Characterization of a New Type of Phosphopantetheinyl Transferase for Fatty Acid and Siderophore Synthesis in *Pseudomonas aeruginosa*

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Summary

Phosphopantetheinyl (Ppant)-dependent carrier proteins are part of fatty acid synthases (primary metabolism), polyketide synthases and non-ribosomal peptide synthetases (secondary metabolism). For these proteins to become functionally active, they need to be primed with the 4’-phosphopantetheine moiety of Coenzyme A by a dedicated phosphopantetheine transferase (PPTase). Most organisms that employ more than one Ppant-dependent pathway also have more than one PPTase. Typically, one of these PPTases is optimized for the modification of carrier proteins of primary metabolism and rejects those of secondary metabolism (AcpS-type PPTases), while the other, Sfp-type PPTase, efficiently modifies carrier proteins involved in secondary metabolism. We present here a new type of PPTase, the carrier protein synthase of P. aeruginosa, an organism that harbors merely one PPTase, namely PcpS. Gene deletion experiments clearly show that PcpS is essential for growth of P. aeruginosa and biochemical data indicates its association with both fatty acid synthesis and siderophore metabolism. At first sight, PcpS is a PPTase of the monomeric Sfp-type and was consequently expected to have catalytic properties typical for this type of enzymes. However, in vitro characterization of PcpS with natural protein partners and non-cognate substrates revealed that its catalytic properties differ significantly from those of Sfp. Thus, the situation in P. aeruginosa is not simply the result of the loss of an AcpS-type PPTase. PcpS exhibits high catalytic efficiency with the carrier protein of fatty acid synthesis and shows a reduced, however, significant conversion rate of the carrier proteins of non-ribosomal peptide synthetases from their apo to holo form. This association with enzymes of primary and secondary metabolism indicates that PcpS belongs to a new sub-class of PPTases.
### Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| ACP          | acyl carrier protein |
| AcpS         | PPTase involved in FAS, acyl carrier protein synthase |
| ArCP         | aryl carrier protein |
| CoA          | Coenzyme A |
| CP           | Ppant dependent carrier protein |
| EntD         | PPTase involved in enterobactin production |
| FAS          | fatty acid synthase |
| IPTG         | isopropyl-β-D-thiogalactopyranoside |
| NRPS         | nonribosomal peptide synthetase |
| NTA          | nitrilotriacetic acid |
| PCP          | peptidyl-carrier protein |
| PcpS         | PPTase of *Pseudomonas aeruginosa* |
| PKS          | polyketide synthase |
| Ppant        | 4’-phosphopantetheine |
| PPTase       | 4’-phosphopantetheine transferase |
| Sfp          | PPTase involved in surfactin production |
| TCA          | trichloroacetic acid |
| TFA          | trifluoroacetic acid |
Introduction

4'-phosphopantetheine (Ppant)-dependent carrier proteins (CP) are the central entity in fatty acid synthases (FAS), polyketide synthases (PKS), and non-ribosomal peptide synthetases (NRPSs) (1,2). The superfamily of CP includes the acidic acyl- and aryl carrier proteins (ACPs, ArCPs) and the neutral peptidyl carrier proteins (PCPs). They can be part of a larger polypeptide chain or exist as distinct proteins but always fulfill the same job: during the multistep assembly of the product, the reaction intermediates of the growing acyl- or polypeptide chain remain covalently tethered to the Ppant cofactor moiety of these proteins. This moiety is about 20 Å in length and enables the bound intermediates to move between the reaction centers of multifunctional proteins. The thioester linkage that is used to bind the intermediates and final product is energy-rich which facilitates cleavage after the final step of the assembly. After ribosomal synthesis, however, the carrier protein exists only in the inactive apo-form. The Ppant moiety is posttranslationally transferred from Coenzyme A to a conserved serine residue of the CP in a Mg$^{2+}$-dependent reaction by a dedicated Phosphopantetheine transferase (PPTase), thus converting it to the active holo-CP (3) (Fig 1).

Many organisms utilize more than one Ppant-dependent pathway. For instance, aside from fatty acids, B. subtilis and E. coli produce the cyclic lipopeptide surfactin and the catecholic siderophore enterobactin, respectively (4,5). Both are products of the secondary metabolism of these organisms and were name giving when the associated PPTases, Sfp and EntD, were discovered. The conversion of ACPs of fatty acid synthesis (primary metabolism) is catalyzed by a second PPTase, namely the acyl carrier protein synthase (AcpS), that does not recognize the CPs of the NRPS systems (1,6). It has been shown that EntD does not cross-interact with primary metabolism while B. subtilis can sustain fatty acid synthesis even if AcpS is not present (7).

The substrate spectrum of the PPTases has, apart from the difference in size, lead to a classification into three groups. The name-giving prototype of the first group is AcpS of
Escherichia coli. PPTases of this group have a narrow substrate specificity, are about 120 aa in size and were shown to act as homotrimers (8,9). AcpS of *E. coli* modifies only ACPs of FAS and type II PKS (10). Recently, these results were further supported by the characterization of other AcpS-type PPTase, especially AcpS of *B. subtilis* (7). PPTases of the second, Sfp-group, are about twice the size of AcpS, have an extraordinarily broad substrate spectrum and are active as monomers. Sfp has proven to recognize every CP tested so far, including PCPs of NRPSs and ACPs of FASs and PKSs. PPTases of the third group act as integrated domains on their cognate ACP of type I FAS, as is the case in *Saccharomyces cerevisiae*, for example (11) (Fig 2).

Since Sfp has been shown to accept ACPs of FAS as substrate, the question remained whether the presence of a second, AcpS-type PPTase, would be essential for the survival of *B. subtilis*. Although many organisms contain both a PPTase of the AcpS type and one of the Sfp-type, some of the recently sequenced organisms seem to have lost its AcpS in the course of evolution (table 1). Mootz *et al.* simulated this loss by the deletion of AcpS in an sfp⁺-strain of *B. subtilis* (7). The phenotype of this mutant was that of the wild-type but further *in vitro* characterization of Sfp revealed that the catalytic efficiency was low with the ACP of primary metabolism. This PPTase is evidently optimized for the modification of CPs of secondary metabolism.

*Pseudomonas aeruginosa*, a gram negative human pathogen, is a major origin of infection in patients with burns but is also known to cause pneumonia and ocular diseases (12). *Pseudomonas spp.* secret toxic compounds such as elastase, proteases or cyanide and are very resistant to treatment with antimicrobial agents (13,14). Two siderophores, namely pyoverdin (pvd) and pyochelin (pch), which are produced during iron-limiting growth conditions, are associated with the high virulence of *P. aeruginosa* (15-17). Disruption of the synthesis of these two non-ribosomally produced peptides has proven to significantly reduce the virulence of this organism (18). This would make the PPTase responsible for the
modification of the *pvd*- and *pch*-NRPSs an excellent target for the development of new antibiotics.

Our blast searches using *E. coli* EntD and *B. subtilis* AcpS as probes revealed that *P. aeruginosa* harbors merely one putative orf encoding a PPTase (PA1165, accession number AAG04554) of 242 aa (27 kDa) with a pI of 6.77. According to these characteristics this enzyme ought to belong to the Sfp-type PPTases but exhibits only minor similarity to Sfp (13.9%) (Fig 2). In contrast to Sfp, this PPTase is not clustered with any NRPS. We report here on the genetic and biochemical characterization of the PPTase of *P. aeruginosa* in vivo and in vitro with its natural substrates. The PPTase was renamed according to its function as *Pseudomonas aeruginosa* carrier protein synthase (PcpS). As we will show, this enzyme is of special interest because it represents a new type of PPTase, essential for the modification of both the CPs of primary and secondary metabolism. Unlike Sfp, that exhibits poor catalytic efficiency with the ACP of FAS and high efficiency with CPs of secondary metabolism, PcpS behaves like a PPTase of primary metabolism toward ACP substrates.
Experimental procedures

General techniques

_E. coli_ was grown on LB medium. Antibiotics were used at the following concentrations, ampicillin 100 µg/mL, kanamycin 25 µg/mL, gentamycin 10 µg/mL. For _E. coli_ techniques, such as transformation and plasmid preparation, standard protocols were used (19). Vent polymerase (New England Biolabs, Schwalbach, Germany) or Pwo polymerase (Roche, Mannheim, Germany) was used to amplify gene fragments for cloning and expression purposes. Oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany). [³H]-CoA was purchased from Hartmann Analytics (Braunschweig, Germany).

For complementation and interaction experiments (Matchmaker Two Hybrid System 3, Clontech, Heidelberg, Germany), _S. cerevisiae_ was grown on synthetic dropout (SD) minimal medium. Yeast extracts were prepared as described in the manufacturers manual for the Matchmaker Two Hybrid System 3 (PT3247-1, Clontech, Heidelberg, Germany).

Antibodies against GAL4 DNA BD (Clontech, Heidelberg, Germany) and c-Myc (Clontech, Heidelberg, Germany) were used for western blot analysis that was carried out according to the guidelines for the ECL kit (Amersham Biosciences, Upsalla, Sweden) with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (IgG) (Sigma, Deisenhofen, Germany) as secondary antibody.

For mating experiments, _P. aeruginosa_ PA01 was grown on LB medium. Antibiotics were used at the following concentrations: carbenicillin: 500 µg/mL, gentamycin: 150 µg/mL. Sucrose was added to solid medium at 50 mg/mL.

Construction of plasmids

All plasmids and strains used in this study are summarized in table 2.
Construction of pQE70-PA1165 – The PA1165 gene encoding the *P. aeruginosa* carrier protein synthase (PcpS) was amplified by PCR with oligonucleotides 5'-ATAGCATGCAGCCTGATCAACGACCGTCTC-3' and 5'-ATAAGATCTTGCGCACCAG (restriction sites are underlined) from chromosomal DNA of *P. aeruginosa* PA01 and, after restriction digest of the amplified fragment, ligated into the *SphI* and *BglII* sites of pQE70. The resulting plasmid pQE70-PA1165 encodes the recombinant PcpS with a C-terminal tag RSHHHHHH.

Construction of pQE70-PA2966 – The PA2966 gene encoding the acyl carrier protein (ACP) was amplified by PCR with oligonucleotides 5'-ATAGCATGGCATGCACCATCGAAGAACGCG-3' and 5'-ATAAGATCTTTGCTGTGAGCAACGATG-3' from chromosomal DNA of *P. aeruginosa* PA01. The amplified fragment was digested with *SphI* and *BglII* and ligated into the *SphI* and *BglII* sites of pQE70. The resulting plasmid pQE70-PA2966 encodes the recombinant ACP with a C-terminal tag RSHHHHHH.

Construction of pQE70-pchEArCP – The fragment containing the ArCP domain from the first module (pchE1) of pyochelin synthetase E was amplified by PCR with oligonucleotides 5'-ATAGCATGCATCTGCCCCCCGATTCCCG-3' and 5'-ATAAGATCTTTTGCTGAGCAACGATG-3' from chromosomal DNA of *P. aeruginosa* PA01. The amplified fragment was digested with *SphI* and *BglII* and ligated into the *SphI* and *BglII* sites of pQE70. The resulting plasmid pQE70-pchEArCP encodes the excised recombinant ArCP (residues 1-87 of pchE, 10.5 kDa) with a C-terminal tag RSHHHHHH.

Construction of pQE70-pvdD1-PCP – The fragment containing the PCP domain from the first module (pvdD1) of pyoverdin synthetase D was amplified by PCR with oligonucleotides 5'-ATAGCATGCAATCGAGCGCCGGTACG-3' and 5'-ATAAGATCTTTTGCTGAGGCAACGATG-3' from chromosomal DNA of *P. aeruginosa* PA01. The amplified fragment was digested with *SphI* and *BglII* and ligated into the *SphI* and *BglII* sites of pQE70. The resulting plasmid pQE70-pvdD1-PCP encodes the excised recombinant PCP (residues 1-87 of pvdD1, 10.5 kDa) with a C-terminal tag RSHHHHHH.
BglII sites of pQE70. The resulting plasmid pQE70-pvdD1-PCP encodes the excised recombinant pvdD1-PCP (residues 1067-1140 of pvdD, 9.3 kDa) with a C-terminal tag RSHHHHHHH.

Construction of BS-LYS5::HIS3– using oligonucleotides 5’-
GATGCGGCCGCCTGAGTCGAACAATGCCTTACG-3’ and 5’-
CCGCTCGAGATGCAATCTGATGATGGCGG-3’, a 1445 bp fragment was amplified by PCR from chromosomal DNA of S. cerevisiae GSY155 that contained 229 bp upstream and 397 bp downstream of the LYS5 orf. This fragment was digested with NotI and XhoI and ligated into the NotI and XhoI restriction sites of pBluescript KS (Stratagene, Heidelberg, Germany) generating plasmid BS-LYS5. The disruption plasmid BS-LYS5::HIS3 was constructed by replacing the internal NcoI/EcoRV of LYS5 with an NcoI/blunt-ended fragment of pJJ215 (20) containing the HIS3 gene.

Construction of pGBKT7-LYS5– The LYS5 gene encoding the S. cerevisiae Lys5 PPTase was amplified by PCR using oligonucleotides 5’-
CCGCTCGAGTTAAAAACCATCATTTC-3’ and 5’-
GGGAATTCGTTAAAACGACTGAAGTA-3’ from chromosomal DNA of S. cerevisiae GSY155. Following restriction digest with EcoRI and XhoI, the resulting fragment was ligated into the EcoRI and SalI restriction sites of pGBKT7 (Clontech, Heidelberg, Germany).

Construction of pGBKT7-entD– The entD gene encoding the E. coli EntD PPTase was amplified by PCR using oligonucleotides 5’-
CCGCTCGAGTTAAAAACCATCATTTC-3’ and 5’-
GGGAATTCGTTAAAACGACTGAAGTA-3’ from chromosomal DNA of E. coli Top10F.

The resulting fragment was digested with EcoRI and XhoI and ligated into the EcoRI and SalI restriction sites of pGBKT7 (Clontech, Heidelberg, Germany).

Construction of pGBKT7-PA1165– The PA1165 gene encoding PcpS was amplified by PCR using oligonucleotides 5’-CGGAATTCATCGCGCCATGAACGACCG-3’ and 5’-
CCGCTCGAGTCAGGCAGCCGACCAGCCACC-3’ from chromosomal DNA of \textit{P. aeruginosa} PA01. The resulting fragment wasdigested with \textit{EcoRI} and \textit{XhoI} and ligated into the \textit{EcoRI} and \textit{SalI} restriction sites of pGBKTK7.

\textit{Construction of pGBKTK7-pchEArCP} and \textit{pGBKTK7-pvdD1-PCP} - these plasmids were constructed by first amplifying the coding sequence with the primers 5’-
CAGAATTCATTAAAGAGGAGCAATTGGAGCATGC-3’and 5’-
GGGCTCGAGTTAGTGATGGTGATGGTGATG-3’ from pQE70-pvdD1-PCP and pQE70-pchEArCP respectively and then ligating a \textit{MunI/XhoI} fragment into \textit{EcoRI/SalI} sites of pGBKTK7.

\textit{Construction of pGADT7-PA1165} - this plasmid was generated by subcloning an \textit{EcoRI/XhoI} fragment of \textit{PA1165} (used for pGBKTK7-PA1165) into the \textit{EcoRI/XhoI} sites of pGADT7 (Clontech, Heidelberg, Germany).

\textit{Construction of p\textDelta pcPS} – a 2904 bp fragment was amplified by PCR from chromosomal DNA of \textit{P. aeruginosa} PA01 that contained 1119 bp upstream and 1057 bp downstream of the \textit{PA1165} orf using primers 5’-ATAGAATTCGGTTGAGCCGATCTTGC-3’ and 5’—
ATAAAGCTTCTTTGCTGGCCGAATGG-3’. This fragment was digested with \textit{EcoRI} and \textit{HindIII} and ligated into the \textit{EcoRI} and \textit{HindIII} restriction sites of pEX18Ap (21) generating plasmid pEX18Ap-5’pcpS3’. This plasmid served as template in an inverse PCR using primers 5’-ATAAAGCTTCTCGAGGCGTTCCCGCGGCGT-3’ and 5’-
ATACATATGCCCACCAGTCACGTGGCG-3’. The resulting fragment was digested with \textit{SpeI} and \textit{NdeI} and ligated with a fragment containing the \textit{aacC1} gene (conferring gentamycin resistance) that had been amplified by PCR from pX1918G (22) with primers 5’-
ATACATATGCGGTTCGGCCAGCGGCAA-3’ and 5’-
ATACATATGCTCCGAACAACTCCCGCGGC-3’ and treated with \textit{SpeI} and \textit{NdeI}, to give the disruption plasmid p\textDelta pcPS.
Overproduction and purification of recombinant proteins

*E. coli* M15-pREP4 was transformed with pQE70-PA1165, pQE70-PA2966 and pQE70-pchEArCP to give strains RF6, RF7 and RF9, respectively, for the production of the His<sub>6</sub> fusion proteins PcpS, ACP and ArCP. 5 mL of an overnight culture of RF6, RF7 and RF9 in LB were inoculated into 500 mL of the same medium. The culture was grown at 37 °C and 300 rpm. Expression was induced by addition of 1 mM IPTG (final concentration) at an A<sub>600</sub> of 0.7 and the culture was allowed to grow for an additional 3 h before being harvested by centrifugation at 4,500 g and 4°C. The cells were resuspended in buffer A (50 mM Hepes, 300 mM NaCl (pH 8.0)), and disrupted by three passages through a cooled French pressure cell. The resulting crude extract was centrifuged at 36,000 g at 4°C for 30 min. Protein purification using Ni<sup>2+</sup>-affinity chromatography was carried out as previously described (7). The presence of the respective protein in the fractions was detected using SDS-polyacrylamide gel electrophoresis analysis (15% Laemmli gels). Fractions containing the desired protein were pooled and subsequently concentrated using Vivaspin (Vivascience AG, Hannover, Germany) with a molecular weight cut off of 5 kDa in the case of all carrier proteins and a cut off of 10 kDa in the case of PcpS. The concentrated fractions were applied to a Superdex™ G75 26/60 gel filtration column (Amersham Biosciences, Sweden) that had been equilibrated with 50 mM Tris/HCl (pH 7.0); 4 mL fractions were collected. Those fractions containing the desired protein were pooled and concentrated as described above, brought to 10 % glycerol (v/v) and stored at −80 °C. In the case of PcpS, all buffers contained 30 % glycerol. TycC3-PCP, AcpS from *B. subtilis*, Sfp of *B. subtilis* and ACP from *B. subtilis*, hereafter referred to as PCP, AcpS, Sfp and B.s.-ACP, respectively, were produced and purified as previously described (6,7). Protein concentrations were determined based on the calculated extinction coefficient at 280 nm: PcpS-His<sub>6</sub> 38,220 M<sup>−1</sup> cm<sup>−1</sup>; ACP-His<sub>6</sub> 1,280 M<sup>−1</sup> cm<sup>−1</sup>; ArCP-His<sub>6</sub> 13,020 M<sup>−1</sup> cm<sup>−1</sup>.
Determination of the Oligomeric State of PcpS

A Superdex™ G75 26/60 gel filtration column (Amersham Biosciences, Sweden) was calibrated using the following proteins (numbers in parentheses indicate the size of the protein and the amount used): aprotinin (6.5 kDa, 3 mg), cytochrome c (12.4 kDa, 2 mg), carboanhydrase (29 kDa, 2 mg), albumin-egg (45 kDa, 5 mg). Determination of the void volume, V₀, of the column was carried out using ferritin (450 kDa, 5 mg). Isocratic elution at 1 mL/min of the proteins was performed with buffer 50 mM Tris/HCl (pH 7.0) using an Äkta purifier system (Amersham Biosciences, Sweden); absorbance at 220 nm was monitored. The retention times, V, of the proteins were: aprotinin, 247.14 mL; cytochrome c, 214.97 mL; carboanhydrase, 186.21 mL; albumin-egg, 163.15 mL; ferritin, 108.96 mL (V₀). The V/V₀ of the proteins were plotted against the log(kDa) and a linear fit was applied. 5 mg of the His₆-tagged PcpS were injected onto the same column and eluted in the same manner. The retention time of the His₆-tagged PcpS was 185.67 mL.

Radioassay for the Detection of Posttranslational Modification Activity of PcpS.

PcpS activity was assayed using a radioactive assay method essentially as described previously (7). This method measures the incorporation of the [³H]-labeled 4'-phosphopantetheine group from [³H]-CoA into apo-enzymes. Reaction mixtures (in duplicate) containing 50 mM Tris/HCl (pH 8.8), 10 mM MgCl₂, 64.1 µM ACP, ArCP or B.s.-ACP, 20 µM CoA, 200 nM [³H]-CoA (specific activity: 40 Ci/mmol, 0.95 mCi/mL), and 112 nM AcpS, 120 nM Sfp or PcpS were incubated at 37 °C for 30 min. Reactions were stopped by the addition of 0.8 mL of ice-cold 10 % TCA (w/v) and 15 µL BSA (25 mg/mL). Precipitated protein was collected by centrifugation at 13,000 rpm and 4 °C for 15 min. The pellet was washed twice with 0.8 mL of ice-cold TCA (w/v) and resuspended in 180 µL formic acid. The resulting suspension was mixed with 3.5 mL Rotiszint Eco Plus scintillation fluid (Roth,
Karlsruhe, Germany) and counted using a 1900CA Tri-Carb liquid scintillation analyzer (Packard, Dreieich, Germany).

**Kinetic analysis of PcpS.**

For kinetic studies, the amount of holo-carrier protein formed was determined by an HPLC method essentially as described previously (7). 800 µL reaction mixtures contained the apo-carrier protein (1.5-250 µM ACP, 2.2-200 µM B.s-ACP, 1-154 µM ArCP and 0.9-150 µM PCP), 75 mM MES/NaAc pH 6.5, 10 mM MgCl₂ and 1-500 µM CoA. The reaction was started by the addition of PcpS to a final concentration of 5.15-34.3 nM; reaction mixtures were incubated at 37 °C for 10-30 min. The reaction was stopped and the protein precipitated by the addition of 10 % TCA. Precipitated protein was collected by centrifugation at 13,000 rpm and 4 °C for 30 min using a microcentrifuge. The pellet was resuspended in 120 µL of 50 mM Tris/HCl pH 8.8. In the case of PCP, a 3-100 µL sample of this solution was injected onto an analytical reversed phase HPLC column (Nucleosil C18, 250 mm, 5 µm, 300 Å, Macherey&Nagel, Germany) that had been equilibrated with 60 % solvent A (0.1 % trifluoroacetic acid). Absorbance at 220 nm was monitored. Apo- and holo-PCP could be separated by applying a 13.5 mL linear gradient to 57.3 % solvent B (acetonitril in 0.1% trifluoroacetic acid) followed by a 0.9 mL linear gradient to 95 % solvent B (flowrate 0.9 mL/min and temp. 45 °C). Under these conditions, holo-PCP migrates faster than apo-PCP (10.52 and 12.15 min, respectively). The amount of holo-PCP formed was determined by comparing the peak area of the holo-PCP formed with those of both apo- and holo-PCP and subtracting the amount of holo-PCP that was already present after the heterologous expression of the protein in *E. coli* (see Results).

In the case of all other carrier proteins, a 24.3 mL linear gradient 5 to 70 % solvent B (to 60 % in the case of ArCP) followed by a 2.7 mL linear gradient to 95 % solvent B at 0.9 mL/min and 45 °C was applied. The retention times of the respective holo- and apo-carrier proteins...
were: ACP: 18.74 and 20.53 min; B.s.-ACP: 21.02 and 21.76 min; ArCP: 24.13 and 24.84 min. The amount of holo-carrier protein formed was determined as described for PCP.

Buffers MES/NaAc (for pH 4.5-6.5) and Tris/HCl (for pH 7.0-9.0) were used at 75 mM final concentration for the determination of the optimum pH for PPTase activity.

For the determination of $K_m$ and $k_{cat}$ values of PcpS for apo-ACP, apo-B.s.-ACP, apo-ArCP and apo-PCP, reaction mixtures (in triplicate) contained 5 µM CoA, 1.5-250 µM apo-ACP, 2.2-200 µM apo-B.s.-ACP, 1-154 µM apo-ArCP, 0.9-150 µM apo-PCP and 5.15 nM PcpS in the case of ACP and B.s.-ACP and 34.3 nM in the case of the latter two. The $K_m$ of PcpS for CoA was determined by varying the CoA-concentration between 1-500 µM while the apo-ACP-concentration was kept constant at 200 µM.

Kinetic constants were determined by a Michaelis-Menten fit of the data sets derived from the HPLC-method (PPTase rate vs. substrate concentration).

**Deletion of the PA1165 gene encoding PepS**

The vector pΔpcpS was used for gene deletion experiments, the sacB-based strategy described by Hoang *et al.* (21) was employed. Mating procedure and isolation of potential mutants was done essentially as described by Hoang *et al.* (21) with minor modifications.

The mutated or the wild type PA1165 genes were identified by PCR. Oligonucleotides 5'-ATAGAATTCTCGTTGAGCCCGATCTTGC-3' and 5'-ATAAAGCTTCTTTGCCTGGCCGAATGG-3' were used to amplify a ca. 3 kb fragment in both cases. Digestion of this fragment with Agel yields two 1.5 kb fragments in case of the wild type gene, while digestion with SpeI yields a 1 kb and a 2 kb fragment confirming the presence of the gentamycin cassette in this fragment.
Results

Complementation of a LYS5 deletion by PcpS – The ability of PcpS to complement the S. cerevisiae PPTase Lys5, encoded by LYS5, in vivo was used as a test to determine whether orf PA1165 codes for a PPTase. The LYS5 deletion strain was constructed essentially as described previously (23). The haploid strain GSY155 was transformed with a PCR fragment of BS-LYS5::HIS3 to give strain AY61. The integration of HIS3 in the chromosomal LYS5 gene was confirmed by PCR from genomic DNA (not shown) and by loss of growth on lysine dropout plates (Fig 3A). Strain AY61 was transformed with pGBK7-LYS5, pGBK7-entD, pGBK7-PA1165, pGBK7-lamC and the empty vector pGBK7. The resulting strains were streaked on plates lacking lysine/tryptophane as a test for complementation or, as a control, on plates lacking histidine/tryptophane or tryptophane, respectively. The plates were incubated at 30 °C for two days. As shown in figure 3A, neither the strain transformed with the empty plasmid nor strain AY61-pGBK7-lamC that expresses the human lamC, which is known not to form complexes nor to interact with most other proteins (24), could grow on plates lacking lysine. The control strain AY61-pGBK7-LYS5, however, shows that the PPTase Lys5 can complement itself, as expected. The ability of E. coli EntD and PcpS to complement the ∆LYS5 strain was verified by streaking strains AY61-pGBK7-entD and AY61-pGBK7-PA1165 on plates lacking lysine as shown in figure 3A.

The pGBK7 vector expresses proteins fused to amino acids 1-147 of the GAL4 DNA binding domain (DNA BD). In addition, the vector contains a c-Myc epitope tag that is N-terminally fused to proteins expressed from this vector. In order to demonstrate that functional GAL4 DNA BD fusion proteins are made in the yeast strains, strains AY61-pGBK7, AY61-pGBK7-LYS5, AY61-pGBK7-entD, AY61-pGBK7-PA1165 and AY61-pGBK7-lamC were grown in liquid medium. Western blot analysis using anti-GAL4 BD or anti-c Myc-antibodies revealed that protein extracts of strains AY61-pGBK7-LYS5, AY61-pGBK7-entD, AY61-pGBK7-PA1165 and AY61-pGBK7-lamC (lanes 2-5, respectively, in Fig 3B)
contained the desired GAL4 BD-Myc tag-protein fusions; the control, AY61-pGBK7, shows that only the Gal4 BD and the Myc tag can be detected in this strain carrying the empty vector (Fig 3B, lane 1).

Complementation of Lys5 in vivo characterized PcpS as a functional PPTase, however, does not define its substrate specificity. We therefore applied an in vivo and an in vitro approach to test putative protein substrates of PcpS (see below).

Overproduction and Purification of P. aeruginosa PcpS, ACP and ArCP – All proteins were produced as C-terminal His6 tag fusion proteins and purified using Ni2+-NTA affinity-chromatography followed by gel filtration (see experimental procedures). SDS-PAGE analysis (not shown) revealed two bands in the case of ACP resulting from partial apo- to holo-conversion of the protein by an E. coli PPTase during expression as described previously for other CPs (7). B. subtilis ACP and PCP were produced and purified as described previously (6,7). Per liter of cell culture, 5 mg PcpS, 44 mg ACP and 19 mg ArCP with a purity of >99% were obtained. Interestingly, PcpS precipitated after Ni2+-NTA purification. We have since added 30% glycerol to all buffers during the purification process. Production of pvdD1-PCP in E. coli M15 failed as judged by SDS-PAGE with subsequent western blot analysis using a penta-His antibody (not shown).

Determination of the oligomeric state of PcpS - A Superdex™ G75 26/60 gel filtration column (Amersham Biosciences, Sweden) was calibrated as described in the experimental procedures section. The V/V0 of the proteins used to calibrate the column was plotted against their log(kDa) and a linear fit was applied (not shown). The V/V0 of the His6-tagged PcpS eluted from the same column was 1.704, indicating an apparent size of 27 kDa. This is in agreement with the size of the monomeric, His6-tagged, protein (calculated size: 27.8 kDa).
Posttranslational modification of carrier protein substrates by PcpS

(a) in vivo studies on PcpS/carrier protein partnerships using the yeast two hybrid system– Judging from the size (242 aa, 27 kDa) and the pI, it was assumed that orf PA1165 codes for an Sfp-type PPTase. It was therefore expected to be able to modify CPs of the secondary metabolism of P. aeruginosa. For this purpose, the matchmaker two hybrid system 3 (Clontech, Heidelberg, Germany) was used to test putative protein substrates of PcpS in vivo. Both pchEArCP and pvdD1-PCP were PCR amplified from the respective pQE70 constructs (table 2) and cloned into the pGBK7 vector. S. cerevisiae AH109 was transformed with either pGADT7-PA1165 or, as a control, with the empty pGADT7, and with pGBK7-pchEArCP or pGBK7-PvdD1-PCP. Transformands were streaked on plates lacking leucine/tryptophane (control) or on plates lacking leucine/tryptophane/histidine/adenine (test for activation of the reporters), and incubated at 30 °C until colonies were visible. As a positive control, AH109 cells were transformed with pGBK7-p53 and pGADT7-SV40TAg. Murine p53 encoded by the former and SV40 large T-antigen encoded by the latter were chosen because they are known to interact in yeast two hybrid systems (25,26). As a test for autoactivation of PcpS, S. cerevisiae AH109 was transformed with pGBK7-p53 and pGADT7-PA1165. All strains created were able to grow on plates lacking leucine/tryptophane as would be expected of AH109 strains harboring both a pGADT7 and a pGBK7 construct (not shown). On plates lacking, in addition to this, histidine and adenine, activation of the reporter was observed in the positive control (not shown) and strains AH109-pGADT7-PA1165 that carried either pGBK7-pchEArCP or pGBK7-pvdD1-PCP showing a clear interaction between these two CPs and PcpS (not shown). The interaction between PcpS and the former was also shown in vitro (see below) but pvdD1-PCP failed to express in E. coli and was therefore not available for the in vitro characterization of PcpS. The negative control using the empty vector pGADT7 as the second plasmid did not grow on these plates, as expected. Also, autoactivation of PcpS was not
observed (not shown). This experiment revealed that, as would be expected of an Sfp-type PPTase, PcpS was indeed able to modify ArCP and pvdD1-PCP, both of which are CPs of the siderophore metabolism of *P. aeruginosa*.

(b) *in vitro* studies using [3H]-labeled CoA – Because PcpS seemed to be the only PPTase present in *P. aeruginosa*, we expected this enzyme to also recognize and modify other CPs, especially the ACP of FAS. To assess whether the recombinant CPs are substrates of PcpS, a radio-assay was applied. We assumed that both PcpS and Sfp would be able to modify all CPs described above while we expected AcpS, being a PPTase of primary metabolism, to be more selective in this respect. However, it is worth mentioning that, although ArCP is normally part of an NRPS, it is nevertheless an acidic protein much like an ACP. Thus, the outcome of this experiment was not quite as clear as figure 4 shows. As expected, PcpS and Sfp recognize and modify *B.s.*-ACP, ACP and ArCP. AcpS, on the other hand, efficiently modifies its natural substrate *B.s.*-ACP but fails to convert ArCP to its *holo* form, proving once again that it does not cross-interact with secondary metabolism. This is the first time that AcpS from *B. subtilis* was tested with other recombinant ACPs of FAS. Although this is a mere qualitative assay, AcpS seems to modify ACP to almost the same degree as does PcpS.

These *in vitro* studies revealed that PcpS efficiently recognizes and modifies the ACP of primary metabolism in addition to CPs of secondary metabolism, so there would be no need for a supplementary AcpS-type PPTase in *P. aeruginosa*. We consequently went about to gather quantitative information on the modification of these CPs.

**Biochemical characterization of PcpS** – An HPLC-assay was applied to determine the catalytic efficiency of *P. aeruginosa* PcpS. The ratios of apo- to *holo*-CP after heterologous production in *E. coli* were as follows: ACP: 66% to 34%, ArCP: 100% to 0%, *B.s.*-ACP: 92% to 8%, PCP: 95% to 5%.
Before the analysis of PPTase activity towards these substrates, the pH profile of PcpS was determined. Figure 5A shows that the enzyme has a clear pH optimum at pH 6.5 with ACP, exhibiting less than 27% and 62% activity at pH 5 and 7, respectively. This pH was therefore chosen for the determination of $K_m$ and $k_{cat}$ values.

Kinetic constants were determined through a Michaelis-Menten fit of the experimental data sets. Kinetic constants of PcpS, Sfp and AcpS that were determined in this study or elsewhere, are summarized in table 3. For the determination of the kinetic constants for CoA, we varied its concentration between 1 and 500 µM while the ACP concentration was kept at 200 µM. Saturation, however, was reached very early at 5 µM and PcpS was in fact inhibited by higher concentrations of CoA (Fig 5B). Normal Michaelis-Menten behavior was observed if only the velocity values for CoA-concentrations ranging from 0.5 to 5 µM were used for the determination of kinetic constants. In all further experiments, the CoA concentration was therefore kept at 5 µM. The Michaelis-Menten constant for CoA concentrations between 0.5-5 µM was $1.1 \pm 0.3$ µM with the $k_{cat}$ being $168 \pm 13.8$ min$^{-1}$. This is the first time that a PPTase is shown to be inhibited by CoA, neither Sfp nor AcpS of *B. subtilis* or *E. coli* show this phenomenon (3,4,7). In comparison with the values determined for other PPTases, PcpS has $K_m$ and $k_{cat}$ values for CoA that are very close to the values determined for Sfp. As described for other ACPs (6,7), two $K_m$ and $k_{cat}$ could be determined for ACP. This was first seen with AcpS of *S. pneumoniae* (9) and was also confirmed for AcpS and Sfp of *B. subtilis*. (7) The kinetic constants of PcpS and AcpS for their respective cognate apo-ACP are comparable (table 3), while Sfp exhibits $K_m$ values (1.4 µM and 38 µM, table 3) that are increased by a factor of about 4.2 and 1.8, respectively for low and high apo-ACP concentrations, and $k_{cat}$ values that are diminished by a factor of at least 13 ($1.7$ min$^{-1}$ for low ACP concentrations) and 10 ($12.5$ min$^{-1}$ for high ACP concentrations) compared to the former two PPTases (table 3). We also determined two kinetic constants for apo-ArCP and apo-*B.s.*-ACP. The outcome of this experiment for the latter was not surprising, however, ArCPs are part of NRPS systems.
like PCPs and this incident has not been encountered with any other PPTase. In comparison with values determined for Sfp, the $k_{cat}$ values of PcpS for ArCP are low (0.9 min$^{-1}$ and 6.5 min$^{-1}$, respectively, for low and high apo-ArCP concentrations). For instance, the $k_{cat}$ value of Sfp for apo-EntB-ArCP (4) was determined to be ten times higher at 65 min$^{-1}$. PcpS also modifies the non-cognate B.s.-ACP. While the $K_m$ value for low B.s.-ACP concentrations (5.9 µM) is increased by a factor 12, the $K_m$ for high concentrations (37.8 µM) is 1.8 times lower compared to its cognate ACP (table 3). The $k_{cat}$ values are about twice as high and 0.9 times as high (50.9 min$^{-1}$ and 108.3 min$^{-1}$), respectively, for low and high B.s.-ACP concentrations.

So it seems that P. aeruginosa ACP (59% identity with B.s.-ACP) is not a special case but that PcpS exhibits only slightly lower catalytic efficiency with non-cognate ACPs.

Interestingly, we could also determine two kinetic constants for apo-PCP. First saturation was reached between 1 and 10 µM apo-PCP with a $K_m$ of 1.1 ± 0.2 µM and a $k_{cat}$ of 0.6 min$^{-1}$.

When the apo-PCP concentration was raised to 25 µM, the velocity values began to increase. The second $K_m$ value between 25 and 150 µM apo-PCP was 66.8 ± 3.7 µM with the $k_{cat}$ being 3.0 ± 0.1 min$^{-1}$. Like for ArCP, especially the $k_{cat}$ values of PcpS for PCP are unusually low for this type of PPTase while the second $K_m$ is comparatively high. For comparison, the same values of Sfp for PCP are 4.5 µM and 96 min$^{-1}$ (table 3).

Deletion of PA1165 – To further substantiate the in vivo role of PcpS, especially concerning fatty acid and siderophore metabolism, we attempted a knockout of the corresponding gene, PA1165. For this purpose, we decided to use the reliable knockout system based on the pEX18Ap suicide vector, which has previously been used successfully (21). In the suicide vector p∆pcpS, a gentamycin resistance cassette replaces the PA1165 gene. The cassette is flanked by two ca. 1kb fragments of homologous DNA. In addition, the vector contains an ampicillin/carbenicillin resistance cassette and a sacB gene for counterselection on a chromosomally integrated vector by a single crossover event (see experimental procedures).
After the mating experiment, several hundred gentamycin resistant mutants were isolated, 50 of which were chosen for further analysis and found to be carbenicillin resistant, which indicates a single crossover event in all isolated mutants. The presence of the gentamycin cassette replacing the PA1165 gene and the wild type PA1165 gene was confirmed by PCR in 10 mutants. PCR and restriction analysis (see experimental procedures) confirmed the presence of the wild type and the gentamycin-cassette disrupted gene in all strains tested (not shown). The complete suicide vector was evidently integrated into the genome by a single crossover event. As expected, these mutants were also sensitive to growth on sucrose. We selected for possible double crossover mutants on LB medium containing sucrose and gentamycin. We obtained spontaneous sucrose and gentamycin resistant mutants of each of the ten initially sucrose sensitive mutants. The isolated spontaneous mutants also lost carbenicillin resistance, which is another indication for a possible double crossover event by deprivation of the vector. These findings suggest the replacement of the wild type gene by the resistance cassette. However, in this case, PCR and restriction analysis of these mutants' DNA (not shown) revealed the presence of the wild type and the mutated PA1165 gene in all mutants. The outcome of these experiments utilizing this reliable knockout system strongly indicates that the PA1165 gene is essential for growth of P. aeruginosa and cannot be deleted.
**Discussion**

Posttranslational modification is absolutely essential for Ppant-dependent CPs to be functionally active, be it as distinct proteins like ACPs (primary metabolism) or as integrated domains of NRPSs and PKSs such as PCPs or ArCPs (secondary metabolism). The PPTase responsible for the modification of CPs of primary metabolism is usually AcpS whereas CPs of secondary metabolism are modified by a second, Sfp-type, PPTase. For this reason, most organisms harbor two PPTases (table 1) if they produce a secondary metabolite via a NRPS or PKS. Some of the recently sequenced organisms, however, seem to have sorted out their PPTase of primary metabolism. We present here the characterization of PcpS of *P. aeruginosa*, the first example of a PPTase that has to serve primary as well as secondary metabolism, *in vivo* and *in vitro* with natural substrates.

A recent study has shown that deletion of *acpS* in a *B. subtilis* strain that also possesses a functional Sfp-gene has no negative effect on the organism (7). The growth curves of the mutant strain showed no difference compared to wild type. Moreover, the production of the secondary metabolite surfactin in this strain was not impaired, indicating that Sfp is catalytically competent enough for the modification of all CPs present. In the light of these results it seems plausible that organisms harboring an AcpS-type PPTase in addition to an Sfp-type PPTase would gradually expel this dispensable ballast. Our blast searches in the *P. aeruginosa* genome using AcpS of *B. subtilis* and *E. coli*, Sfp and EntD as probes, revealed that this organism seems to have merely one PPTase, termed PcpS. Orf PA1165 encodes PcpS, a protein of about 27 kDa (242 aa) with an isoelectric point of 6.77 that shows the typical core motifs of PPTases (Fig 2). This data lead to the assumption that orf *PA1165* encodes PcpS of the Sfp-type. However, alignments with other PPTases revealed that the sequence similarity of PcpS to the well characterized "prototype" PPTases Sfp is merely 13.9% (Fig 2). This lead us to suspect that this PPTase may represent a special case in the pool of Sfp-type PPTases.
Mootz et al. established a genetic system to test for PPTase activity by complementation of \textit{S. cerevisiae} Lys5 (23). Essentially the same approach was used in this study to show that orf \textit{PA1165} does in fact code for a PPTase. \textit{LYS5} encodes Lys5, a PPTase that is essential for posttranslational modification of the \(\alpha\)-amino adipate reductase (Lys2) with the Ppant cofactor. PcpS as well as \textit{E. coli} EntD and \textit{S. cerevisiae} Lys5 were expressed in a \(\Delta\textit{LYS5}\) strain of \textit{S. cerevisiae}. The ability of these PPTases to complement Lys5 \textit{in vivo} was verified by streaking these strains on plates lacking lysine. As figure 3A shows, both PcpS and EntD can modify Lys2 to its active \textit{holo} form thereby conferring the ability to synthesize lysine on the \textit{LYS5} deletion strain which characterized them as PPTases.

Although for the best characterized Sfp-type PPTase, Sfp, no exemplary CP is known that is not accepted as substrate, the enzyme does differentiate between CPs of primary and secondary metabolism \textit{in vitro} (7). As can be seen in table 4, the catalytic efficiency of Sfp with PCP is 21.6 \(\mu\text{M}^{-1}\text{min}^{-1}\). It is worth to stress that the tested PCP is not a natural substrate of Sfp, but the enzyme has been characterized with the excised PCPs of module 1 and 2 of surfactin synthetase B (4). The catalytic efficiency with those PCPs was even higher at 80 \(\mu\text{M}^{-1}\text{min}^{-1}\) and 31 \(\mu\text{M}^{-1}\text{min}^{-1}\), respectively. The situation is quite different with CPs of primary metabolism. Catalytic efficiencies of Sfp with \textit{B.s.}-ACP are as low as 0.3 \(\mu\text{M}^{-1}\text{min}^{-1}\) (table 4), which is also the case for other ACPs it has been tested with (4). These facts show that Sfp is clearly an enzyme of secondary metabolism which accepts CPs of primary metabolism, albeit with dramatically reduced efficiency. The opposite is true for AcpS. Here, catalytic efficiency with \textit{B.s.}-ACP is high (110 \(\mu\text{M}^{-1}\text{min}^{-1}\) and 1.8 \(\mu\text{M}^{-1}\text{min}^{-1}\), respectively, for low and high \textit{B.s.}-ACP concentrations, table 5) but ArCPs and PCPs are rejected by this enzyme.

Organisms that carry both types of PPTases seem to have selected one PPTase responsible for primary metabolism and have optimized the other one for the modification of CPs of secondary metabolism. In \textit{E. coli} this idea extends even further if the two PPTases AcpS and EntD are taken into account. While the kinetic efficiency pattern for AcpS of \textit{E.}...
coli and B. subtilis is the same, EntD has been shown not to cross-interact with primary metabolism (1). It has been argued that the justification for the existence of two different PPTases could be a defined regulation of the CoA pool (27). The other explanation, that AcpS-type PPTase cannot recognize type I CPs for steric or size reasons, has recently been ruled out (6).

P. aeruginosa is a gram negative organism with PcpS as the sole PPTase that exhibits high similarity to E. coli EntD. If this PPTase behaved like EntD, the organism would not be able to survive without an additional AcpS-type PPTase. We first determined the protein partners of PcpS and found that it recognizes, in contrast to AcpS, both ACPs and ArCP (Fig 4) as well as pvdD1-PCP as shown in the yeast two hybrid system. This is in accord with the broad substrate specificity that would be expected of this PPTase. Also, the pH-optimum determined is very close to that of Sfp. The interpretation up to this point was that we are faced with the same situation found for the acpS deletion mutant of B. subtilis described above. However, our first attempt to determine the Michaelis-Menten constant of PcpS for CoA revealed that this enzyme is inhibited by higher concentrations of this cofactor which has not been found for any other PPTase (Fig 5B). Moreover, further characterization of the enzyme showed that it is evidently optimized for the activation of CPs from primary metabolism. The catalytic efficiency with its natural ACP is 32.5 µM⁻¹min⁻¹ and 2.6 µM⁻¹min⁻¹, respectively, while the efficiencies with ArCP are 1.1 µM⁻¹min⁻¹ and 0.13 µM⁻¹min⁻¹ (table 4). This corresponds to a 30-fold and 20-fold drop in catalytic efficiency, respectively, compared to ACP.

The enzyme also efficiently recognizes and modifies non-cognate CPs. After several attempts failed to express the P. aeruginosa PCPs of the pyoverdin and pyochelin NRPSs in E. coli, we turned to the well characterized excised TycC3-PCP for the characterization of PcpS. Nevertheless, both the PCP of the first module of the pyoverdin synthetase D (pvdD1-PCP) and ArCP were successfully tested for interaction with PcpS in a yeast two-hybrid
system (see Results). In vitro, the situation for the TycC3-PCP is the same as for ArCP: catalytic efficiency is low at 0.04 \( \mu \text{M}^{-1} \text{min}^{-1} \) and 1.1 \( \mu \text{M}^{-1} \text{min}^{-1} \). B.s.-ACP, on the other hand, is modified at 8.6 \( \mu \text{M}^{-1} \text{min}^{-1} \) and 2.9 \( \mu \text{M}^{-1} \text{min}^{-1} \); especially the last value for high (25-206 \( \mu \text{M} \)) concentrations of B.s.-ACP is the same (within experimental error) as for the natural ACP. The situation is almost the exact reverse found for Sfp.

Another, very notable difference between PcpS and Sfp was that we could determine two \( K_m \) and \( k_{cat} \)-values for all carrier proteins tested. This phenomenon has first been described for AcpS of \textit{S. pneumoniae} (9) and also recently for AcpS of \textit{B. subtilis} (7) with B.s.-ACP. By contrast, saturation at low PCP and ArCP concentrations with increasing velocity values at higher concentrations has never been encountered with any other PPTase. It has been proposed that this is due to the fact that AcpS acts as a homotrimer, holding three active sites, and may therefore exhibit allosteric regulation (9). This sort of regulation is not conceivable for the monomeric Sfp and PcpS because they only have a single active site (28). Another explanation for this behavior may be that CPs undergo a conformational change which is pH-dependent (29) and concentration-induced (6). This may account for the values determined for Sfp with ACPs but points to a difference in the way substrate recognition is carried out by PcpS compared to Sfp.

The chromosome of \textit{B. subtilis} MR168 carries the sequence information for the \textit{dhb} NRPS cluster. This NRPS usually produces the catecholic siderophore bacillibactin (30). \textit{B. subtilis} MR168, however, contains a defective \textit{sfp} gene, thus all carrier proteins of the NRPSs remain in the inactive \textit{apo}-form and no siderophore is produced. \textit{P. aeruginosa} produces two siderophores, namely pyoverdin and pyochelin that contribute to the high virulence of this organism (18). Both are synthesized non-ribosomally and thus production relies on posttranslational \textit{apo} to \textit{holo} conversion of the corresponding NRPS templates catalyzed by PcpS. In the light of the above stated facts, PcpS represents an excellent target for antimicrobial agents. The selective inhibition of this enzyme may be sufficient to get
infections caused by *P. aeruginosa* under control. Two big advantages are connected with this approach: first, because catalytic efficiency is low with CPs of secondary metabolism but at least 20 fold higher with the ACP of FAS, it is possible that only siderophore synthesis is affected by partial inhibition of PcpS. This would leave Pseudomonas viable, albeit deprived of the pvd and pch siderophores which are associated with the high virulence of this organism (18). Because primary metabolism would not or only slightly be influenced, it is unlikely that the organism becomes resistant to the inhibitor. Second, if the agent is selective enough to inhibit Sfp-type PPTases only, other organisms that are beneficial to the host would not be killed off as is the case with many antibiotics.

Characterization of PcpS *in vivo* using a yeast two hybrid system and *in vitro* with several different CPs has shown that PcpS is a PPTase capable of modifying CPs of primary and secondary metabolism. This, however, does not answer the question whether PcpS plays an essential functional role in both fatty acid and siderophore metabolism. To prove this we have attempted a disruption of the corresponding gene, *PA1165*. For this purpose, the pΔpcpS vector was used in mating experiments where the *PA1165* gene was replaced by a gentamycin resistance cassette and flanked by homologous DNA. However, subsequent PCR and restriction analysis of the conjugents revealed the presence of both the mutated and the wild type gene in all mutants that were gentamycin sensitive and carbenicillin and sucrose resistant, indicating that the gene encoding PcpS is essential (see Results). Consequently, PcpS ought to possess a functional role in fatty acid synthesis. In addition, the blast searches did not unveil the presence of a second Sfp-type PPTase indicating that PcpS is also needed for siderophore metabolism.

Our data shows that the sole PPTase of *P. aeruginosa*, PcpS, although related to *E. coli* EntD and Sfp of *B. subtilis*, has significantly different catalytic properties than other enzymes of this superfamily of PPTases. From these *in vitro* data we propose that PcpS is the first representative of a new sub-class of PPTases that putatively have a similar structure as
Sfp-type PPTases but were evolutionary selected for high catalytic efficiency with CPs of primary metabolism. A crystal structure of PepS would be an important contribution to make a decision in this case.

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Literature

1. Lambalot, R. H., Gehring, A. M., Flugel, R. S., Zuber, P., LaCelle, M., Marahiel, M. A., Reid, R., Khosla, C., and Walsh, C. T. (1996) *Chem Biol* 3(11), 923-936

2. Walsh, C. T., Gehring, A. M., Weinreb, P. H., Luis, E. N., and Flugel, R. S. (1997) *Curr Op Chem Biol* 1, 309-315

3. Flugel, R. S., Hwangbo, Y., Lambalot, R. H., Cronan, J. E., Jr., and Walsh, C. T. (2000) *J Biol Chem* 275(2), 959-968

4. Quadri, L. E., Weinreb, P. H., Lei, M., Nakano, M. M., Zuber, P., and Walsh, C. T. (1998) *Biochemistry* 37(6), 1585-1595

5. Gehring, A. M., Mori, I., and Walsh, C. T. (1998) *Biochemistry* 37(8), 2648-2659

6. Mofid, M. R., Finking, R., and Marahiel, M. A. (2002) *J Biol Chem* 277(19), 17023-17031

7. Mootz, H. D., Finking, R., and Marahiel, M. A. (2001) *J Biol Chem* 276(40), 37289-37298

8. Parris, K. D., Lin, L., Tam, A., Mathew, R., Hixon, J., Stahl, M., Fritz, C. C., Seehra, J., and Somers, W. S. (2000) *Structure Fold Des* 8(8), 883-895

9. McAllister, K. A., Peery, R. B., Meier, T. I., Fischl, A. S., and Zhao, G. (2000) *J Biol Chem* 275(40), 30864-30872

10. Gehring, A. M., Lambalot, R. H., Vogel, K. W., Drueckhammer, D. G., and Walsh, C. T. (1997) *Chem Biol* 4(1), 17-24

11. Fichtlscherer, F., Wellein, C., Mittag, M., and Schweizer, E. (2000) *Eur J Biochem* 267, 2666-2671

12. Bodey, G. P., Bolivar, R., Fainstein, V., and Jadeja, L. (1983) *Rev Infect Dis* 5, 279-313

13. Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M. T. G., Camara, M., Haas, D., and Williams, P. (2001) *J Bacteriol* 183(22), 6676-6683
14. Köhler, T., and Pechère, J. C. (2001) *Clin Microbiol Infect* **7**(s5), 7-10
15. Patel, H. M., and Walsh, C. T. (2001) *Biochemistry* **40**(30), 9023-9031
16. Cox, C. D., and Adams, P. (1985) *Infect Immun* **48**, 130-138
17. Cox, C. D., and Graham, R. (1979) *J Bacteriol* **137**, 357-364
18. Takase, H., Nitanai, H., Hoshino, K., and Otani, T. (2000) *Infect Immun* **68**(4), 1834-1839
19. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
20. Jones, J. S., and Prakash, L. (1990) *Yeast* **6**, 363-366
21. Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J., and Schweizer, H. P. (1998) *Gene* **212**, 77-86
22. Schweizer, H. P., and Hoang, T. T. (1995) *Gene* **158**, 15-22
23. Mootz, H. D., Schörgendorfer, K., and Marahiel, M. A. (2002) *FEMS Microbiol Lett* **213**(1), 51-57
24. Ye, Q., and Worman, H. J. (1995) *Experimental Cell Res.* **219**, 292-298
25. Li, B., and Fields, S. (1993) *FASEB J.* **7**, 957-963
26. Iwabuchi, K., Li, B., Bartel, P., and Fields, S. (1993) *Oncogene* **8**, 1693-1696
27. Fischl, A., and Kennedy, E. (1990) *J Bacteriol* **172**(9), 5445-5449
28. Reuter, K., Mofid, M. R., Marahiel, M. A., and Ficner, R. (1999) *Embo J.* **18**, 6823-6831
29. Flaman, A. S., Chen, J. M., Van Iderstine, S. C., and Byers, D. M. (2001) *J Biol Chem* **276**(38), 35934-35939
30. May, J. J., Wendrich, T. M., and Marahiel, M. A. (2001) *J Biol Chem* **276**(10), 7209-7217
31. Weber, T., Baumgartner, R., Renner, C., Marahiel, M. A., and Holak, T. A. (2000) *Structure Fold Des* **8**(4), 407-418
Figure legends

Figure 1

**Priming reaction in NRPS, PKS and FAS by PPTases.** PPTases transfer the 4'phosphopantetheinyl moiety from coenzyme A onto the hydroxyl side chain of a conserved serine residue of the carrier protein, thus converting it from the inactive apo- to the active holo-form.

Figure 2

**Partial Sequence alignment of PcpS, EntD and Sfp.** The bar at the top symbolizes *S. cerevisiae* fatty acid synthase (FAS) 1 and 2 (not true to scale) with their catalytic domains (abbreviations are: AT, acetyl transferase; ER, enoyl reductase; DH, dehydratase; MAL/PAL, malonyl/palmityl transferase; KR, β-ketoacyl reductase; KS, β-ketoacyl synthase; PPT, PPTase). The size in aa is given on top of the bars. The bars underneath FAS represent Sfp-type PPTases and AcpS-type PPTases, respectively; the numbers on top of the bars show the approximate size in aa. Highly conserved regions (according to Lambalot *et al.* (1)) are shaded in gray. Highly conserved residues in the partial sequence alignment below are boxed. Also shown is the % similarity of PcpS toward *E. coli* EntD as well as Sfp and AcpS from *B. subtilis*.

Figure 3

**PcpS complements a LYS5 deletion strain and has PPTase activity in vivo.**

A) *S. cerevisiae* ΔLYS5 cells were transformed with plasmid pGBK7, pGBK7-LYS5, pGBK7-entD, pGBK7-PA1165 (PcpS), or pGBK7-lamC, streaked on minimal medium plates lacking lysine/tryptophane (test for complementation), or as a control on plates lacking histidine/tryptophane or tryptophane respectively. The plates were incubated at 30°C for two days.
B) \( \Delta LYS5 \) cells carrying plasmid pGBK7 (lane 1), pGBK7-LYS5 (lane 2), pGBK7-entD (lane 3), pGBK7-PA1165 (PcpS) (lane 4), or pGBK7-lamC (lane 5) were grown in liquid medium lacking tryptophane. Extracts were taken and analyzed by SDS gel electrophoresis and immunoblotting with anti-Gal4 DB or anti-c Myc-antibodies.

**Figure 4**

**Protein partners of PcpS, AcpS and Sfp.** The selectivity of PcpS, AcpS and Sfp toward \( B.s. \)-ACP, ACP and ArCP was determined by the radioactive assay. Reaction mixtures including 50 mM Tris/HCl (pH 8.8), 64.1 \( \mu \)M ACP, ArCP or \( B.s. \)-ACP, 20 \( \mu \)M CoA, 200 nM \([^{3}H]\)-CoA, and 112 nM AcpS, 120 nM Sfp or PcpS were incubated at 37 °C for 30 min.

**Figure 5**

**pH profile of PcpS and substrate inhibition of PcpS by CoA.**

**A)** PPTase activity was measured by the HPLC assay at different pH values. Reaction mixtures including 36 \( \mu \)M apo-ACP, 5.15 nM PcpS, 500 \( \mu \)M CoA and 75 mM buffer were incubated at 37 °C for 10 min. MES/NaAc-buffers were used for pH 4.5-6.5 and Tris-HCl-buffers for pH 7.0-8.5 at a final concentration of 75 \( \mu \)M.

**B)** Reaction mixtures contained 200 \( \mu \)M ACP and 5.15 nM PcpS while the concentration of CoA was varied between 1 and 500 \( \mu \)M. Reactions were incubated at 37 °C for 10 min and the amount of *holo*-ACP formed was determined by an HPLC assay.
## Tables

Table 1: Presence of PPTases of the AcpS- and Sfp-type in selected bacteria

|                | AcpS-type PPTase | Sfp-type PPTase | AcpS-type PPTase | Sfp-type PPTase |
|----------------|------------------|-----------------|------------------|-----------------|
| *A. acolicus*  | +                | -               | +                | +               |
| *B. burgdorferi* | +              | -               | +                | -               |
| *B. halodurans* | +                | +               | +                | +               |
| *B. subtilis*  | +                | +               | -                | +               |
| *C. acetobutylicum* | +          | +               | +                | +               |
| *C. perfringens* | +                | +               | +                | +               |
| *C. pneumoniae* | +                | -               | +                | +               |
| *C. trachomatis* | +               | -               | +                | -               |
| *D. radiodurans* | +              | -               | +                | -               |
| *E. coli*      | +                | +               | +                | +               |
| *H. influenzae Rd* | -          | +               | +                | +               |
| *H. pylori*    | +                | -               | +                | -               |
| *M. genitalium* | +                | -               | +                | +               |
| *M. leprae*    | +                | -               | +                | +               |
| Plasmids                                  | relevant characteristics                                      | origin or reference |
|-------------------------------------------|-------------------------------------------------------------|---------------------|
| pQE70-PcpS                                | Expression plasmid for PcpS                                 | This work           |
| pQE70-PA2966                              | Expression plasmid for ACP                                  | This work           |
| pQE70-pchEArCP                            | Expression plasmid for ArCP                                 | This work           |
| pQE70-pvdD1-PCP                           | Expression plasmid for pvdD1-PCP                             | This work           |
| pQE70-TycC3-PCP                           | Expression plasmid for PCP                                  | Weber et al. (31)   |
| pTZ18-ydcB                                | Expression plasmid for AcpS                                 | Mootz et al. (7)    |
| pQE60-sfp                                 | Expression plasmid for Sfp                                  | Mofid et al. (32)   |
| pQE60-B.s.-ACP                            | Expression plasmid for B.s.-ACP                             | Mootz et al. (7)    |
| pJ215                                     | Plasmid containing an excisable HIS-cassette, a yeast selectable marker | Jones et al. (20)   |
| BS-LYS5::HIS3                             | Disruption plasmid for LYS5                                 | This work           |
| pGBK7-LYS5                                | Control plasmid for the complementation of Lys5             | This work           |
| pGBK7-entD                                | Plasmid for the complementation of Lys5 by EntD             | This work           |
| pGBK7-PA1165                              | Plasmid for the complementation of Lys5 by PcpS             | This work           |
| pGBK7-pchEArCP                            | Plasmid for yeast two hybrid system (interaction PcpS-ArCP)  | This work           |
| pGBK7-pvdD1-PCP                           | Plasmid for yeast two hybrid system (interaction PcpS-pvdD1-PCP) | This work           |
| pGBK7-lamC                                | Control plasmid for yeast two hybrid system encoding GAL4-BD-c-Myc-human lamin C | Clontech            |
| pGBK7-p53                                 | Control plasmid for yeast two hybrid system encoding GAL4-BD-c-Myc-murine p53 | Clontech            |
| pGADT7-SV40TAg                             | Control plasmid for yeast two hybrid system encoding GAL4-BD-c-Myc-SV40 large T-antigen | Clontech            |
| pEX18Ap                                   | Ap^S, oriT^T, sacB^T, gene replacement vector with MCS from pUC18 | Hoang et al. (21)  |
| pX1918G                                   | xylEGmR fusion cassette-containing plasmid                   | Schweizer et al. (22) |
| pΔpcpS                                    | Disruption plasmid for PA1165                               | This work           |

| Strains                                   | relevant characteristics                                      | origin or reference |
|-------------------------------------------|-------------------------------------------------------------|---------------------|
| S. cerevisiae GSY155                      | MATa ura3-52 leu2Δ1 his3Δ200 trp1Δ63                         | Schlenstedt et al. (33) |
| S. cerevisiae AY61                        | MATa ura3-52 leu2Δ1 his3Δ200 trp1Δ63                         | This work           |
| LYS5::HIS3                                |                                                             | Clontech            |
| S. cerevisiae AH109                       | MATa ura3-52 leu2-3,112 Δ1 his3Δ200 trp1-901 gal4Δ, gal80Δ, LYS2::GAL1 UAS-GAL1 TATA-HIS3, GAL2 UAS-GAL2 TATA-ADE2, URA3::MEL1 UAS-MEL1 TATA-lacZ | Clontech            |
| E. coli RF6                               | M15, pQE70-PcpS                                            | This work           |
| E. coli RF7                               | M15, pQE70-PA2966                                          | This work           |
| E. coli RF9                               | M15, pQE70-pchEArCP                                         | This work           |
### Table 3: kinetic constants of PcpS, AcpS and Sfp

| Substrate          | \( K_m \) [µM] | \( k_{cat} \) [min\(^{-1}\)] |
|--------------------|----------------|-----------------------------|
| \( \text{PcpS} \)  |                |                             |
| \( \text{AcpS} \)  |                |                             |
| \( \text{Sfp} \)   |                |                             |
| \( \text{apo-ACP} \) (1.6-12.5 µM) | 1.1 ± 0.3 | a n.dm. | n.dm. | 35.7 ± 2.1 | n.dm. | n.dm. |
| \( \text{apo-ACP} \) (23-234 µM) | 60.4 ± 10.3 | n.dm. | n.dm. | 154.7 ± 9.3 | n.dm. | n.dm. |
| \( \text{apo-ACP} \) (2.2-25 µM) | 5.9 ± 1.1 | b 0.2 ± 0.3 | b 1.4 ± 0.3 | 50.9 ± 3.2 | b 22 ± 2 | b 1.7 ± 0.1 |
| \( \text{apo-ACP} \) (25-206 µM) | 37.8 ± 6 | b 68 ± 11 | b 38 ± 8 | 108.3 ± 5 | b 125 ± 9 | b 12.5 ± 1 |
| \( \text{apo-PCP} \) (0.9-12 µM) | 1.1 ± 0.2 | c n.d. | n.d. | 0.6 | n.d. | n.d. |
| \( \text{apo-PCP} \) (25-150 µM) | 66.8 ± 3.7 | n.d. | d 4.5 ± 1 | 3.0 ± 0.1 | n.d. | d 96 ± 4 |
| \( \text{apo-ArCP} \) (1-10 µM) | 0.8 ± 0.1 | n.d. | n.dm. | 0.9 | n.d. | n.dm. |
| \( \text{apo-ArCP} \) (21-155 µM) | 50.3 ± 1.4 | n.d. | n.dm. | 6.5 ± 0.1 | n.d. | n.dm. |
| \( \text{CoA*} \) (1-5 µM) | 1.1 ± 0.3 | b 5.4 ± 1.5 | c 0.7 | 168.9 ± 13.8 | b 109 ± 4.8 | e 102 |

* 1-500 µM in the case of AcpS, 0.5-50 µM in the case of Sfp

**a** n.dm., not determined

**b** Mootz et al. (7)

**c** n.d., not detectable

**d** Mofid et al. (6), apo-PCP concentration 2-140 µM

**e** Quadri et al. (4)
Table 4: catalytic efficiency of PcpS, AcpS and Sfp

| Substrate       | PcpS  | AcpS  | Sfp   |
|-----------------|-------|-------|-------|
| ApcO             | 32.5  | a n.dm. | n.dm. |
| (1.6-12.5 μM)    |       |       |       |
| ApcO             | 2.6   | n.dm. | n.dm. |
| (23-234 μM)      |       |       |       |
| ApcO-B.s.-ACP    | 8.6   | 110   | 1.2   |
| (2.2-25 μM)      |       |       |       |
| ApcO-B.s.-ACP    | 2.9   | 1.8   | 0.3   |
| (25-206 μM)      |       |       |       |
| ApcO-PCP         | 0.5   | b n.d. | c n.d.|
| (0.9-12 μM)      |       |       |       |
| ApcO-PCP         | 0.04  | n.d.  | 21.6  |
| (25-150 μM)      |       |       |       |
| ApcO-ArCP (1-10 μM) | 1.1   | n.d.  | n.dm. |
| ApcO-ArCP (21-155 μM) | 0.13  | n.d.  | n.dm. |
| CoA              | 154   | 20    | 146   |

a n.dm., not determined
b n.d., not detectable
c Sfp exhibits only one $K_m$ and $k_{cat}$
d 2-140 μM in the case of Sfp
Figures

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Characterization of a new type of phosphopantetheinyl transferase for fatty acid
and siderophore synthesis in Pseudomonas aeruginosa
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