Molecular Cloning and Sequence Determination of the lpd Gene Encoding Lipoamide Dehydrogenase from Pseudomonas fluorescens

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The lpd gene encoding lipoamide dehydrogenase (dihydrolipoamide dehydrogenase; EC 1.8.1.4) was isolated from a library of Pseudomonas fluorescens DNA cloned in Escherichia coli TG2 by use of serum raised against lipoamide dehydrogenase from Azotobacter vinelandii. Large amounts (up to 15% of total cellular protein) of the P. fluorescens lipoamide dehydrogenase were produced by the E. coli clone harbouring plasmid pCJB94 with the lipoamide dehydrogenase gene. The enzyme was purified to homogeneity by a three-step procedure. The gene was subcloned from plasmid pCJB94 and the complete nucleotide sequence of the subcloned fragment (3610 bp) was determined. The derived amino acid sequence of P. fluorescens lipoamide dehydrogenase showed 84% and 42% homology when compared to the amino acid sequences of lipoamide dehydrogenase from A. vinelandii and E. coli, respectively. The lpd gene of P. fluorescens is clustered in the genome with genes for the other components of the 2-oxoglutarate dehydrogenase complex.

INTRODUCTION

Lipoamide dehydrogenase (dihydrolipoamide dehydrogenase; EC 1.8.1.4) is the flavoprotein component (E3) of the multifunctional complexes that catalyse the oxidative decarboxylation of pyruvate (pyruvate dehydrogenase complex; PDC), 2-oxoglutarate (2-oxoglutarate dehydrogenase complex; OGDC) and branched-chain oxoacids (branched chain oxoacid dehydrogenase complex; BCOADC) to acyl-CoA (Williams, 1976; Sokatch et al., 1981). Lipoamide dehydrogenase catalyses the oxidation of reduced lipoil groups that are covalently bound to the dihydrolipoamide acyltransferase components (E2) of the complexes.

Lipoamide dehydrogenase belongs to the family of FAD-containing pyridine nucleotide oxidoreductases. Other members of this family are glutathione reductase (Williams, 1976), mercuric reductase (Fox & Walsh, 1982), thioredoxin reductase (Holmgren, 1980) and trypanothione reductase (Shames et al., 1986). Common characteristics of these enzymes are (1) they are all dimeric and (2) they all contain one redox-active disulphide bridge per subunit which participates in catalysis.

From several organisms genes encoding lipoamide dehydrogenase have been cloned and sequenced: from Escherichia coli (Stephens et al., 1983) and recently from man (small-cell carcinoma) and pig (adrenal medulla) (Otulakowski & Robinson, 1987), from bakers' yeast (Browning et al., 1988; Ross et al., 1988) and from Azotobacter vinelandii (Westphal & de Kok, 1988).

Abbreviations: PDC, pyruvate dehydrogenase complex; OGDC, 2-oxoglutarate dehydrogenase complex; BCOADC, branched-chain oxoacid dehydrogenase complex; ORF, open reading frame.

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In *E. coli* and *A. vinelandii* only one copy of the *lpd* gene is present. In *E. coli* the gene belongs to the *ace* operon together with the genes encoding pyruvate dehydrogenase (EC 1.2.4.1) (E1) and dihydrolipoamide acetyltransferase (EC 2.3.1.12) (E2) of the PDC (Langley & Guest, 1977; Guest et al., 1981); in *A. vinelandii*, on the other hand, the *lpd* gene was found to be located downstream of the gene encoding dihydrolipoamide succinyltransferase (EC 2.3.1.61) (E2) of the OGDC (Westphal & de Kok, 1988; Hanemaaijer et al., 1988). In both organisms the *lpd* genes can be transcribed independently from the genes for the other components in the respective clusters by use of separate promoters. In *Saccharomyces cerevisiae* (Dickinson et al., 1986) and in man, PDC and OGDC contain identical lipoamide dehydrogenases. In man this lipoamide dehydrogenase is also found in BCOADC (Otulakowski et al., 1988).

In *Pseudomonas putida* and *P. aeruginosa*, on the other hand, two different lipoamide dehydrogenases have been found (Sokatch et al., 1981; McCully et al., 1986) and their respective genes have been mapped in *P. putida* (Sykes et al., 1985). One lipoamide dehydrogenase (*lpd-val*), encoded by *lpdv*, is part of the BCOADC; the second (*lpd-glc*), encoded by *lpdg*, is part of the OGDC, of the glycine oxidation system (Sokatch & Burns, 1984) and most likely of the PDC. The *lpdv* gene is part of a cluster comprising the genes encoding the BCOADC and the *lpdg* gene is part of the cluster encoding the OGDC.

Sequence homology between carboxy-terminal peptides containing the active-site histidine residue of *lpd-val* from *P. aeruginosa* and *P. putida* and lipoamide dehydrogenase from *E. coli* has already been demonstrated by McCully et al. (1986) and extends to other lipoamide dehydrogenases. Therefore, a detailed structure–function analysis of lipoamide dehydrogenases, some of them cooperating in different complexes and others being specific for one complex, is very interesting. Of great value to this is the determination of the three-dimensional structure of *A. vinelandii* lipoamide dehydrogenase, which is in progress (Schierbeek et al., 1989). Here we report on the molecular cloning and sequence determination of the gene encoding lipoamide dehydrogenase of the OGDC of *P. fluorescens*. Some general properties of the highly expressed, purified enzyme are described as well.

**METHODS**

**Bacterial strains, vectors and growth conditions.** *E. coli* TG2 (Gibson, 1984), a recA version of TG1 [Δ(lac-pro) thi supE [Res- Mod- (k)] F′(traD36 proA+B+ lacIqZAM15)] was used throughout as a host for cloning and grown in YT medium. The *P. fluorescens* strain used in this study was that described by Howell et al. (1972) and was grown in YT-medium or in minimal medium containing 20.0 mM-K₂HPO₄, 20.0 mM-NH₄Cl, 80.0 mM-NH₄NO₃, 85 μM-MgSO₄, 15.5 mM-p-hydroxybenzoate and 0.1 mg FeCl₃ l⁻¹, pH 7.0. Plasmids pUC9 (Vieira & Messing, 1982), pUC18 and pUC19 (Yanisch-Perron et al., 1985) and pTZ18R (Pharmacia) were used for cloning; M13mp9 and M13mp18 were used for nucleotide sequencing (Norlander et al., 1983; Yanisch-Perron et al., 1985).

**Materials.** Restriction endonucleases were obtained from Anglian Biotechnology, Boehringer or Bethesda Research Laboratories (BRL). T4 DNA ligase and *E. coli* DNA polymerase I (Klenow fragment) were obtained from BRL. Universal sequencing primer, 7-deaza-dGTP and calf intestinal phosphatase were from Boehringer. Low- and high-gelling-temperature agarose were from Seakem. [α-³²P]dATP (3000 Ci mmol⁻¹; 11.1 TBq mmol⁻¹) was purchased from New England Nuclear (NEN). Goat anti-rabbit IgG conjugated to alkaline phosphatase was from Promega Biotec. Sera against purified lipoamide dehydrogenase from *A. vinelandii* and *E. coli* were kindly provided by Mr A. H. Westphal (Westphal & de Kok, 1988). Chromatography resin were from Pharmacia. All chemicals used were of analytical grade.

**Isolation of chromosomal and plasmid DNA.** *P. fluorescens* was grown at 30 °C in 1 litre of minimal medium to an OD₆₀₀ of 1.5–2.0. Cells were harvested by centrifugation (10 min, 6000 g) and washed once with 100 ml 20% (w/v) sucrose, 150 mM-NaCl, 100 mM-EDTA, pH 7.6 (SSE). The pellet was resuspended in 20 ml SSE and lysozyme (10 mg) was added, followed by incubation at 37 °C for 1 h. Next, 5 mg proteinase K was added, followed by another incubation at 37 °C for 1 h. The lysate was extracted five times with phenol, phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and repeatedly extracted with chloroform/isoamyl alcohol (24:1, v/v) (Maniatis et al., 1982), until upon centrifugation no pellicle was formed at the interface. Finally, the DNA was precipitated with ethanol, dissolved in 10 mM-Tris/HCl, pH 8.0, 0.1 mM-EDTA and stored at 4 °C with a drop of chloroform added. Large-scale plasmid DNA isolations were performed as described by Westphal & de Kok (1988) and small-scale isolations as described by Birnboim & Doly (1979).
Nucleotide sequence of *P. fluorescens* lpd gene

Construction and screening of a *P. fluorescens* gene library in *E. coli*. Construction of a *P. fluorescens* gene library in *E. coli* and screening for clones that produce *P. fluorescens* lipoamide dehydrogenase was performed essentially according to the procedure used for the isolation of the *A. vinelandii* lpd gene as described by Westphal & de Kok (1988) with the following slight modifications. Instead of 9–23 kb partial Sau3AI fragments, 7–15 kb Sau3AI fragments of genomic *P. fluorescens* DNA were ligated into BamHI-digested pUC9 and instead of 1351-labelled protein A, goat anti-rabbit IgG conjugated to alkaline phosphatase was used for detection of bound antibodies. As a source of primary antibodies, serum raised against *A. vinelandii* lipoamide dehydrogenase was used. Plasmid pCJB94 was isolated from a clone that appeared positive upon initial screening and which produced high amounts of lipoamide dehydrogenase. The lpd gene was subcloned from this plasmid. Therefore, plasmid pCJB94 was partially digested with restriction enzyme Sau3AI, the digested DNA was fractionated on a 1.2% (w/v) low-gelling-temperature agarose gel and fragments of 34–40 kb were isolated and ligated into the BamHI site of pTZ18R. After transformation of *E. coli* TG2 cells with the recombinant plasmids, the same screening procedure was applied as described above. Out of several positive clones *E. coli* TG2(pJB201) was chosen for sequence analysis of the cloned gene.

Nucleotide sequence determination and analysis. The insert of plasmid pJB201 was isolated and digested with several restriction enzymes. The resulting fragments were cloned in M13-derived vectors for sequence determination with the dideoxy chain-termination method of Sanger et al. (1977) according to the strategy displayed in Fig. 1. Because of the high G + C content, 7-deaza-dGTP was used instead of dGTP. Sequence data were compiled and analysed with a VAX computer using the programs of Staden (1982, 1984).

Western blotting. This was done as described by Westphal & de Kok (1988) with the same modification, with respect to detection of bound antibodies, as mentioned above.

Southern blotting. Digests of *P. fluorescens* DNA and plasmids pCJB94 and pJB201 were fractionated in a 0.6% (w/v) agarose gel in 89 mM-Tris/borate, 2 mM-EDTA, pH 8.3, followed by transfer of the DNA to nitrocellulose according to Southern (1975). Isolated insert of pJB201 was nick-translated using [32P]dATP and used as a probe (Rigby et al., 1977). Hybridization was carried out at 65 °C for 16 h in 6× SSC, 5× Denhardt’s solution and 0.1% (w/v) SDS (Maniatis et al., 1982). The blot was washed at 65 °C in buffers containing 0.1% (w/v) SDS and 6× SSC, 1× SSC and 0.1× SSC, respectively, each wash lasting 45 min.

Enzyme purification. *P. fluorescens* lipoamide dehydrogenase was isolated from *E. coli* TG2(pCJB94), applying essentially the same procedure as used for the isolation of lipoamide dehydrogenase from *A. vinelandii* cloned in *E. coli* TG2 (Westphal & de Kok, 1988). Briefly, cell-free extract was treated with protamine sulphate to remove nucleic acids and large complexes. After centrifugation, the supernatant was made 50% saturated with
ammonium sulphate, clarified by centrifugation and applied to a Sepharose 6B column for hydrophobic interaction chromatography. The enzyme, firmly bound at the top of the matrix, was eluted with 45% saturated ammonium sulphate in 50 mM-potassium phosphate, pH 7.0, 0.5 mM-EDTA. FPLC analytical gel filtration was done as described by Van Berkel et al. (1988).

Lipoamide dehydrogenase activity was assayed as described by Van den Broek (1971) using 1 M-potassium phosphate, pH 7.0, instead of 0.8 M-sodium citrate, pH 6.5, as the buffer.

**RESULTS AND DISCUSSION**

Isolation of *E. coli* clones that produce *P. fluorescens* lipoamide dehydrogenase

Genomic *P. fluorescens* DNA was partially digested with restriction endonuclease Sau3AI. Fragments of 7–15 kb were isolated and ligated into the BamHI site of pUC9. The recombinant plasmids were used to transform *E. coli* TG2. Of the resulting clones, 1900 were screened with antiserum raised against lipoamide dehydrogenase from *A. vinelandii*. One clone, *E. coli* TG2(pCJB94), reacted strongly with the antiserum: this clone had a bright yellow appearance, suggesting that a large amount of a flavoprotein was produced. Cell-free extract of *E. coli* TG2(pCJB94) showed a lipoamide dehydrogenase activity which was 30-fold higher than that of cell-free extract of *E. coli* TG2(pUC9). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of this cell-free extract revealed large amounts of a polypeptide with an apparent molecular mass of 56 kDa which was not detectable in *E. coli* TG2(pUC9) (results not shown).

The *P. fluorescens* lipoamide dehydrogenase produced by *E. coli* TG2(pCJB94) was purified from cell-free extract of this clone following essentially the same procedure as described by Westphal & de Kok (1988) for purification of lipoamide dehydrogenase from *A. vinelandii* cloned in *E. coli* (Table 1). The *P. fluorescens* lipoamide dehydrogenase was eluted from the Sepharose 6B column at 45% ammonium sulphate saturation instead of 25% as found for the *A. vinelandii* enzyme. This indicates that *P. fluorescens* lipoamide dehydrogenase is less hydrophobic than *A. vinelandii* lipoamide dehydrogenase. SDS-PAGE of the purified protein showed a polypeptide with an apparent molecular mass of 56 kDa that was virtually free of contaminating polypeptides (Fig. 2a, lane 2). The mobility of this purified polypeptide on SDS-PAGE is different from *E. coli* lipoamide dehydrogenase, which has an apparent molecular mass of 50 kDa (Fig. 2a, lane 1).

The subunit composition of the purified enzyme was estimated by gel filtration. The enzyme eluted as one symmetrical peak from a Superose 12 column. The apparent molecular mass of the protein as estimated from the distribution coefficient was 110 kDa, indicating that, like other lipoamide dehydrogenases, the enzyme is a dimer in its native state. The molecular mass as determined here is in agreement with the reported molecular mass of lipoamide dehydrogenase holoenzyme purified from *P. fluorescens* by Scouten & McManus (1971).

The amino acid sequence of the 22 amino-terminal residues of the purified protein was determined by automated Edman degradation. This sequence was different from the N-terminal sequence of *E. coli* lipoamide dehydrogenase, but was found to coincide with the sequence derived from the nucleotide sequence of the gene found in the cloned *P. fluorescens* fragment (vide infra), omitting the initiating formylmethionine. This demonstrates that the purified lipoamide dehydrogenase was synthesized under the direction of the cloned *P. fluorescens* DNA fragment.

Western blots of purified lipoamide dehydrogenases from *E. coli*, *A. vinelandii* and *P. fluorescens* [obtained from *E. coli* TG2(pCJB94)] were incubated with antiserum raised against lipoamide dehydrogenases from *E. coli* or *A. vinelandii* (Fig. 2b, c). Cloned *P. fluorescens* lipoamide dehydrogenase reacted with antiserum against *A. vinelandii* lipoamide dehydrogenase (Fig. 2c) but not with serum against the *E. coli* enzyme (Fig. 2b). It can also be concluded from Fig. 2 that purified *P. fluorescens* lipoamide dehydrogenase as isolated from *E. coli* TG2(pCJB94) was not detectably contaminated with endogenous *E. coli* lipoamide dehydrogenase.

A purification table of a large-scale isolation of the cloned *P. fluorescens* lipoamide dehydrogenase is presented in Table 1. From this table it can be estimated that the cloned enzyme accounts for approximately 15% of the total cellular protein of *E. coli* TG2(pCJB94).
Nucleotide sequence of *P. fluorescens* lpd gene

![Image](https://microbiologyresearch.org/)

Fig. 2. Electrophoretic profiles of purified lipoamide dehydrogenases in a 12.5% (w/v) polyacrylamide gel with 0.1% SDS. (a) Staining with Coomassie brilliant blue R; (b, c) Western blots of purified lipoamide dehydrogenases incubated with antisera against *E. coli* lipoamide dehydrogenase (b) or *A. vinelandii* lipoamide dehydrogenase (c). Lanes 1, *E. coli* lipoamide dehydrogenase purified from isolated pyruvate dehydrogenase complex; lanes 2, *P. fluorescens* lipoamide dehydrogenase purified from *E. coli* TG2(pCJB94); lanes 3, purified *A. vinelandii* lipoamide dehydrogenase; lanes 4, sample buffer; lane M: molecular mass markers. The band at approximately 60 kDa in all four lines in (c) results from reaction of antiserum against *A. vinelandii* lipoamide dehydrogenase with some component present in the sample buffer. The bands at 100 kDa and 40 kDa in *E. coli* lipoamide dehydrogenase represent residual pyruvate dehydrogenase and a degradation product of the latter.

Table 1. Purification of *P. fluorescens* lipoamide dehydrogenase from *E. coli* TG2(pCJB94)

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Specific activity* (U mg⁻¹)</th>
<th>Total activity* (U)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>860</td>
<td>5525</td>
<td>19.2</td>
<td>107 500</td>
<td>100</td>
</tr>
<tr>
<td>Protamine sulphate supernatant</td>
<td>900</td>
<td>3240</td>
<td>32.7</td>
<td>106 200</td>
<td>98</td>
</tr>
<tr>
<td>50% ammonium sulphate supernatant</td>
<td>1240</td>
<td>936</td>
<td>88.7</td>
<td>83040</td>
<td>77</td>
</tr>
<tr>
<td>Sepharose 6B (pooled fractions)</td>
<td>215</td>
<td>512</td>
<td>127.1</td>
<td>65 575</td>
<td>61</td>
</tr>
</tbody>
</table>

* 1 U = 1 μmol NADH oxidized min⁻¹.

Using standard assay conditions, the specific activity of the purified enzyme (127 U mg⁻¹; 1U = 1 μmol NADH oxidized min⁻¹) is comparable to the specific activity of cloned *A. vinelandii* lipoamide dehydrogenase.

Nucleotide sequence and analysis of the *P. fluorescens* lpd gene

The lpd gene from *P. fluorescens* was subcloned from the 7 kb insert of pCJB94. From one of the resultant clones, *E. coli* TG2(pJB201) (Fig. 1), plasmid was isolated and the 3.6 kb insert used for sequence analysis.

The sequencing strategy is shown in Fig. 1; all sequence data were derived from gel-readings from at least two independently sequenced fragments. The final 3610 bp sequence was compiled...
Fig. 3. Nucleotide sequence of the *P. fluorescens* ipd gene with the derived amino acid sequence in one-character code. Indicated are a putative ribosome-binding site, RBS (overlined) and three possible terminators, A, B, and C (arrows). Asterisks represent stop codons. The initiating formylmethionine is omitted.
from overlapping sequence files, 78% of the data (93% of the coding region of the lpd gene) being derived from gel-readings of both strands. The insert of pJB201 (3610 bp) was found to comprise two open reading frames (ORFs), one between bp 99 and 1532 and the second between bp 1863 and 2990. The first ORF (bp 99–1532; Fig. 3) could be identified as coding for lipoamide dehydrogenase. Identification was based on (1) the extensive homology of the derived amino acid sequence of the polypeptide encoded by this gene with lipoamide dehydrogenase from both *A. vinelandii* (Westphal & de Kok, 1988) and *E. coli* (Stephens et al., 1983) showing 84% and 42% homology, respectively (Fig. 4), and (2) the identity of the derived amino-terminal amino acid sequence with that of the protein purified from *E. coli* TG2(pCJB94).

The derived amino acid sequence of *P. fluorescens* lipoamide dehydrogenase (Fig. 3) contains 477 amino acid residues excluding formylmethionine. The calculated molecular mass is 50033 Da (50830 Da when FAD is included). Apparently, the molecular mass as determined by SDS-PAGE (56 kDa) is overestimated by 10%. A similar observation was reported for lipoamide dehydrogenase from *A. vinelandii* (Westphal & de Kok, 1988).

Upstream of the *lpd* gene no consensus *E. coli*-type promoter sequence could be identified in pJB201. Expression of *P. fluorescens* lipoamide dehydrogenase from plasmid pJB201 is dependent on the *lacZ* promoter of the cloning vector. This was confirmed by cloning the insert in inverted direction towards the *lacZ* promoter in pUC19. The start codon AUG is preceded by a potential ribosome-binding site (bp 87–94).

The nucleotide sequence downstream of the *lpd* gene contains three regions of dyad symmetry. Free energy calculations (Tinoco et al., 1973) for the mRNA transcripts of the regions of dyad symmetry suggest that they may form very stable stem-and-loop structures: region A, \(\Delta G = -109 \text{kJ mol}^{-1}\); region B, \(\Delta G = -84 \text{kJ mol}^{-1}\); and region C, \(\Delta G = -148 \text{kJ mol}^{-1}\). The indicated stem-and-loop structures might serve as transcription terminators.

**Homology between lipoamide dehydrogenases**

The primary structures of lipoamide dehydrogenases from *E. coli* (Stephens et al., 1983), *A. vinelandii* (Westphal & de Kok, 1988) and *P. fluorescens* (this study) are aligned in Fig. 4. Indications about functional domains in lipoamide dehydrogenases were first obtained by Rice et al. (1984). They compared the primary structures of *E. coli* lipoamide dehydrogenase and human glutathione reductase and fitted the primary structure of lipoamide dehydrogenase into the three-dimensional structure of glutathione reductase. Schierbeek et al. (1989) determined the three-dimensional structure of lipoamide dehydrogenase from *A. vinelandii* by X-ray crystallography. The overall folding of the polypeptide chain of *A. vinelandii* lipoamide dehydrogenase appears to be very similar to that of glutathione reductase, with the same four-domain structure. In the primary structure several features appear to be conserved in the enzymes: the ADP-binding folds of the cofactors FAD and NAD(P)\(^+\) (positions 5–35 and 182–210, respectively, relative to *P. fluorescens* lipoamide dehydrogenase), the region around the active-site disulphide bridge (positions 44–60) and the region around the active-site histidine (positions 448–455). These conserved regions are indicated in Fig. 4; they are also conserved in *P. fluorescens* lipoamide dehydrogenase.

Besides the primary structures of the bacterial lipoamide dehydrogenases shown in Fig. 4, sequences of eukaryotic lipoamide dehydrogenases have been published: those of porcine adrenal medulla and human small-cell carcinoma (Otlakowski & Robinson, 1987) and of bakers’ yeast (Browning et al., 1988; Ross et al., 1988). Also in the primary structures of these eukaryotic lipoamide dehydrogenases the above-mentioned features are conserved (not shown).

**Localization of lpd genes in the genome: *P. fluorescens*, *E. coli* and *A. vinelandii* compared**

In pCJB94 the *lpd* gene is preceded by the genes for the other two components of the OGDC (Fig. 1). Immediately upstream of the *lpd* gene, the gene for dihydrolipoamide succinyltransferase (*sucB*) was detected; this gene has now been subcloned and sequenced and is highly homologous to the dihydrolipoamide succinyltransferase of *A. vinelandii* (A. H. Westphal, personal communication) and *E. coli* (Spencer et al., 1984). Also the gene for the third component of the OGDC, 2-oxoglutarate dehydrogenase (*sucA*), could be identified (results not
Fig. 4. Comparison of the amino acid sequence of the lipoamide dehydrogenases of *P. fluorescens* (Pflip), *A. vinelandii* (Avlip; Westphal & de Kok, 1988) and *E. coli* (Eclip; Stephens et al., 1983). Identical residues are indicated by asterisks. Boxed residues indicate the ADP-binding site of FAD (I), the redox-active disulphide bridge (II), the ADP-binding site of NAD+ (III) and the region around the active-site histidine (IV).
the lpd gene large ORFs are found in both organisms (position 1863–2990 in the sequence of pJB201, prtX in Fig. 1) which might encode polypeptides that are 85% homologous when compared to each other. This indicates that the organization of the OGDC clusters of A. vinelandii and P. fluorescens is very similar. Formal proof that, as in A. vinelandii, no lpd gene is present in the PDC cluster of P. fluorescens, has to await the analysis of the genes of the PDC cluster.

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