family of (de)carboxylases and contains prenylated FMN (prFMN), K⁺, and, unlike other UbiD-like enzymes, Fe²⁺ as cofactors. The latter is suggested to be involved in oxygen-independent electron-transfer during oxidative prFMN maturation. Either oxidation to the Fe³⁺-state in air or removal of K⁺ by desalting resulted in >95% loss of both, prFMN and decarboxylation activity suggesting the presence of an active site prFMN/Fe²⁺/K⁺-complex in PCD. The PCD-catalyzed reaction was essentially irreversible: neither carboxylation of benzoyl-CoA in the presence of 2 M bicarbonate, nor an isotope exchange of phthaloyl-CoA with ¹³C-bicarbonate was observed. PCD differs in many aspects from prFMN-containing UbiD-like decarboxylases and serves as a biochemically accessible model for the large number of UbiD-like (de)carboxylases that play key roles in the anaerobic degradation of environmentally relevant aromatic pollutants.

Introduction

Phthalic acid esters (PAE) are annually produced at the million ton scale, and are mainly used as plasticizers incorporated into high-molecular weight polymers (Mersiowsky et al., 2001; Cousins et al., 2003; Net et al., 2015). PAE are classified as xenobiotics that easily migrate into the environment during the production, transport and disposal of plastics. They negatively impact human health and the environment, particularly due to their endocrine disrupting activity (Caldwell, 2012; Gao and Wen, 2016).

Biodegradation by microorganisms is considered the main way of PAE elimination from the environment (Cousins et al., 2003; Huang et al., 2013; Gao and Wen, 2016). The initial steps of PAE degradation are identical under oxic and anoxic conditions and comprise the hydrolysis of PAE to phthalate and alcohols (Liang et al., 2008; Gao and Wen, 2016). Aerobic microorganisms employ ring-hydroxylating dioxygenases for the degradation of phthalate to protocatechuate, a key intermediate of aerobic aromatic degradation. The degradation of the latter is well-studied and depends on ring-cleaving dioxygenases (Nomura et al., 1992; Vaillancourt et al., 2006).

In the absence of oxygen, microbial phthalate degradation has to follow completely different principles and is considered a rate limiting step of PAE degradation (Gao and Wen, 2016). Only very recently, the previously obscure anaerobic phthalate degradation has been elucidated in studies with a number of denitrifying β -proteobacteria that use phthalate and nitrate as their only source of energy and cell carbon (Junghare et al., 2016; Ebenau-Jehle et al., 2017). In extracts of *Thauera*, *Azoarcus* and 'Aromatoleum' strains, phthalate was first activated to phthaloyl-CoA by a succinyl-CoA-dependent CoA transferase, followed by decarboxylation to benzoyl-CoA (Figure 1) (Junghare et al., 2016; Ebenau-Jehle et al., 2017). In these denitrifying organisms, benzoyl-CoA is then dearomatized by ATP-dependent class I benzoyl-CoA reductases to a cyclic dienoyl-CoA (Kung et al., 2010; Boll et al., 2014; Buckel et al., 2014). The latter is subsequently channeled into the well-established benzoyl-CoA degradation pathway (Rabus et al., 2005; Fuchs et al., 2011; Schmid et al., 2015). Differential proteome analyses in *T. chlorobenzoica*, *A. aromaticum*, and *Azoarcus* sp. PA01 revealed phthalate-

induced gene clusters. They contained genes putatively encoding proteins for uptake and activation of phthalate together with genes that were proposed to code for a phthaloyl-CoA decarboxylase (PCD, *ubiD*-like) and an enzyme involved in maturation of the putative flavin cofactor of PCD (*ubiX*-like) (Fig. 1) (Junghare et al., 2016; Ebenau-Jehle et al., 2017).

The UbiD enzyme family derives from 3-octaprenyl-4-hydroxybenzoate carboxy-lyase involved in ubiquinone biosynthesis (Cox et al., 1969). The structure and mechanism of a functional UbiD-like decarboxylase was recently elucidated using Fdc1 (Payne et al., 2015). This enzyme catalyzed the decarboxylation of ferulate or cinnamate and contained a prenylated FMN cofactor (prFMN) in the active site. At this cofactor reversible decarboxylation was achieved by covalent catalysis via an unprecedented 1,3-dipolar cycloaddition (Payne et al., 2015) (see also Supporting Information Fig. S5). A second component, UbiX, acts as a prenyltransferase that modifies its bound FMN (Lin et al., 2015) using dimethylallyl-monophosphate as co-substrate (White et al., 2015). The tetracyclic prFMN formed is then transferred from UbiX to the cofactor-free UbiD component, followed by an dioxygen-dependent oxidative activation step to the mature prFMN (White et al., 2015). A recent study suggested variations for the activation of prFMN-containing enzymes (Marshall et al., 2017).

UbiD-like proteins have been shown to play essential roles in the anaerobic degradation of benzene, naphthalene, phenol, terephthalate and many other aromatic compounds (Schühle and Fuchs, 2004; Holmes et al., 2011; Luo et al., 2014; Nobu et al., 2015; Meckenstock et al., 2016; Rabus et al., 2016). The proposed phthaloyl-CoA decarboxylating enzyme of denitrifying bacteria differs from other studied UbiD-like enzymes as it decarboxylates a thioester substrate; unlike the studied Fdc1 or hydroxybenzoate decarboxylases it catalyzes C–C cleavage of a non-phenolic arylcarboxylate which is much more difficult to achieve. In this work we report on the purification of PCD from extracts of *T. chlorobenzoica* grown with phthalate and nitrate. The cofactors and the encoding gene were identified, and the molecular, kinetic and spectroscopic properties were determined. Based on the results obtained, we propose a model for oxygen-independent maturation and provide a rational for oxygen-sensitivity of PCD.

Results

***In vitro* activity, purification and molecular properties**

PCD activity was determined in extracts from *T. chlorobenzoica* grown with phthalate and nitrate. In anaerobically conducted *in vitro* assays substrate consumption and product formation was followed by UPLC (ultra-performance liquid chromatography) analysis coupled to diode array detection and/or quadrupole time-of-flight mass spectrometry (Q-TOF) analysis after electrospray ionization (ESI) (for MS spectra see Supporting Information Fig. S1AB). In initial PCD-activity monitoring experiments, cell extracts, phthalate (0.5 mM), succinyl-CoA (0.5 mM) and the intrinsic phthalate succinyl-CoA:phthalate CoA transferase (SCPCT) present in cell extracts were used for phthaloyl-CoA formation. Using this setup, maximal PCD activity was 21 nmol min⁻¹ mg⁻¹.

Purification of PCD was carried out under anaerobic conditions starting from the 100,000×*g* supernatant of extracts from cells grown with phthalate and nitrate. During initial enrichment experiments using DEAE anion-exchange chromatography, PCD (elution at 200 mM KCl) and SCPCT (elution at 400 mM KCl) activity were separated, yielding fractions containing only either of the activities. For this reason either chemically synthesized phthaloyl-CoA or the SCPCT-enrichment together with its substrates phthalate and succinyl-CoA were added to activity assays. Surprisingly, PCD activity was reproducibly 4-fold higher in the presence of saturating SCPCT (0.5–1 U mL⁻¹, approximately 20-fold excess compared to PCD activity), phthalate and succinyl-CoA (0.5 mM each) compared to chemically synthesized phthaloyl-CoA (0.5 mM). Notably, the presence of SCPCT in assays that were started with phthaloyl-CoA had no stimulating effect suggesting a complex mode of interaction between PCD and SCPCT. For easier handling, the chemically synthesized phthaloyl-CoA was routinely used in further studies, if not otherwise stated.

PCD was purified by DEAE anion-exchange chromatography followed by size exclusion chromatography. In the first step, the highest specific activity was obtained with the fraction eluting at 200 mM KCl (104 nmol min⁻¹ mg⁻¹), while it was significantly lower in the fractions eluting at 100

mM KCl ($15 \text{ nmol min}^{-1} \text{ mg}^{-1}$) and 1 M KCl ($<0.5 \text{ nmol min}^{-1} \text{ mg}^{-1}$). Size exclusion chromatography revealed three major peaks in the elution profile (Fig. 2B). Only the peak eluting at 10.7 min contained PCD activity ($122 \text{ nmol min}^{-1} \text{ mg}^{-1}$). SDS-polyacrylamide gel analysis of the fractions obtained during PCD purification revealed the enrichment of a single protein band ($>95\%$ purity) migrating around 60 kDa (Fig. 2A). The protein band was excised from the gel, tryptically digested, and analyzed by UPLC/ESI-Q-TOF. The only proteins identified were Tchl_RS15505/Tchl_RS15550 (70% coverage) that had previously been identified as phthalate-induced UbiD-like proteins (Ebenau-Jehle et al., 2017). Both proteins share 100% identical amino acid sequences and are not distinguishable; they most likely result from a recent gene duplication. The deduced molecular mass of the Tchl_RS15505/Tchl_RS15550 gene products is 58.9 kDa, fitting to the experimentally determined mass of the enriched protein band. From 700 mg soluble protein 32 mg PCD were purified with a recovery of 27% suggesting that PCD represents approximately 17% of the soluble protein from *T. chlorobenzoica* grown with phthalate (Table 1).

The apparent molecular mass of PCD was determined by gel filtration (Fig. 2B), and triplicate analyses gave a value of 359 ± 8 kDa (mean value $\pm$ standard deviation of triplicate analyses). This result suggests an α_6 - composition and is in line with hexameric UbiD from *E. coli* (Marshall et al., 2017) but differs from the previously reported dimeric compositions of UbiD-like Fdc1 from yeast or *Aspergillus niger* (Bhuiya et al., 2015; Payne et al., 2015).

Determination of flavin and metal cofactors

Purified PCD exhibited a faint yellow color; for the UV/vis spectrum see Fig. 2C. For cofactor extraction, 1.2 to 2.8 mg of purified PCD were denatured by addition of equal volumes of acetonitrile, centrifuged, and the supernatant was analyzed by UPLC coupled to ESI-Q-TOF or diode array detection. A compound with a molecular mass of 525.1751 ± 0.0008 Da was identified fitting to the mass reported for the catalytically active azomethide ylide form of prFMN extracted from Fdc1 (Supporting Information Fig. S2A) (Payne et al., 2015). Fragmentation of this ion by collision induced dissociation resulted in the formation of daughter ions which could be assigned to characteristic

prFMN fragments (Supporting Information Fig. S2BC). The UV/visible spectrum of isolated prFMN markedly differed from the enzyme-bound form with absorbance peaks at 218, 284 and 353 nm at an intensity ratio of 6.12:3.36:1 (Fig. 2D). Quantification of prFMN was carried out by ESI-Q-TOF analysis. Using FMN as standard 0.9±0.1 prFMN per PCD monomer were determined (after careful content determination of the commercially available FMN standard by UPLC ESI/Q-TOF analyses). The amount of prFMN was also correlated to PCD activity during purification. As indicated in Table 1, the ratio of prFMN content/PCD activity remained constant during the entire purification procedure indicating a parallel enrichment of the active site prFMN cofactor and PCD activity.

UbiD-like enzymes have been reported to contain divalent cations (Jacewicz et al., 2013; Payne et al., 2015; Marshall et al., 2017). ICP-MS analysis of PCD revealed 1.8±0.2 (mean of three replicates±standard deviations) mol Fe per mol enzyme, while for Mn, Co, Ni, Cu, Zn, Mo, Mg and Ca the values obtained were either below the detection limit and/or not significantly different from the buffer blanks. The spectrophotometrically determined Fe-content was 1.7±0.2 mol per mol enzyme and in line with ICP-MS analysis.

Electron paramagnetic resonance (EPR) spectroscopy

The prFMN cofactor of Fdc1 directly binds via the phosphate group to Mn²⁺ in an octahedral coordination sphere (Payne et al., 2015). The failure to detect Mn in PCD suggests that Fe adopts the role of Mn²⁺ in the Fe²⁺-state. To test this hypothesis, PCD was subjected to EPR spectroscopic analyses. The enzyme as isolated exhibited a minor radical spectrum at 77 K (<0.05 spins per enzyme) (Fig. 3A). Only a very minor signal at g=4.3 that may be assigned to a Fe³⁺-species was observed (Fig. 3B). Oxidation of the enzyme either in air or by ferricyanide (2 mM) resulted in a twofold increase of the radical species (not shown). This finding suggests that around 8–10% of prFMN PCD was present in the inactive, reduced state that could be oxidized to the inactive radical form. Most importantly, a strong EPR-signal at g=4.3 appeared upon oxidation fitting perfectly to a S=5/2 high-spin system of a tetrahedrally coordinated, enzyme-bound Fe³⁺-species (Meyer et al., 1995) (Fig. 3B). Notably, no such signal was observed in a stock solution with hexacoordinated ferricyanide (2 mM). Reduction of PCD

as isolated by cyanoborohydride gave no change in the EPR spectrum (not shown). However, oxidation of cyanoborohydride-reduced enzyme in air resulted in a ≈ 25 -fold increase of the radical species as reported for Fdc1 (Fig. 3A). This oxidation was accompanied with a marked purple color accompanied with a characteristic UV/visible spectrum (Fig. 3C), highly similar to that reported for prFMN of Fdc1 treated in the same manner (Payne et al., 2015).

Oxidative and reductive inactivation

In agreement with previous cell extract activity determinations, purified PCD was oxygen-sensitive with a half-life in air of 13 ± 3 min (Supporting Information Fig. S3) (Ebenau-Jehle et al., 2017). No change in hexameric subunit composition was observed upon oxygen-exposure. Notably, the oxygen-stable Fdc1 and UbiD contain under cellular conditions redox-insensitive Mn^{2+} or Mg^{2+} cofactors that are involved in prFMN binding via ligation of its phosphate group (Payne et al., 2015, Marshall et al. 2017). As the prFMN cofactor of PCD in its active imine form is not prone to further oxidation, we tested the possibility whether the oxygen-sensitivity of PCD can be rationalized by a loss of prFMN as a result of the oxidation to the Fe^{3+} -state. After 2 h incubation in air and a desalting step in KCl-containing buffer, PCD had lost more than 95% of prFMN compared to an anaerobically incubated control; it was accompanied with a comparable loss of decarboxylation activity (Table 2). Notably, MS-analysis of the prFMN released from air-treated PCD revealed no significant differences in total ion chromatograms as from the prFMN released from the isolated enzyme, indicating no direct effect of dioxygen on prFMN. An almost identical oxidative inactivation and loss of prFMN was observed with ferricyanide (2 mM), indicating that inactivation cannot be assigned to a specific effect of dioxygen. The inactivation by oxygen was only partially reversible: re-reduction of air-oxidized enzyme with reduced methylene blue (1 mM, without removal of released prFMN by desalting) recovered 28% activity. With 20 mM sodium-EDTA, a Fe-complexing reagent, a decrease in activity was observed albeit only to 50%. As EDTA is known to complex Fe^{3+} with much higher affinity than Fe^{2+} , PCD was first oxidized in air, followed by EDTA treatment and a desalting step. After this procedure PCD completely lost activity ($<0.2\%$ compared to an anaerobic control without EDTA

treatment). When anaerobically incubated enzyme was desalted in KCl-free buffer a similar loss of activity and prFMN was observed as during incubation in air (Table 2). This finding is in line with previous work, where a protecting effect of K^+ towards oxygen-damage was reported (Ebenau-Jehle et al., 2017).

Similar to Fdc1, PCD was sensitive to cyanoborohydride due to the reduction of the essential imine form of prFMN (Payne et al., 2015). Re-oxidation in air resulted in the formation of the inactive radical form as evidenced by the typical purple color of PCD and the EPR- and UV/vis spectroscopic properties of the radical species (Fig. 3AC).

Kinetic properties

PCD activity measured with chemically synthesized phthaloyl-CoA followed a Michaelis-Menten curve (Supporting Information Fig. S4) with apparent values for $K_m = 121 \pm 27 \mu\text{M}$ and $V_{\max} = 35 \pm 5 \text{ nmol min}^{-1} \text{ mg}^{-1}$; extrapolation with the 4-fold higher values in the presence of SCPCT gives $V_{\max} = 140 \text{ nmol min}^{-1} \text{ mg}^{-1}$). Next to phthaloyl-CoA, 3-F-phthaloyl-CoA was decarboxylated to 3-F-benzoyl-CoA with 16% of phthaloyl-CoA activity (for MS spectra see Supporting Information Fig. S1CD). In agreement with earlier cell extract assays, 2-cyanobenzoyl-CoA, 2-nitrobenzoyl-CoA, 2-methylphthaloyl-CoA, and maleoyl-CoA were virtually not converted by purified PCD (<2% of the rate obtained with phthaloyl-CoA) (Ebenau-Jehle et al., 2017).

The reverse reaction of PCD, the carboxylation of benzoyl-CoA to phthaloyl-CoA was tested. Even in the presence of 2 M bicarbonate and 1 mM benzoyl-CoA and up to 8 h incubation time, virtually no phthaloyl-CoA formation was observed in UPLC/MS analyses (detection limit $\approx 0.05 \mu\text{M}$). UbiD-like enzymes typically catalyze an isotope exchange of $^{13/14}\text{C}$ -labelled CO_2 and their carboxylated substrates/products (Schühle and Fuchs, 2004; Mouttaki et al., 2012). In the presence of 0.5 mM ^{13}C - HCO_3^- and 0.2 to 1 mM non-labeled phthaloyl-CoA no formation of ($2\text{-}^{13}\text{C}$)-phthaloyl-CoA was observed. In summary, the inability of detecting neither the reverse reaction nor the isotope exchange suggests that PCD reaction is essentially irreversible.

Discussion

In this work PCD, the key enzyme of anaerobic phthalate degradation, was purified and characterized in the active form from the wildtype of a denitrifying model organism. It represents a novel member of the growing class of carboxylases/decarboxylases belonging to the UbiD-enzyme family. It differs from the recently characterized prFMN-containing enzymes Fdc1 and UbiD by (i) the oxygen sensitivity, (ii) the nature of the metal cofactor, (iii) the nature of the CoA-ester substrate and the reaction catalyzed, (iv) the inability to catalyze the reverse reaction or an $^{13}\text{CO}_2$ -isotope exchange, and, most probably, (v) the mode of oxidative prFMN maturation. The properties of PCD are summarized in Table 3.

The catalytic number (0.14 s^{-1}) of PCD is unusually low, possibly reflecting the difficulty of the reactions catalyzed (see below). However, *T. chlorobenzoica* compensates the intrinsically low catalytic activity of PCD by a high cellular expression of the encoding gene making PCD the most abundant cellular protein (17%, see Fig. 2A). With $21 \text{ nmol min}^{-1} \text{ mg}^{-1}$, the resulting specific activity in cell extracts is in the range of dearomatizing benzoyl-CoA reductase ($20\text{-}60 \text{ nmol min}^{-1} \text{ mg}^{-1}$) (Boll and Fuchs, 1995; Kuntze et al., 2011; Tiedt et al., 2016), the next enzyme in phthalate catabolism which is usually considered a bottleneck in the anaerobic catabolism of aromatic compounds. Taken into account the three-fold higher doubling time during growth with phthalate vs. benzoate (Ebenau-Jehle et al., 2017), the activities determined in this work are in agreement with the observed growth parameters. Unexpectedly, the activity of PCD was higher in the presence of phthaloyl-CoA forming CoA transferase plus their substrates than with chemically synthesized phthaloyl-CoA. This phenomenon may be explained by an activity stimulating interaction of PCD and the CoA transferase, though no co-purification of both enzymes was observed. Notably, succinyl-CoA and phthalate in the absence of SCPCT had no effect on PCD activity (not shown).

The flavin cofactor of PCD was identified as prFMN and corroborates the concept that all UbiD-like enzymes contain identical, prenylated FMN cofactors (Leys and Scrutton, 2016). This work demonstrated the first isolation of the prFMN cofactor in its active form from a wild type holo-enzyme. The characteristic UV/vis spectra and the high resolution mass spectrum leave little doubt that prFMN from PCD was identified in the catalytically relevant azomethide ylide form. In previous studies this prFMN form was detected in a UbiD-like enzyme either after co-expression with a UbiX-like component or by *in vitro* reconstitution with UbiX, FMN and dimethylallyl-monophosphate (Payne et al., 2015; Marshall et al., 2017).

Phthalate-induced *ubiX*-like genes are located next to both homologous genes encoding PCD (*Tchl_RS15555* and *Tchl_RS15510*), and there remains little doubt that the products act as dimethylallyl-monophosphate dependent prenyltransferases as reported earlier for the UbiX-like Pad1 from *Pseudomonas aeruginosa* (White et al., 2015). Though a loose complex between UbiX-like prenyltransferase and the UbiD-like decarboxylase may occur during prFMN cofactor transfer from UbiX to apo-UbiD, the data obtained in this work rather rule out a permanent complex between PCD and their appropriate UbiX component; they are in full agreement with results from other UbiD/UbiX two-components systems (Payne et al., 2015; Marshall et al., 2017).

PCD is so far the only UbiD-like enzyme reported that contains Fe^{2+} as redox-sensitive metal instead of the usually found $\text{Mn}^{2+}/\text{Mg}^{2+}$ that are redox-inactive under standard cellular conditions. Based on the structures of Fdc1 and UbiD (Payne et al., 2015; Marshall et al., 2017) Fe^{2+} and K^+ are predicted to directly ligate the phosphate functionality of prFMN in PCD. The observed loss of prFMN upon Fe^{2+} -oxidation or upon K^+ -removal by desalting agrees with such a scenario. Amino acid comparisons indicate that the three amino acids involved in divalent metal binding are conserved in PCD from *T. chlorobenzoica* and from other organisms (N170, H189 and E235, *T. chlorobenzoica* numbering); moreover, R171, Q183, E279, and E284 are conserved amino acids binding prFMN (Fig. 4).

The question rises why PCD uses a redox-sensitive divalent metal for prFMN ligation thereby risking oxygen-damage of an enzyme that makes almost 20% of the cellular protein. Notably, *T. chlorobenzoica* is a facultative anaerobe that faces rapid changes of steep oxygen-gradients. The Fe^{2+} -cofactor could play a role in the oxidative maturation of prFMN to the catalytically active imine form in UbiD-like enzymes, which in aerobic organisms is accomplished with oxygen as electron acceptor (Payne et al., 2015; Marshall et al., 2017). In the absence of oxygen, the Fe-cofactor in its 3^{+} -state could serve as primary electron acceptor for prFMN oxidation during its insertion into apo-UbiD, thereby generating the Fe^{2+} -state ready to ligate the matured prFMN. As Fe-determinations reproducibly pointed to rather two than one Fe per PCD, two-electron oxidation of prFMN could result in the reduction of two Fe^{3+} sites; such a scenario would be independent of an external electron acceptor such as dioxygen. A second $\text{Fe}^{2+/3+}$ could be ligated by four cysteines in the C-terminal domain of PCD in a tetrahedral, rubredoxin-like manner (Herriott et al., 1970). These cysteines are strictly conserved in all predicted PCDs but are missing in other characterized UbiD-like enzymes from aerobes (Fig. 4). Therefore, it is tempting to speculate whether all PCD-like enzymes contain Fe^{2+} rather than $\text{Mn}^{2+}/\text{Mg}^{2+}$ as a result of Fe^{3+} reduction during prFMN maturation. The only isolated and characterized UbiD-like enzyme from an anaerobic organism other than PCD is phenylphosphate carboxylase from *Thauera aromatica* (Lack and Fuchs, 1992). In this enzyme the conserved cysteines present in PCDs are missing suggesting that variations with regard to metal content/binding may exist in UbiD-like enzymes from anaerobes. With phenylphosphate carboxylase, a stimulating effect of Mn^{2+} and K^{+} was reported, though no metal was found in the purified enzyme (Schühle and Fuchs, 2004). These findings indicate that the metal(s) of this enzyme are easily lost during sample preparation. However, once the prFMN is matured to the oxidized form in phenylphosphate carboxylase, the prFMN/ $\text{Me}^{2+}/\text{K}^{+}$ -complex may be reconstituted with a redox-inactive divalent cation such as Mn^{2+} together with K^{+} .

Among the studied UbiD-like decarboxylases acting on cinnamate, (*E*)-ferulate, or 3-octaprenyl-4-hydroxybenzoate (Payne et al., 2015; Marshall et al., 2017), and carboxylases acting on phenylphosphate, catechylphosphate or naphthalene (Schühle and Fuchs, 2004; Ding et al., 2008;

Mouttaki et al., 2012), PCD is unique with regard to its CoA ester substrate. Moreover, it represents the so far only biochemically accessible enzyme of the UbiD-family that cleaves a C–C-bond of a non-phenolic arylcarboxylate. The function of the CoA ester moiety for stabilizing a proposed negatively charged anionic transition state after C–C bond cleavage is unclear. The 1,3-dipolar cycloaddition to the olefinic cinnamate double bond is highly plausible and supported by experimental evidence (Payne et al., 2015). However, such a reaction is much more difficult to achieve with a non-activated aromatic system as it would involve thermodynamically unfavorable dearomatization steps (Supporting Information Fig. S5). The difference between PCD and other UbiD-like enzymes is also reflected by its virtual inability to catalyze both, reverse benzoyl-CoA carboxylation and isotope exchange between phthaloyl-CoA and $^{13}\text{CO}_2$. Taken these observations together, the mechanism of PCD is expected to differ from the established prFMN-dependent decarboxylation of cinnamate, ferulate or related compounds, and suggests that a functional diversity of prFMN-dependent catalysis exists.

PCD represents a model for (de)carboxylases involved in the degradation of many environmentally problematic aromatic compounds in anaerobic bacteria including phenols and non-substituted aromatic hydrocarbons such as benzene or naphthalene. Further studies of PCD will help to gain detailed insights into the function of (de)carboxylases acting on aromatic substrates from anaerobic bacteria that, due to the slow growth and the limited biomass available, are much more difficult to study.

Experimental procedures

Cultivation of bacteria and preparation of cell extracts

Thauera chlorobenzoica strain 3CB-1 (DSM-18012), was grown anaerobically at 30 °C in a 200 L bioreactor (Bioengineering AG, Wald, Switzerland) which was operated in a continuous fed-batch mode using a 0.5 M phthalate and 1.8 M NaNO₃ stock solution. Cells were harvested in the exponential growth phase at an OD₅₇₈ of 3.5. Harvested cells were kept frozen in liquid nitrogen until use. Cell extracts were prepared under anaerobic conditions as described (Ebenau-Jehle et al., 2017).

Synthesis of CoA thioesters

Phthaloyl-CoA and maleoyl-CoA were synthesized from the corresponding anhydrides and coenzyme A, 3-fluorophthaloyl-CoA was synthesized starting from 3-fluorophthalate followed by anhydride formation using ethylchloroformate (Junghare et al., 2016). Benzoyl-CoA was synthesized from benzoic anhydride and coenzyme A by a different method reported earlier (Schachter and Taggert, 1953). 2-cyanobenzoyl-CoA, 2-nitrobenzoyl-CoA, and phthaloyl-CoA methyl ester were synthesized starting from esterification of the acids with N-hydroxysuccinimide (Blecher et al., 1981). Synthesis of the CoA esters from succinimidylesters was identical to the synthesis of benzoyl-CoA from benzoic anhydride.

LC/MS and LC/diode array analyses

Metabolites were analyzed by LC/MS using a Waters Acquity I-class UPLC with a Waters C18 HSS T3 column (2.1 mm x 100 mm, 1.8 µm particle size) either coupled to a Waters Synapt G2-Si HDMS ESI/Q-TOF system or a Waters Acquity photo diode array detector. For analysis of CoA thioesters, an 8 min linear gradient of 2 to 30 % acetonitrile in 10 mM ammonium acetate pH 6.8 at a flow rate of 0.35 mL min⁻¹ was applied, for analysis of prFMN/FMN a 20 min linear gradient of 5 to 25 % acetonitrile/0.1 % formic acid (v/v) in water/0.1 % formic acid (v/v) was used at a flow rate of 0.35 mL min⁻¹. Both, CoA esters and prFMN/FMN were measured in MS positive mode with a capillary voltage of 3 kV, 150 °C source temperature, 450°C desolvation temperature, 1000 L min⁻¹ N₂

desolvation gas flow and 100 L min⁻¹ N₂ cone gas flow. Collision induced dissociation of precursor ions was performed using a collision energy ramp from 10 to 50 V. LC-UV/visible analyses were conducted on a Waters Acquity H-class UPLC with a Knauer Eurospher 100-2 C18 column (2 mm x 100 mm, 2 µm particle size). CoA esters were separated by the same gradient and same flow rate as described above with 10 mM potassium phosphate buffer pH 7.0. Evaluation of LC/MS metabolite data was performed using MassLynx (Waters); for evaluation of LC-UV/visible data MassLynx or Empower (Waters) was used. For protein identification, 5 µg of purified PCD was loaded onto an SDS gel. The band migrating at around 59 kDa was excised, cysteine residues were reduced using dithiothreitol and alkylated by treatment with iodoacetamide. After in-gel digestion with trypsin (Sigma-Aldrich), the resulting peptides were separated on a Waters Acquity I-class UPLC with a Waters Peptide CSH C18 column (2.1 mm x 150 mm, 1.7 µm particle size) with a gradient from 1 to 40 % ACN/0.1 % formic acid (v/v) in water/0.1 % formic acid (v/v) at a flow rate of 0.04 mL⁻¹ min⁻¹. The source conditions used were the same as described above except that the source temperature was lowered to 80 °C, the desolvation temperature to 400 °C and the desolvation gas flow to 800 L h⁻¹. The instrument was operated in positive HD-MS^E mode. The resulting spectra were analyzed with ProteinLynx Global Server (Waters) by matching with the UniProt database of *Thauera chlorobenzoica* (minimal fragment ion matches per peptide = 3, minimal fragment ion matches per protein = 3, minimal peptide matches per protein = 7, false discovery rate 3%).

Enzyme assays

Enzyme assays were anaerobically performed in a glove box (95% N₂, 5% H₂) at 30°C in 100 mM Tris/HCl pH 7.5 with 100 mM KCl added. Enzymatic reactions were started by the addition of CoA ester substrate. Proteins were precipitated in 0.8 M HCl/8 % acetonitrile (v/v). Product formation was quantified based on calibration curves of standards.

Inhibition assays

Inhibition assays were performed anaerobically or aerobically (depending on the experiment) in the standard buffer at pH 7.5; constant pH values were always controlled. If necessary, the enzyme was desalted using a PD MiniTrap G-25 desalting column (GE Healthcare) after equilibration with 100 mM Tris/HCl pH 7.5 with or without 100 mM KCl. Inhibition assays were carried out

Purification of PCD and determination of native mass

All steps were carried out under anoxic conditions (95% N₂, 5% H₂). Around 7 g of phthalate grown cells (wet mass) were suspended in 30 mL 50 mM MOPS/KOH 10 mM KCl pH 7.5 (buffer A) and lysed using a French pressure cell. After ultracentrifugation, 25 mL of the supernatant was applied to a 50 mL DEAE-Sepharose column (GE Healthcare) at a flow rate of 3 mL⁻¹ min⁻¹. Fractions were eluted with 100 mM, 200 mM and 1 M KCl in buffer A. Size exclusion chromatography was conducted on a 24 mL Superdex 200 Increase gel filtration column (GE Healthcare) in buffer A containing 100 mM KCl at a flow rate of 0.5 mL min⁻¹. The column was calibrated under the same conditions with standards of known molecular mass (thyroglobulin, ferritin, alcohol dehydrogenase, carboanhydrase, cytochrome C).

Determination of kinetic properties

K_m and V_{max} of purified PCD were determined by adding 0.025 to 1 mM phthaloyl-CoA to the enzyme assay. The enzymatic activities were fitted to a Michaelis-Menten curve (GraphPad Prism 6). Oxygen inactivation was tested by pre-incubating the reaction mixture in the presence or absence of oxygen followed by addition of the CoA ester substrate; the assay was then performed in the absence of oxygen. PCD half-life in air was calculated by fitting a one-phase exponential decay curve (GraphPad Prism 6).

Determination of cofactors

Prenylated FMN was extracted by precipitating 1.2 to 6 mg purified protein with 50% aqueous acetonitrile (v/v) followed by incubation for 10 min at 70 °C (18,20). After centrifugation the

supernatant was freeze-dried; the product was dissolved in 0.4 to 2 mL 0.1% formic acid/10% acetonitrile (v/v) in water. Absolute prFMN concentrations were estimated in reference to a calibration curve using FMN as a standard. For identification of metal cofactors by inductively coupled plasma mass spectrometry (ICP-MS), 1.2 to 2.8 mg purified protein were freeze dried as well as an equal volume of buffer without protein as blank. The obtained powder was dissolved in 1 mL deionized water (MilliQ Element, Merck Millipore). After addition of ultrapure nitric acid, hydrogen peroxide and spikes of internal standards (^{103}Rh), samples were treated with a microwave program (room temperature to 235°C, 30 min; 235°C 30 min; cooldown 120 min). The attained clear solution was filled to 10 mL and measured using an ICP-sector field mass spectrometer (ELEMENT XR, Thermo Scientific) with specific instrumental parameters (medium resolution, $R = 4200$; triple detector mode; measurement window 125%; 32 samples per peak; sample time 0.01 sec; integration time 0.24 sec; 7 replicates per measurement). For evaluation, the signals were correlated with calibration curves. Detection limits were estimated as 3 times the standard deviation of measurements of 10 procedural blanks. Spectrophotometric determination of Fe was by the method of Hennessy et al., 1984 using ferene as complexing reagent.

EPR spectroscopy

PCD (200 to 250 μM in 100 mM MOPS/KOH pH 7.5 + 10 mM KCl) as isolated was frozen in liquid nitrogen and transferred to an EPR tube. Oxidized samples were prepared either by incubation for 2 h in air, or by addition of 2 mM ferricyanide. Reduced samples were obtained upon addition of 5 mM cyanoborohydride; reoxidation of reduced sample was carried out by incubation in air until the purple color virtually fully developed (approximately 5 min). If necessary, all steps were performed under anaerobic conditions. EPR measurements were conducted using a Bruker ELEXYS E580 X-band EPR spectrometer at 77 K. EPR parameters of individual measurements are given in the figure legends.

Amino acid sequence alignment

Amino acid sequences were obtained from the NCBI database and aligned using the ClustelW algorithm.

Acknowledgements

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Table 1. Purification of PCD from extracts of *T. chlorobenzoica* cells grown with phthalate and nitrate (7 g wet mass).

Enrichment step	Total protein (mg)	Total activity (nmol min ⁻¹)	Specific activity (nmol min ⁻¹ mg ⁻¹)	Ratio prFMN/activity*	Enrichment factor (-fold)	Recovery (%)
100.000×g supernatant	700	14700	21	1	1	100
DEAE-Sephadex	92	9568	104	0.97	4.9	65
Gel filtration	32	3904	122	0.91	5.8	27

*PrFMN was relatively quantified by ESI/Q-TOF and normalized for the amount of protein used for analyses relative to activity measurements.

Table 2. Inhibition assays of PCD.

Condition	Activity* (%)	prFMN content**[§] (%)
Anaerobic as isolated / desalting + 100 mM KCl	100	100
Anaerobic as isolated / desalting – KCl	6	8
2 h in air / desalting + 100 mM KCl	1	4
2 h in air / reduction by 1 mM reduced methylene blue	28	n.d.
2 h in air + 20 mM EDTA / desalting + 100 mM KCl	<0.2	n.d.
30 min reduction by 1 mM cyanoborohydride	40	n.d.
30 min reduction by 20 mM cyanoborohydride	<0.2	n.d.

*Desalting in the presence of 100 mM KCl in the buffer recovered ≈80% of the initial activity; [§]prFMN was quantified by ESI/Q-TOF. n.d. = not determined

Table 3. General properties of PCD from *T. chlorobenzoica*.

Property	Value
Reaction catalyzed	Phthaloyl-CoA + H ⁺ → Benzoyl-CoA + CO ₂
Kinetic parameters	K_m (phthaloyl-CoA) = 121 ± 27 μM; V_{max} = 140 ± 5 nmol min ⁻¹ mg ⁻¹ k_{cat} = 0.14 s ⁻¹ per PCD subunit (30° C)**
Native molecular mass	359 ± 8 kDa
Subunit mass and suggested composition	α ₆ (59 kDa)
Cellular content	16-18% of soluble protein
Organic cofactor	prFMN: 0.9±0.2 per PCD subunit*
Metal cofactors	Fe ²⁺ : 1.8±0.2 per PCD subunit (ICP/MS), 1.7±0.2 per PCD subunit (spectrophotometrically); K ⁺
Alternative substrate converted	3-F-phthaloyl-CoA: 16% of phthaloyl-CoA
Alternative substrates not converted	Phthalate, 2-cyanobenzoyl-CoA, 2-nitrophthaloyl-CoA, 2-methylphthaloyl-CoA, maleoyl-CoA: all <2% of phthaloyl-CoA
Inactivation by	Cyanoborohydride, air, ferricyanide, Na-EDTA

*prFMN was quantified by ESI/Q-TOF using FMN as standard. **Values normalized for the 4-fold higher values obtained using SCPCT/succinyl-CoA phthalate instead of phthaloyl-CoA

FIGURE LEGENDS

Fig. 1. Enzymatic steps and gene cluster involved in anaerobic phthalate degradation. A.

Enzymatic steps and putative gene products involved in *T. chlorobenzoica* anaerobic phthalate degradation. **B.** Phthalate-induced gene cluster in *T. chlorobenzoica* with locus tags indicated. Annotations: (1) UbiX-like FMN prenyltransferase, (2) UbiD-like phthaloyl-CoA decarboxylase, (3) Nudix family hydrolase, (4) IclR family transcriptional regulator, (5) type III CoA transferase subunit, (6) type III CoA transferase subunit, (7) TRAP transporter substrate-binding protein, (8) C4 dicarboxylate ABC transporter, (9) hypothetical protein. Hatched arrows: genes encoding putative transposases/integrases; black arrows: no function in anaerobic phthalate degradation assumed. The genes Tchl_RS15550 and Tchl_RS15505 share 100% deduced amino acid sequence identity; in this work they were identified to encode PCD.

Fig. 2. Purification, determination of molecular mass und UV/vis spectroscopy of PCD. A.

SDS gel of protein fractions obtained during PCD enrichment: Lane 1, cell extract; lane 2, fraction from DEAE-Sepharose eluting at 100 mM KCl; 3, DEAE fraction eluting at 200 mM KCl containing >80% of PCD activity; 4, DEAE fraction eluting at 1 M KCl; 5, size exclusion chromatography fraction eluting at 10.7 mL; 6, size exclusion chromatography fraction eluting at 13.6 mL. Protein applied was 10 µg (lane 1) and 2 µg (lane 2-6). **B.** Gel filtration of the 200 mM KCl fraction obtained during DEAE-Sepharose chromatography, and determination of the apparent molecular mass. PCD activity was only detected in the fractions of the peak eluting at 10.7 mL (grey bar). **C.** UV/visible spectrum of PCD as isolated (3 mg mL⁻¹). **D.** UV/visible spectrum of prFMN cofactor as recorded by diode array detection during UPLC analysis; Au = arbitrary units.

Fig. 3. EPR and UV/vis spectroscopy of PCD. **A.** EPR signals at the $g=2$ region at 77 K: (---) as isolated, (—) after reduction by cyanoborohydride and incubation in air. **B.** EPR signals at the $g=4.3$ region at 77 K: (---) as isolated, (—) after incubation in air EPR parameters: microwave frequency 9.44 GHz, microwave power 5.1 mW ($g=2.0$ signal) and 20.2 mW ($g = 4.3$ signal), modulation amplitude 1.5 mT. **C.** UV/visible spectrum of PCD (3 mg mL⁻¹) after reduction by cyanoborohydride and incubation in air.

Fig. 4. Alignment of amino acid sequences involved in metal binding and cysteine-rich motif in selected UbiD-like enzymes. The numbering refers to PCD from *T. chlorobenzoica*. Amino acids involved in Me²⁺- (magenta) and prFMN- (green) binding are highlighted; four cysteines proposed to be involved in binding a second Fe²⁺ in PCDs are marked in yellow. PCD = phthaloyl-CoA decarboxylases from *T. chlorobenzoica* (PCDTchl, WP_075149174), and the phthalate-induced gene products from *Aromatoleum aromaticum* (PCDAro, WP_011255033), *Azoarcus* PA01 (PCDPA01, WP_053419403), and *Desulfosarcina cetonica* (PCDceto, WP_054696789); PPC = phenylphosphate carboxylase subunits A and B from *Thauera aromatica* (PPCATaro, CAC12690; PPCBTaro, CAC12688); TPDSaro = terephthalate decarboxylase from *Syntrophorhabdus aromaticivorans* (SynarDRAFT_0374), Fdc1 = ferulate decarboxylase from yeast (Fdc1Yeast, AJV10846) and *Aspergillus niger* (Fdc1Aspg, XP_001390534); UbiDEcoli = 3-octaprenyl-4-hydroxybenzoate carboxy-lyase from *E. coli* (WP_072651860).

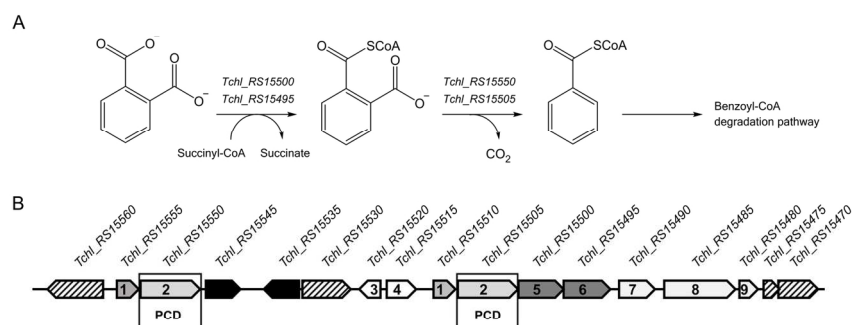


Fig. 1

76x42mm (600 x 600 DPI)

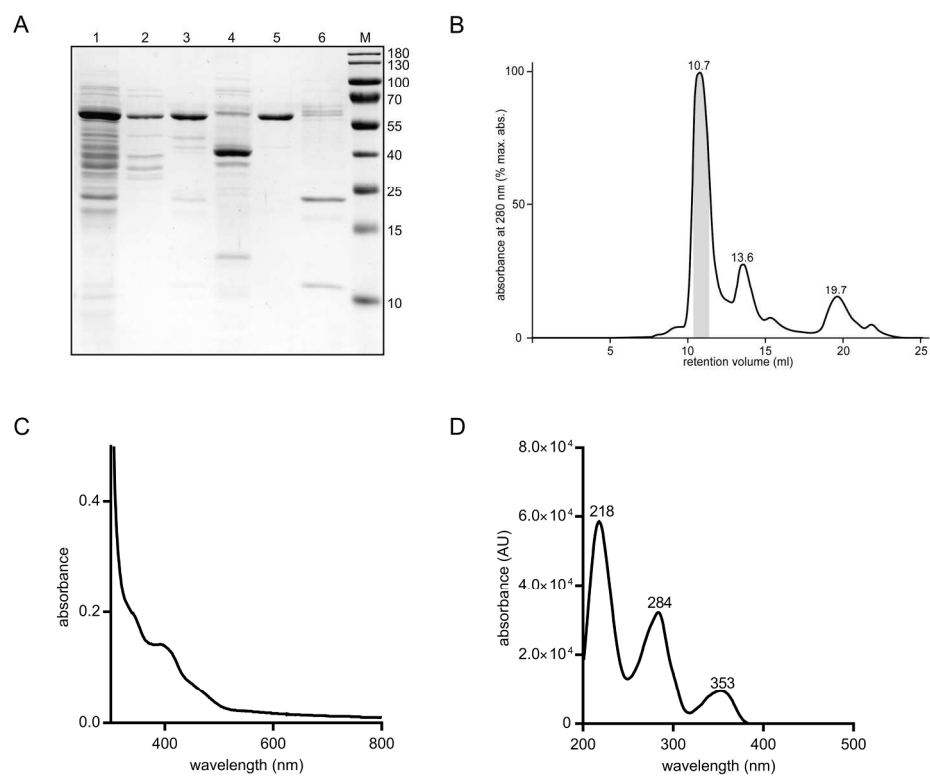


Fig. 2

116x93mm (600 x 600 DPI)

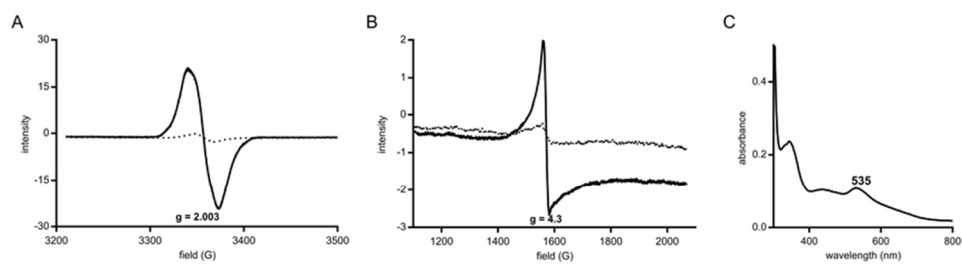


Fig. 3

37x9mm (600 x 600 DPI)

1. PCDTchl 120 VQEEVLIT-DLDDVNKWLTAIRHTPLETETMTIGSGISCVVGPYFDGGSHIGYNNMFRWGNVGTFTISPGSMWQVMTE
2. PCDARO 128 VQEEVIT-DDLDVNKWLTAIRHTPLETETMTIGSGISCVVGPYFDGGSHIGYNNMFRWGNVGTFTISPGSMWQVMTE
3. PCDPA01 138 VQEEVIT-DDLDVNKWLTAIRHTPLETETMTIGSGISCVVGPYFDGGSHIGYNNMFRWGNVGTFTISPGSMWQVMTE
4. PCDcto 148 VQEHVIT-DDLDVNKWLTAIRHTPLETETMTIGSGISCVVGPYFDGGSHIGYNNMFRWGNVGTFTISPGSMWQVMTE
5. PCDTaro 158 VQEHVIT-DDLDVNKWLTAIRHTPLETETMTIGSGISCVVGPYFDGGSHIGYNNMFRWGNVGTFTISPGSMWQVMTE
6. PCDTaro 168 VLENVLSGDKVDLNLMPFVPTFP-LDGGRYIGTMVSVVLRDPTGEVLTGYMQMLDDKRCGVLLPKRGERIMKK
7. TPDsaro 178 CKENIMLGDEVDLHQFPMPLIHE-GDGGRYVGTWVVIINKDPEETNWOVWGMVMAHTKNWLTGVPFTISQPAFIMNK
8. Fdc1Yeast 188 CKTHILSEEKIHLSLPTTPYLHV-SDGGKYLOTYGMWILQTPDKKWTWSIA GMVVDKHHITGLVIFPI IROIADS
9. Fdc1Aspg CKENSILDDSEFDLTELVPPLIHK-SDGGKYLOTYGMHIVQSPDGTWTWSIA AMVHDKNHLTGLVIFPI IROIADS
10. UbiDEcoli CQKIVSGDDVDLNRIPIMTCWP--EDAAFLITWGLTVTRGPHKERO LGIYQQLIGKNKLIMRWLSRGGALDIQE

1. PCDTchl 198 H--YKDDDEPIPLTMCFGVPPSCTFYVAGAGFDYAILPKGCD 236 IGIAGAIQGSPPVRLVKCRTI-DAYTLADA EYVLEGYL
2. PCDARO 206 H--YKDDDEPIPLTMCFGVPPSCTFYVAGAGFDYAILPKGCD 236 IGIAGAIQGSPPVRLVKCRTI-DAYTLADA EYVLEGYL
3. PCDPA01 216 H--YKDDDEPIPLTMCFGVPPSCTFYVAGAGFDYAILPKGCD 236 IGIAGAIQGSPPVRLVKCRTI-DAYTLADA EYVLEGYL
4. PCDcto 226 H--YKDDDEPIPLTMCFGVPPSCTFYVAGAGFDYAILPKGCD 236 IGIAGAIQGSPPVRLVKCRTI-DAYTLADA EYVLEGYL
5. PCDTaro 236 Y--YLPREGGCPFATAIGLSPLAAMAAACA----PSPPIP- PELTGMLAGEPVRVLKCCETN-DLEVPPADAEIIEGV
6. PCDTaro 246 Y--AKMGKKMPAAAIIGCDPLIFMSGT-----MHKKGASDFDITGTVRGQQAEEFLMAPLT-GLVPPAGAEIIVLEGEI
7. TPDsaro 256 S--YLPYKMPMPVAIVLGADPLSHIATAG-----IKPYEN--AENVGGLRGQAVELVOCETS-DLMVPAHA EIVIEGEV
8. Fdc1Yeast WAAIGKANEIPFAICFGVPPAA ILVSSMP----IPEGVS SDYVGA ILGESVPPVCCETN-DLMVPAHA EIVIEGEV
9. Fdc1Aspg WKKEGRS-DVPWALAFGVPPAAIMASSMP----IPDGVTSAGYVGA MTSSLELVKCDTN-DLVVPATSEIVLEGT
10. UbiDEcoli WCAAHFGERFPVSVALGADPATILGAVTP----VPDTLTSYAFAGLLRGTKTEVVKCISN-DLEVPPADAEIIVLEGEYI

1. PCDTchl 275 HPRDKRYTAESAAADIQGRFHFPEWAGYMGKAYKAPTTHVTAITMRRRESKPIIFPLGVHTADDANIDTSVRESA I
2. PCDARO 285 HPRDKRYTAESAAADVQGRFHFPEWAGYMGKAYKAPTTHVTAITMRRRESKPIIFPLGVHTADDANIDTSVRESA I
3. PCDPA01 295 HPRDKRYTAESAAADVQGRFHFPEWAGYMGKAYKAPTTHVTAITMRRRESKPIIFPLGVHTADDANIDTSVRESA I
4. PCDcto 305 KPKDRRFATTEAKIGKQGAWFHPEWAGYMGKAYKAPTTHVTAITMRRRESKPIIFPLGVHTADDANIDTSVRESA I
5. PCDTaro 315 LPDYKVBGPFGYTG-----YRT-SPRDFRVTFRVDAITYRNATMTISN-MGVPEQDEGOLLRSFSLGLEL
6. PCDTaro 325 DPNAFLEGPFAVTG-----YVDELHKPIPKPVLEVQOILHRNSFILWATG-QGRPVTDVHMLLATRTATL
7. TPDsaro 335 MPDQIADCGPFGYFG-----YRS-ETMCACGAVKVTAVTVESKPIVAMIA-LCTEPDDSSAAASATAAAMV
8. Fdc1Yeast SLTDTHLGPFGMHG-----YVFKSQGHPCPLYTVKAMS YRDNA ILPVSN-PGLCTDETHLTIGSLVATEA
9. Fdc1Aspg SISETGPGPFGMHG-----YIFPGDTHLGA KYKVNRIYRNNAIMPMS-CGRLTDETHLTIGSLVAAAEI
10. UbiDEcoli EQGETAPGPYGDHTG-----YNEVDSFPVFTVTHITQREDAIYHSTY-TGRPPDEPAVLG--VALNEV

1. PCDTchl 355 FALCERLQPG-IVQNVHIPYMTDWGGCI IQVKK---RNQIEEGWQRNFLAAILA CSQG---MRLAIAVSEVDVDIYSM
2. PCDARO 364 FALCERLQPG-IVQNVHIPYMTDWGGCI IQVKK---RNQIEEGWQRNFLAAILA CSQG---MRLAIAVSEVDVDIYSM
3. PCDPA01 374 FALCERLQPG-IVQNVHIPYMTDWGGCI IQVKK---RNQIEEGWQRNFLAAILA CSQG---MRLAIAVSEVDVDIYSM
4. PCDcto 383 FELCERLQPG-IEDVSTPYETMDWGGCI IQVKK---RNQIEEGWQRNFLAAILA CSQG---MRLAIAVSEVDVDIYSM
5. PCDTaro 391 ETELEQMRIPG IQSVCMPESTGRFWSVSVKQA-----YAGIAMOIAQLAFGSKLGPW-FHVMVMVDDQTDIFINW
6. PCDTaro 401 KKLMLRHGVPTVDVYVPEEGVTHLIVIGVT-----KGGTETTRKVLVDYFTLRRIIM-TNKHIVVDADVDVFN
7. TPDsaro 408 KELAIESGLP-ILDAFMPYEAQALWLLKVDLKLGLAKTTSEEFCKKVGDIYRTKVGFI-VHEIILVADDIDILNF
8. Fdc1Yeast RKLCQQNDLP-ITDAFPEFESQVTHVAVRVDLKLGLAKTTSEEFCKKVGDIYRTKVGFI-VHEIILVADDIDILNF
9. Fdc1Aspg FVPLILQKQFPEIVDFYLPPEGCSYRLAVVTIKKQ-----YAGHAKRVMGMVWSFRQFMYTKFVIVCDDVDVNDARDW
10. UbiDEcoli

274x171mm (300 x 300 DPI)