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# NOTES

## A Ferredoxin, Designated FdxP, Stimulates *p*-Hydroxybenzoate Hydroxylase Activity in *Caulobacter crescentus*

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**A gene, *fdxP*, was identified upstream of the *rrnA* gene in *Caulobacter crescentus* and shown to encode ferredoxin II (FdII) by insertional inactivation. FdII is homologous to a class of [2Fe-2S] ferredoxins typified by putidaredoxin. Furthermore, reconstitution assays demonstrated that FdII was able to promote *p*-hydroxybenzoate hydroxylase activity in ferredoxin-depleted extracts. Thus, biodegradation of *p*-hydroxybenzoate may be ferredoxin dependent in *C. crescentus*.**

*Caulobacter crescentus* is a gram-negative, freshwater bacterium that has been studied extensively because it differentiates during its normal cell division cycle (for recent reviews, see references 7 and 17). Each cell division results in a mature stalked cell and an immature swarmer cell. The stalked cell is capable of starting a new cell cycle immediately. However, the motile swarmer cell must go through a maturation process, during which it sheds its flagellum and synthesizes a stalk, before it can initiate a new cell cycle. In nature, caulobacters are ubiquitous and thrive in low-nutrient conditions. In addition, MacRae and Smit (14) have demonstrated that caulobacters are a major component of trickling filter systems in sewage treatment plants. Furthermore, they have been shown to be capable of degrading aromatic compounds (4, 5, 19). Thus, they may play an important role in cleaning polluted environments.

As part of our effort to assess metabolic genes and their regulation in *C. crescentus*, we have been analyzing the genes that encode ferredoxins. Ferredoxins are small, acidic iron-sulfur proteins that serve as electron carriers in several metabolic processes, including photosynthesis, nitrogen fixation, the biodegradation of aromatic compounds, and various reactions involved in fermentative metabolism (for a recent review, see reference 3). They are widely distributed, being found in bacteria, plants, and animals. Bacterial ferredoxins are polypeptides ranging in mass from 6,000 to 13,000 Da that contain various iron-sulfur clusters at their active sites. They are classified based on the type of iron-sulfur cluster(s) that they contain and the similarity of their amino acid sequences. Ferredoxins in the same subgroup have common structural properties, such as the number and type of FeS clusters and the spacing of the critical cysteines in their apoproteins (3). Many bacteria possess two or more ferredoxins which function in different biochemical processes, and we have demonstrated that *C. crescentus* synthesizes two ferredoxins, designated FdI and FdII (23). In this paper, we report the identification and characterization of a ferredoxin gene, *fdxP*, in *C. crescentus*. Furthermore, we demonstrate that FdII is encoded by the *fdxP* gene and that it stimulates the activity of *p*-hydroxybenzoate hydroxylase.

The DNA sequence of the promoter region of the *rrnA* gene in *C. crescentus* was published previously by Amemiya (2). During our study of *C. crescentus* promoter regions, a computer analysis of this DNA sequence demonstrated that a protein coding region was present immediately upstream of the *rrnA* gene promoter (Fig. 1). This protein coding region is 321 bp in length and would encode a protein with 106 amino acid residues and a predicted molecular weight of 11,236. The protein would be acidic, containing 18 acidic amino acid residues and 9 basic amino acid residues. Furthermore, when we compared the deduced amino acid sequence with the GenBank database, we found that it is homologous to a group of ferredoxins with a [2Fe-2S] active cluster. This group includes ferredoxins from bacteria (8, 13, 22), chickens (12), and humans (15). The highest similarity (45.3% identity at the amino acid level), however, is with putidaredoxin, a [2Fe-2S] ferredoxin from the bacterium *Pseudomonas putida* (13) (Fig. 2). Therefore, we named this *C. crescentus* ferredoxin gene *fdxP*. More recently, a third [2Fe-2S] ferredoxin has been discovered in *Rhodobacter capsulatus* that was shown to be 60% identical to the *C. crescentus* FdxP protein (16).

Bacterial [2Fe-2S] ferredoxins can be divided further into two major types. One type of [2Fe-2S] ferredoxin has an active site similar to that of chloroplast-type ferredoxins. Ferredoxins in this group usually have four conserved cysteine residues to coordinate with the two iron atoms. They are typically arranged in the pattern -Cys-X4-Cys-X2-Cys-X29-Cys-. These ferredoxins are important for the reduction of nitrate in halobacteria (24) and for nitrogen fixation in *Rhodobacter* spp. (8). A second type of [2Fe-2S] ferredoxin, typified by putidaredoxin, was shown to function in the biodegradation of camphor (8, 13), benzene (6), and toluene in *P. putida* (21) and in the degradation of naphthalene in *Pseudomonas* sp. strain NCIB 9816 (9, 10). These ferredoxins have the conserved four cysteines arranged in a pattern -Cys-X5-Cys-X2-Cys-X36/37-Cys-, and the protein encoded by the *fdxP* gene matches this pattern perfectly (Fig. 2). Since *C. crescentus* has been shown to be capable of degrading aromatic compounds (4, 5, 19), the homology between the *fdxP* gene product and putidaredoxin suggests that the *fdxP* gene product functions in the metabolism of aromatic compounds.

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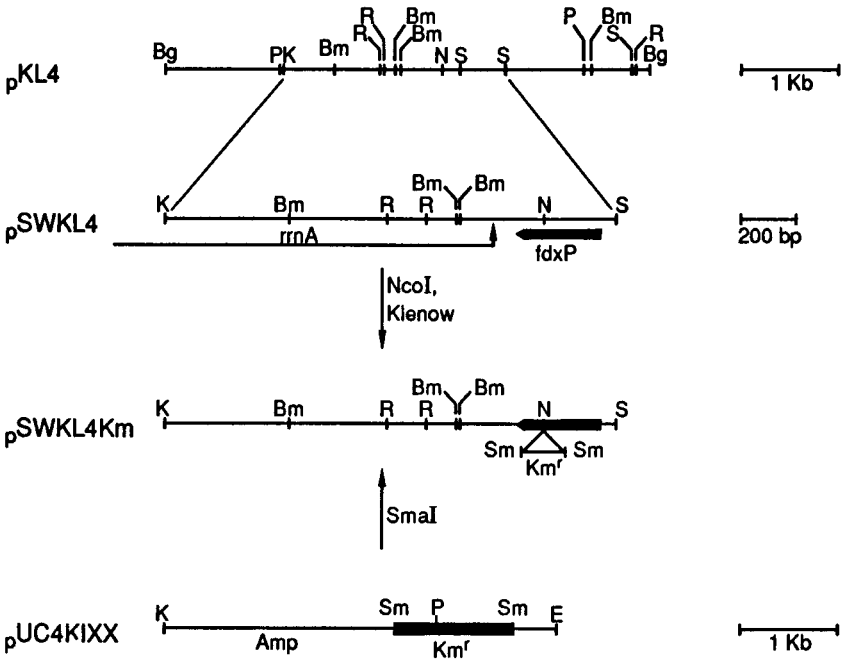


FIG. 1. Diagrams showing the *rrnA* region and the construction of an insertional inactivation of the *fdxP* gene. Plasmid pKL4 was obtained from N. Ohta (18). The diagram of pSWKL4Km is not drawn to scale. Some of the restriction sites were omitted for clarity. The vertical arrow is the transcriptional start site for the *rrnA* gene. Abbreviations: Bg, *Bgl*II; Bm, *Bam*HI; K, *Kpn*I; N, *Nco*I; P, *Pst*I; R, *Eco*RI; S, *Sal*I; Sm, *Sma*I; Km$^r$, kanamycin resistance gene cassette.

To demonstrate that the *fdxP* gene encoded a ferredoxin, we inactivated the *fdxP* gene of strain LS107 (*bla-6 syn-1000*) (1) by insertion of a kanamycin resistance gene cassette (Fig. 1). When ferredoxins were partially purified from the resulting mutant, SC4058, we found that FdII was not present (Fig. 3). This result proves that FdII is encoded by the *fdxP* gene in *C. crescentus*. When the growth properties of SC4058 were tested, we found that the mutant grew identically to the parent strain on both rich medium and minimal medium with glucose as a carbon source (Fig. 4A). However, SC4058 formed considerably smaller colonies than the parent strain when grown on minimal medium with *p*-hydroxybenzoate as the sole carbon source (Fig. 4B). A mutant generated by a single crossover with the mutated *fdxP* gene, SC4059 (containing both an inactivated and an intact *fdxP* gene), formed colonies the same size as those of the wild type when *p*-hydroxybenzoate was used as a carbon source (data not shown), indicating that the mutant gene product is not inhibitory. Thus, these results demonstrate a role for FdII in the metabolism of *p*-hydroxybenzoate. Contrary to published reports (4), significant growth of wild-type *C. crescentus* with other aromatic compounds as a sole carbon source was not obtained in our laboratory.

To determine if FdII is required for *p*-hydroxybenzoate hy-

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          1                                                    50
Cc FdxP   .....MAKIT YIQHDG...A EQVIDVKPGL TVMEGAVKNN V.PGIDADGG
Pp Fdx    .....MSKVV YVSHDGTRRE ...LDVADGV SLMQAAVSNG I.YDIVGDGG
Ec Fdx    .....MPKIV ILPHQDLGPD GAVLEANSGE TILDAALRNG I..EIEHAGE
HumFdx    SSS..EDKITVHFINRD..GE TLTTKGKVGD SLLDVVVENN LDIDGFGACE

          51                                                   100
Cc FdxP   GACACATCHV YVDEAWLDKT GDKSAMEESM LD.FAENVEP NSRLSCQIKV
Pp Fdx    GSASCATCHV YVNEAFTDKV PAANEREIGM LECVTAELKP NSRLCCQIIM
Ec Fdx    KSCACTTCHC IVREGF.DSL PESSEQEDDM LD.KAWGLEP ESRLSCQARV
HumFdx    GTLACSTCHL IFEDHIYEKL DAITDEENDM LD.LAYGLTD RSRLGCQICL

          101                          129
Cc FdxP   SDALDGLVVR LPESQH*... .........
Pp Fdx    TPELDGIVVD VPDRQW*... .........
Ec Fdx    TDE..DLVVE IPRYTINHAR EH*......
HumFdx    TKSMDNMTVR VPE.TVADAR QSIDVGKTS*
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FIG. 2. Comparison of the deduced amino acid sequences of the *C. crescentus* (Cc) *fdxP* gene product with those of the *P. putida* (Pp), *E. coli* (Ec), and human (Hum) [2Fe-2S] ferredoxins. Vertical bars and colons represent identical and similar amino acid residues, respectively. *C. crescentus* FdxP is 45.7, 40.8, and 36.3% identical to the ferredoxins from *P. putida*, *E. coli*, and human mitochondria, respectively, over the entire polypeptide. Cysteines purported to be the binding sites for the [2Fe-2S] cluster are shaded.

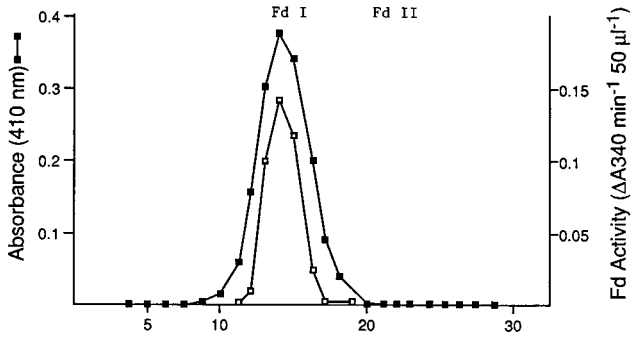


FIG. 3. Characterization of ferredoxins from *C. crescentus* SC4058. The ferredoxins were separated on a Sephadex S-100 column and identified by $A_{410}$ (solid squares). The approximate positions of FdI and FdII from the wild-type strain (23) are indicated. Ferredoxin activity was demonstrated by a reconstitution assay with chloroplast photoreduction (25) (open squares).

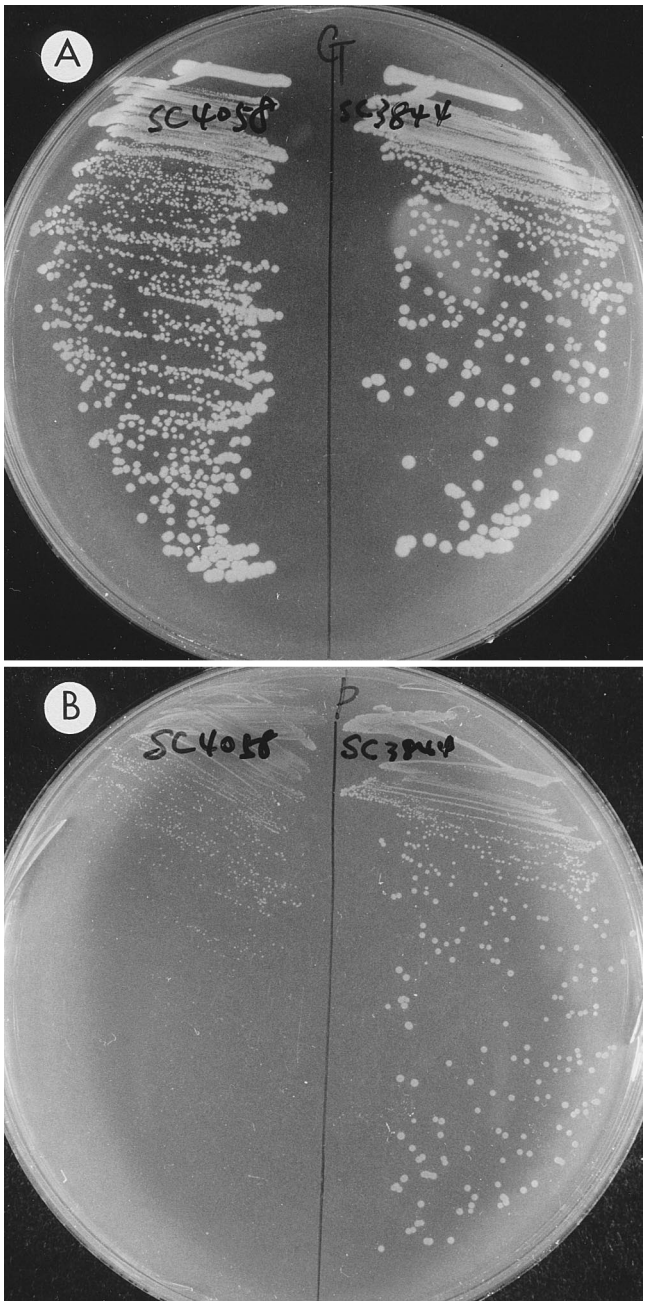


FIG. 4. Growth of SU206 (*fdxP*$^+$) and SC4058 (*fdxP*::Km$^r$) on defined medium with *p*-hydroxybenzoate as the sole carbon source. (A) Bacteria were streaked on the surface of an M2 plate with glucose as the sole carbon source (11) and incubated at 33°C for 5 days. (B) Bacteria were streaked on the surface of an M2 plate with 3 mM *p*-hydroxybenzoate as the sole carbon source and incubated at 33°C for 5 days.

droxylase (PHB-hydroxylase) activity, cell extracts were prepared from the *fdxP* wild-type and mutant strains. When ferredoxins were removed by passing the crude extract through a DEAE-cellulose column at low ionic strength, it was observed that PHB-hydroxylase activity was reduced four- to fivefold in the wild-type strain (Table 1). The activity was restored to wild-type levels by adding back partially purified FdII but not FdI (Table 1). On the other hand, crude extracts of SC4058 had a low level of PHB-hydroxylase activity which changed little after passage through the DEAE-cellulose column. When partially purified FdII was added to the resulting extract, the PHB-hydroxylase activity was restored to approximately the wild-type level. Again, the addition of FdI had no effect. Thus, although *C. crescentus* synthesizes two ferredoxins (23), they function differently in promoting PHB-hydroxylase activity. Furthermore, crude extracts of the *fdxP* mutant had reduced levels of PHB-hydroxylase activity that could be restored by the addition of FdII. This proves that FdII stimulates PHB-hydroxylase activity. It should be noted that there was always a basal level of PHB-hydroxylase activity in the absence of FdII. Thus, there does not seem to be an absolute requirement for FdII. This low level of PHB-hydrolase activity could be sufficient for the slow growth of SC4058 when *p*-hydroxybenzoate is used as a carbon source. To our knowledge, this is the first indication that biodegradation of *p*-hydroxybenzoate may be stimulated by a ferredoxin.

TABLE 1. Activity of PHB-hydroxylase of *C. crescentus*

| Source of extract | PHB-hydroxylase sp act[a] [nmol min$^{-1}$ (mg of protein)$^{-1}$] | | | |
|---|---|---|---|---|
| | Crude | Fd free | Fd free + FdI | Fd free + FdII |
| SU206 | 94 | 21 | 22 | 91 |
| SU4058 | 15 | 10 | 12 | 76 |

[a] Crude extracts, Fd-free extracts, and partially purified FdI and FdII were prepared as described previously (23). PHB-hydroxylase activity was assayed as described previously (20). The results are the average of two separate assays, with a deviation of less than ±10%.

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## REFERENCES

1. **Alley, M. R. K., and L. Shapiro (Stanford University).** Personal communication.
2. **Amemiya, K.** 1989. Conserved sequence elements upstream and downstream from the transcription initiation site of the *Caulobacter crescentus rrnA* gene cluster. J. Mol. Biol. **210:**245–254.
3. **Bruschi, M., and F. Guerlesquin.** 1988. Structure, function and evolution of bacterial ferredoxins. FEMS Microbiol. Rev. **54:**155–176.
4. **Chatterjee, D., and A. Bourquin.** 1987. Metabolism of aromatic compounds by *Caulobacter crescentus*. J. Bacteriol. **169:**1993–1996.
5. **Chatterjee, D., and P. Chatterjee.** 1987. Expression of degradative genes of *Pseudomonas putida* in *Caulobacter crescentus*. J. Bacteriol. **169:**2962–2966.
6. **Crutcher, S., and P. Geary.** 1979. Properties of the iron-sulfur proteins of the benzene diaoxygenase system from *Pseudomonas putida*. Biochem. J. **177:** 393–400.
7. **Dingwall, A., L. Shapiro, and B. Ely.** 1990. Flagella biogenesis in *Caulobacter*, p. 155–176. *In* J. P. Armitage and J. M. Lackie (ed.), Biology of the chemotactic response. Cambridge University Press, Cambridge.
8. **Grabau, C., E. Schatt, Y. Jouanneau, and P. Vignais.** 1991. A new [2Fe-2S] ferredoxin from *Rhodobacter capsulatus*. J. Biol. Chem. **266:**3294–3299.
9. **Haigler, B., and D. Gibson.** 1990. Purification and properties of NADH-ferredoxin$_{NAP}$ reductase, a component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816. J. Bacteriol. **172:**457–464.
10. **Haigler, B., and D. Gibson.** 1990. Purification and properties of ferredoxin$_{NAP}$, a component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816. J. Bacteriol. **172:**465–468.
11. **Johnson, R., and B. Ely.** 1977. Isolation of spontaneously derived mutants of *Caulobacter crescentus*. Genetics **86:**25–32.
12. **Kagimoto, K., J. McCarthy, M. Waterman, and M. Kagimoto.** 1988. Deduced amino acid sequence of mature chicken testis ferredoxin. Biochem. Biophys. Res. Commun. **155:**379–383.
13. **Koga, H., E. Yamaguchi, K. Matsunaga, H. Aramaki, and T. Horiuchi.** 1989. Cloning and nucleotide sequences of NADH-putidaredoxin reductase gene (*camA*) and putidaredoxin gene (*camB*) involved in cytochrome P-450cam hydroxylase of *Pseudomonas putida*. J. Biochem. **106:**831–836.
14. **MacRae, J. D., and J. Smit.** 1991. Characterization of caulobacters isolated from wastewater treatment systems. Appl. Environ. Microbiol. **54:**2078–2085.

15. **Mittal, S., Y. Zhu, and L. Vickery.** 1988. Molecular cloning and sequence analysis of human placental ferredoxin. Arch. Biochem. Biophys. **264:**383–391.
16. **Naud, I., M. Vincon, J. Garin, J. Gaillard, E. Forest, and Y. Jouanneau.** 1994. Purification of a sixth ferredoxin from *Rhodobacter capsulatus*: primary structure and biochemical properties. Eur. J. Biochem. **222:**933–939.
17. **Newton, A., and N. Ohta.** 1990. Regulation of the cell division cycle and differentiation in bacteria. Annu. Rev. Microbiol. **44:**689–719.
18. **Ohta, N., and A. Newton.** Personal communication.
19. **Poindexter, J. S.** 1981. The caulobacters: ubiquitous unusual bacteria. Microbiol. Rev. **45:**123–179.
20. **Shoun, H., T. Beppu, and K. Arima.** 1979. On the stable enzyme substrate complex of *p*-hydroxybenzoate hydroxylase: evidence for proton uptake from the substrate. J. Biol. Chem. **254:**899–904.
21. **Subramanian, V., T. Liu, W. Yeh, C. Serdar, L. Wackett, and D. Gibson.** 1985. Purification and properties of ferredoxin$_{TOL}$. J. Biol. Chem. **260:**2355–2363.
22. **Ta, P. T., and L. E. Vickery.** 1992. Cloning, sequencing, and overexpression of a [2Fe-2S] ferredoxin gene from *Escherichia coli*. J. Biol. Chem. **267:** 11120–11125.
23. **Wang, S. P., P. J. Kang, Y. P. Chen, and B. Ely.** 1995. Synthesis of the *Caulobacter* ferredoxin protein, FdxA, is cell cycle controlled. J. Bacteriol. **177:**2901–2907.
24. **Werber, M., and M. Mevarech.** 1978. Induction of a dissimilatory reduction pathway of nitrate in *Halobacterium* of the Dead Sea. Arch. Biochem. Biophys. **186:**60–65.
25. **Yoch, D.** 1972. The electron transport system in nitrogen fixation by *Azotobacter*. IV. Some oxidation-reduction properties of azotoflanin. Biochem. Biophys. Res. Commun. **49:**335–342.