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Mutagenesis of the gene encoding amicyanin of *Paracoccus denitrificans* and the resultant effect on methylamine oxidation

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The gene encoding the blue-copper protein amicyanin was isolated from a genomic bank of *Paracoccus denitrificans* by using a synthetic oligonucleotide. It is located directly downstream of the gene encoding the small subunit of methylamine dehydrogenase. Amicyanin is transcribed as a precursor protein with a signal sequence, typical for periplasmic proteins. Specific inactivation of amicyanin by means of gene replacement techniques resulted in the complete loss of the ability to grow on methylamine.

Amicyanin; Methylamine dehydrogenase; Gene replacement; Electron transport chain; *Paracoccus denitrificans*

1. INTRODUCTION

Paracoccus denitrificans is capable to grow under a variety of conditions [1]. In the presence of methylamine a specific set of redox carriers is induced, enabling the bacterium to use this C_1 substrate as sole carbon and energy source [2]. Oxidation of methylamine is carried out in the periplasm by methylamine dehydrogenase, an enzyme consisting of 2 identical large and 2 identical small subunits [3]. Each of the small subunits contains a covalently bound PQQ-like cofactor [4]. Electrons are passed from methylamine dehydrogenase to the aa_3-type oxidase. In this electron pathway both amicyanin and cytochrome c_{550} are involved [2,5,6]. However, an alternative way must exist in *P. denitrificans*, since mutants lacking the cytochrome c_{550} were still able to grow on methylamine, although with a lower growth rate [5]. One of the possibilities is that the role of the cytochrome c_{550} is taken over by another electron carrier. In this consideration it is worth knowing that under this specific growth condition two inducible cytochromes *c* were found in the periplasm; cytochrome c_{551i} (also called c_{552}) and cytochrome c_{553i} [7,8]. Another option is an electron transport chain from methylamine to oxygen, in which also amicyanin is by-passed. To investigate the latter possibility, mutants, impaired in the synthesis of amicyanin, were constructed and studied for their ability to grow on methylamine.

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The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession number no. X55665

2. EXPERIMENTAL

A *Paracoccus denitrificans* genomic bank of *Eco*RI fragments was constructed in the pUC19 vector and screened for the amicyanin gene with a 17-mer mixed oligonucleotide essentially as described previously [5]. The sequence was deduced from the N-terminal amino acid sequence of the purified amicyanin [9]. General cloning procedures were done by standard methods [10]. Plasmid DNA was isolated by the cleared-lysate method [11] and purified by using the Qiagen plasmid kit (Diagen GmbH, Düsseldorf, FRG). Chromosomal DNA was isolated as described earlier [5]. DNA fragments were purified from agarose gels by using GeneClean (Bio101, Inc., San Diego, CA). Southern analysis of chromosomal DNA was done by blotting DNA fragments on GeneScreen Plus filters, followed by hybridization with cloned fragments. Subsequent detection of hybrids was done by using an enzyme immunoassay (Boehringer GmbH, Mannheim, FRG). DNA sequencing was in principle performed according to the dideoxy method [12], but with the Klenow polymerase reaction starting from fluorescent labeled M13 primers. Subsequent separation and analysis of the resulting fragments was done on a 370A DNA sequencer (Applied Biosystems, Foster City, CA). Methylamine dehydrogenase activities were determined as described earlier [13], except that the reaction was started with methylamine instead of methanol. Plasmids pGRPd1 and pRVS1 were used successively in gene replacement experiments. Plasmid pGRPd1 was used to introduce an insertional inactivated gene into the chromosome essentially as described previously [5]. Plasmid pRVS1, the construction and use of which will be described in a manuscript in preparation, was used to introduce a simple frame-shift mutation in exchange for the insertional inactivation. Physiological growth characteristics were studied on plates. The composition of the growth media was as used earlier [5] with 100 mM methylamine, 100 mM methanol, 25 mM succinate, or 10 mM choline chloride as the carbon and energy source.

3. RESULTS AND DISCUSSION

Southern analysis of *Paracoccus denitrificans* chromosomal DNA revealed that the amicyanin probe reacted positively on a 0.65 kb *Eco*RI fragment. Fragments of that size were isolated and cloned in pUC19. By using

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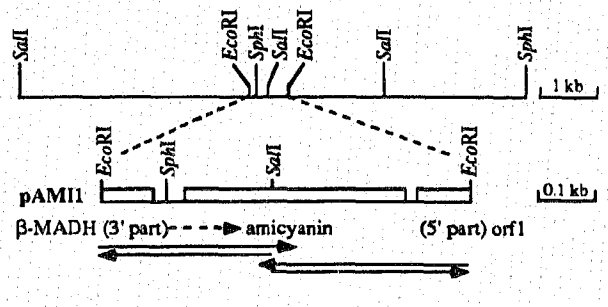

Fig. 1. Physical map and sequencing strategy of a *P. denitrificans* DNA fragment, containing the amicyanin coding region. The direction and extent of the nucleotide sequence are indicated by the direction and length of the arrows. The direction of transcription of the genes is indicated by the direction of the dashed arrow. β-MADH is the small subunit of methylamine dehydrogenase; orf is an open reading frame.

the same mixed oligonucleotide, a positive clone, pAMI1, was isolated and the DNA sequence of the insert was determined and analysed. Location of the amicyanin gene and a map of pAMI1 are shown (Fig. 1). The nucleotide sequence of the *P. denitrificans* chromosome fragment was determined and the amino acid sequences were deduced (Fig. 2). The clone contains the complete amicyanin gene (bp 151 through 543). The gene is preceded by a Shine and Dalgarno sequence, starting at position 141.

The protein sequence reveals a stretch of 10 amino acids, which is identical with the N-terminal amino acid sequence of purified amicyanin [9]. The translational start of the precursor protein is found 26 triplets upstream from the N-terminus of mature amicyanin. This segment of 26 amino acids has all the features of a signal sequence, typical for periplasmic proteins [14]. Amicyanin itself consists of 105 amino acids and has a relative molecular mass of 11 482 Da, the copper not included. The copper-binding site is likely to be formed by a histidine at amino acid position 53 and cysteine, histidine, and methionine at positions 92, 95, and 98 respectively (Fig. 3). The relative positions of these amino acids are in close agreement with the positions of copper ligands, determined in other blue-copper proteins [15,16].

The codon usage is typical for *P. denitrificans* genes; only 13 percent of the codons ends with an A or a T, and the overall G+C content of the gene is 65%. Amino acid sequence of the mature *P. denitrificans* amicyanin is 53% identical with the *Methylobacterium extorquens* AM1 [17] (Fig. 3) and 63% identical with its counterpart in *Thiobacillus versutus*, an organism phylogenetically closely related to *P. denitrificans* [18]. Moreover, an overall DNA homology of 70% was found for the genes encoding amicyanin in *P. denitrificans* and *T. versutus*. Similar values were found for the corresponding 5′ parts of the downstream

small subunit methylamine dehydrogenase (3' region)

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  EcoRI          15                  30                  45                  60                  75
GAA TTC GCC AAC GAC ATC ATC TGG TGC TTC GGC GCC GAG GAC GAT GCC ATG ACC TAT CAC TGC ACG ATC TCG CCC
Glu Phe Ala Asn Asp Ile Ile Trp Cys Phe Gly Ala Glu Asp Asp Ala Met Thr Tyr His Cys Thr Ile Ser Pro

                                                                                        amicyanin
                 90                 110                 130                 150
ATC GTG GGC AAG GCG AGC  TGACGGCGGCCGGGCGCGCATGCCCGCGCCCGGCCTTCATCCCGCATGGAGGAAGCAA  ATG ATT TCT GCG
Ile Val Gly Lys Ala Ser                                                               Met Ile Ser Ala

165                 180                 195                 210                 225
ACC AAG ATC CGC TCG TGC CTG GCG GCC TGC GTC CTG GCG GCA TTC GGC GCG ACG GGC GCC CTG GCC GAC AAG GCG
Thr Lys Ile Arg Ser Cys Leu Ala Ala Cys Val Leu Ala Ala Phe Gly Ala Thr Gly Ala Leu Ala*Asp Lys Ala

240                 255                 270                 285                 300
ACG ATC CCC TCG GAA AGC CCC TTT GCC GCC GCC GAG GTG GCC GAT GGC GCC ATC GTC GTC GAC ATC GCC AAG ATG
Thr Ile Pro Ser Glu Ser Pro Phe Ala Ala Ala Glu Val Ala Asp Gly Ala Ile Val Val Asp Ile Ala Lys Met

315                 330                 345                 360                 375
AAA TAC GAA ACC CCC GAA CTT CAT GTG AAG GTC GGC GAC ACC GTC ACC TGG ATC AAC CGC GAG GCG ATG CCG CAC
Lys Tyr Glu Thr Pro Glu Leu His Val Lys Val Gly Asp Thr Val Thr Trp Ile Asn Arg Glu Ala Met Pro His

390                 405                 420                 435                 450
AAT GTC CAT TTC GTC GCC GGC GTG CTG GGC GAG GCG GCG TTG AAA GGC CCG ATG ATG AAG AAG GAG CAG GCC TAT
Asn Val His Phe Val Ala Gly Val Leu Gly Glu Ala Ala Leu Lys Gly Pro Met Met Lys Lys Glu Gln Ala Tyr

465                 480                 495                 510                 525
TCC CTG ACC TTC ACC GAG GCC GGC ACC TAT GAC TAT CAC TGC ACC CCG CAT CCC TTC ATG CGC GTC AAG GTC GTC
Ser Leu Thr Phe Thr Glu Ala Gly Thr Tyr Asp Tyr His Cys Thr Pro His Pro Phe Met Arg Gly Lys Val Val

                    orf 1 (5' region)
540                 560                 575                 590                 605
GTC GAG TAGCATGTGAAAGGCCGGCCATG TGG ATT CCC TAC GAC ATT CGC GGC TCG CTG AAG CCT GAA TCG CCA GCC GGG
Val Glu                     Met Trp Ile Pro Tyr Asp Ile Arg Gly Ser Leu Lys Pro Glu Ser Pro Ala Gly

620                 635                 650      EcoRI
ACG ATC CGC CTG TCG CGA ACC GAT ACC AGC CCG CGC GAA TTC
Thr Ile Arg Leu Ser Arg Thr Asp Thr Ser Pro Arg Glu Phe
```

Fig. 2. Nucleotide sequence and deduced amino acid sequences of the 3′ region of the small subunit of methylamine dehydrogenase, the amicyanin gene, and the 5′ region of orf1. The signal sequence of amicyanin is in italics, the putative signal sequence cleavage site is indicated by an asterisk. Putative Shine-Dalgarno sequences are underlined, and the inverted repeat is indicated by lines above the joining nucleotides.

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            10         20         30         40         50
Pd  DKATIPSESP FAAAEVADGA IVVDIAKMKY ETPELHVKVG DTVTWINREA
               *  *   *   * *****   ***   * *   *** * **
AM1         AGAL EAVQEAPAGS TEVKIAKMKF QTPEVRIKAG SAVTWTNTEA

            60         70         80         90         100
    MPHNVHFVAG VLGEAALKGP MMKKEQAYSL TFTEAGTYDY HCTPHPFMRG
     ******  *   *    ** *   * **   *   *****  ******* *
    LPHNVHFKSG PGVEKDVEGP MLRSNQTYSV KFNAPGTYDY ICTPHPFMKG

     105
    KVVVE
    *****
    KVVVE
```

Fig. 3. Comparison of amino acid sequences of amicyanin. Pd: Deduced amino acid sequence of amicyanin from *P. denitrificans*. AM1: Amino acid sequence of amicyanin from *M. extorquens* AM1 [17]. Identical residues are indicated by an asterisk.

located open reading frames (M. Ubbink and G.W. Canters, personal communication).

Upstream of the amicyanin gene, the 3′ region of a second gene was identified. The amino acid sequence, deduced from this part of the gene, shows great homology with the C-terminus of the small subunit of *T. versutus* methylamine dehydrogenase [19]. Preliminary sequence data even show that the corresponding C-terminal stretches of 31 amino acids are identical while the DNA sequences deflect only 6% from each other (M. Ubbink and G.W. Canters, personal communication). In the gene encoding the small subunit of methylamine dehydrogenase, the codon for a tryptophan was found at nucleotide number 22 of the DNA sequence. The amino acid at this position of the protein is assumed to be one of two residues involved in the incorporation of a PQQ-like cofactor [19,20].

The genes encoding the small subunit of methylamine dehydrogenase and amicyanin are separated from each other by 54 nucleotides, of which 28 are part of a long inverted repeat (nucleotides 101 through 128). This organization of genes strongly suggests that both proteins are coordinately expressed and acting simultaneously in the periplasm during growth on methylamine. In order to establish that amicyanin is indeed required for electron transport from methylamine dehydrogenase to the aa_3-type oxidase, a mutant was constructed, in which the chromosomal amicyanin gene was interrupted at the *SalI* site by the insertion of a kanamycin gene. Since such a mutation might have effects on the downstream genes, a second mutant was created. In this mutant, the insertionally inactivated gene was replaced by a gene, in which the *SalI* site was filled in. This gives rise to a simple frame-shift mutation in the amicyanin chromosomal gene. The correctness of gene replacements in both mutants was checked by Southern analysis (Fig. 4). As a consequence of the frame-shift mutation, a new transcript could be expected, in which a stop-codon is introduced 52 bp upstream from the original stop-codon. Therefore, the contingent downstream effects of the frame-shift mutation is restricted to a minimum, and the main consequences of the absence of amicyanin alone can be studied properly.

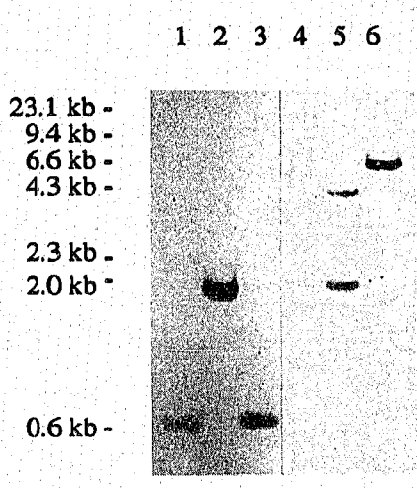

Fig. 4. Blot of *Eco*RI-digested (lanes 1 to 3) and *Sal*I-digested (lanes 4 to 6) chromosomal DNA of the *P. denitrificans* wild-type strain (lanes 1 and 4), the amicyanin insertion mutant strain (lanes 2 and 5) and the amicyanin frame-shift mutant strain (lanes 3 and 6). DNA fragments were detected with the labeled 0.65 kb *Eco*RI fragment of pAMI1. Fragment sizes are indicated at the left.

Physiological studies showed, that both mutations resulted in a complete loss of the ability to grow on methylamine. Neither of them effected heterotrophic growth or growth on methanol. Additional analysis of both mutants with respect to methylamine dehydrogenase activities was not done, since it appeared to be impossible to induce the genes involved in methylamine oxidation by any other way than growth on methylamine alone. This finding seems obvious, but it was found earlier that the genes involved in methanol oxidation in *P. denitrificans* were expressed not only by growth on methanol, but also on methylamine and choline. During the oxidation of all 3 substrates, formaldehyde is formed and this common metabolite was assumed to have an important function in the induction mechanism of this set of genes [13]. However, neither during growth on choline, nor during growth in the presence of both succinate and methylamine, was methylamine dehydrogenase activity observed in the *P. denitrificans* wild-type strain. These results indicate, that methylamine dehydrogenase and amicyanin expression are subject to induction by methylamine itself and to catabolite repression by heterotrophic substrates. Since both mutants were unable to grow on methylamine, no methylamine dehydrogenase activities could be determined.

The results of the experiments described here have clearly indicated, that, during growth of *P. denitrificans* on methylamine, amicyanin is indispensable for electron transport from methylamine to oxygen. Experiments are in progress to investigate the role of the inducible cytochromes c_{551} and c_{553} during methylamine

oxidation in order to elucidate the alternative electron transfer route from methylamine dehydrogenase to the *aa*$_3$-type oxidase in the absence of cytochrome c_{550} but in the presence of amicyanin.

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