Characterization and Nucleotide Sequence of pqqE and pqqF in Methylobacterium extorquens AM1

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Methylobacterium extorquens AM1 pqqEF are genes required for synthesis of pyrroloquinoline quinone (PQQ). The nucleotide sequence of these genes indicates PqqE belongs to an endopeptidase family, including PqqF of Klebsiella pneumoniae, and M. extorquens AM1 PqqF has low identity with the same endopeptidase family. M. extorquens AM1 pqqE complemented a K. pneumoniae pqqF mutant.

Pyroloquinoline quinone (PQQ) is a prosthetic group required by several bacterial dehydrogenases, including methanol dehydrogenase (MDH) of gram-negative methylotrophs and some glucose dehydrogenases (3). PQQ is derived from two amino acids, tyrosine and glutamic acid (12, 24), but the pathway for its biosynthesis is unknown (5, 18).

Genetic studies of PQQ biosynthesis have been performed with a number of methylotrophs: Methylobacterium extorquens AM1 (14, 15), Methylobacterium organophilum DSM760 and XX (1, 14), and Methylobacillus flagellatum (8). In these organisms, the genes for PQQ biosynthesis are required for growth on methanol. Seven PQQ complementation groups, in two clusters, have been identified in M. extorquens AM1: PqqDGCB and PqqEF (14). The nucleotide sequence has been reported for pqqDG and part of pqqC in M. extorquens AM1 (14). Genes for PQQ biosynthesis have also been cloned and sequenced in other organisms that produce PQQ-linked glucose dehydrogenase. In Klebsiella pneumoniae six genes have been identified, pqqABCDEF (13), the first five of which correspond, respectively, to pqqDGCB of M. extorquens AM1 (14). The same five genes, in the same gene order, also exist in Acinetobacter calcoaceticus (9). The product of the first gene is a peptide of 22 to 29 amino acids that contains conserved tyrosine and glutamic acid residues and is believed to be the peptide precursor from which PQQ is derived (10, 13, 25). The products of the other four genes, pqqGCBA in M. extorquens AM1 and pqqBCDE in K. pneumoniae, show no homology with other proteins, and their respective roles in PQQ biosynthesis are unknown (9, 13, 14). A recent study with K. pneumoniae demonstrated that the product of pqqC is required for the final step in the biosynthesis of PQQ, although the biochemical nature of that step was not determined (25). The only pqq gene product that is homologous to a protein of known function is PqqF of K. pneumoniae, which shares identity with a family of endopeptidases (13). It has been suggested that this protein is involved in processing of the peptide precursor during the biosynthesis of PQQ (13).

In this study we present complementation analyses and nucleotide sequence of pqqEF of M. extorquens AM1. M. extorquens AM1 pqqE is functionally equivalent to the PqqF endopeptidase of K. pneumoniae. The putative product of M. extorquens AM1 pqqF also shares some identity with endopeptidases.

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All pqqE mutants (EMS20 and EMS7-17) and pqqF mutants (EMS7-27, EMS7-35, and EMS7-42) were derived from rifamycin-resistant M. extorquens AM1 (15). The K. pneumoniae pqqF mutant, KA216 (pAMH62psi103 pqqF17::Tn10 Kan*), is a derivative of NCTC418 and was provided by P. W. Postma (University of Amsterdam, Amsterdam, The Netherlands). Escherichia coli DH5α was used for DNA manipulation (BRL, Inc.). Plasmids used for complementation of M. extorquens AM1 mutants (Fig. 1) are subclones of pELH2 (14), inserted into pRK310 (Tc’ IncP1) (4). pELH2::TphoA-E1 is described by Morris et al. (14), and a corrected map of the insert in this plasmid is shown in Fig. 1. Other plasmids used for complementation in K. pneumoniae, pEL41(HINDIII-CD), pDN102 (HINDIII-C), and pDN202(HINDIII-E), have been described (14, 15). The mobilizing plasmid used for conjugation was pRK2073 (Sm’) (6). Plasmids used for sequencing were derivatives of pELH2 inserted into pUC19 (26).

M. extorquens AM1 strains were grown at 30°C on minimal medium described previously (7), containing 0.2% (vol/vol) methanol, 0.2% (wt/vol) methylamine, and 0.2% (wt/vol) succinate. K. pneumoniae KA216 was grown at 30°C in minimal medium A (20) with vitamins (22) or on EMB agar (Difco) supplemented with 0.2% (wt/vol) glucose or 0.2% (wt/vol) gluconate. E. coli was grown in L broth (19). Antibiotics were added at the following final concentrations (in milligrams per liter): ampicillin, 100; tetracycline, 12.5; kanamycin, 25; and streptomycin, 10. Bacto-agar, EMB agar, and Peptone were added at the following final concentrations (in milligrams per liter): ampicillin, 100; tetracycline, 12.5; kanamycin, 25; and streptomycin, 10. Bacto-agar, EMB agar, and Peptone were obtained from Difco Laboratories. Chemicals were obtained from Sigma Co.

Plasmids were transferred into M. extorquens AM1 strains by conjugal transfer by using triparental matings as described previously (21). Plasmids were transferred into K. pneumoniae KA216 in a similar manner, except that strains were spotted onto Peptone agar plates (Peptone, 10 g/liter; K2HPO4, 2 g/liter) and transconjugants were plated onto minimal medium A containing either glucose or gluconate and onto glucose EMB agar, each supplemented with tetracycline and kanamycin to select for, respectively, the conjugated plasmid and the Kanr insertion in KA216. For selection of pELH2::TphoA-E1 transconjugants in KA216, kanamycin and ampicillin were used to select for, respectively, the TphoA and an Amp’ plasmid in KA216. Recombinational rescue was scored as described previously (21). Construction of subclones was performed following the methods of Sambrook et al. (19), or as described previously (14, 21). Enzymes were obtained from New England Biolabs and Boehringer-GmbH (Mannheim, Germany).
Sequence was obtained from pqqEF subclones by using the M13 forward and reverse primers designed for pUC19 (U.S. Biochemicals) and by using single-stranded oligonucleotide primers synthesized by the Caltech Microsynthesis facility. Sequencing reactions were performed at the University of California at Los Angeles Sequencing Facility or using Dye Terminator cycle-sequencing kit (Applied Biosystems, Inc.) and by using single-stranded oligonucleotide M13 forward and reverse primers designed for pUC19 (U.S. Biochemicals) and by using single-stranded oligonucleotide M13 forward and reverse primers designed for pUC19 (U.S. Biochemicals).

Complementation analyses of PqqE and PqqF. The complementation groups PqqE and PqqF were previously mapped to the 4.1-kb fragment in pELH2 (14). To further localize these complementation groups, subclones of pELH2 were constructed. Complementation was scored as the ability to restore growth of the respective mutant on methanol; an r in the complementation table indicates recombinational rescue. The open reading frames are indicated at the bottom of the figure as large arrows with gene designations. Abbreviations: B = BamHI, H = HindIII, P = PstI.

**TABLE 1. Complementation of K. pneumoniae KA216(pAMH62/ptsI103, pqqF17/ptyD Kan') with M. extorquens AM1 DNA**

| M. extorquens | M. extorquens | Color of KA216 on EMB glucose |
|---------------|---------------|-----------------------------|
| AM1 clone     | AM1 gene(s)   |                             |
| pELH2         | pqqE          | Purple                      |
| pELH2::TphoA-E1 | pqqF          | White                       |
| pEL41         | pqqDGCA       | White                       |
| pDN102        | pqqCB         | White                       |
| pDN172        | mscQE         | White                       |

Plates (Table 1), indicating that pELH2 is capable of restoring POQ-linked glucose dehydrogenase activity in KA216. Negative controls, KA216 containing M. extorquens AM1 plasmids with mox (genes required for methanol oxidation) and other pqq genes, pEL41, pDN102, and pDN202, did not show a color change on EMB glucose plates (Table 1). The KA216 transconjugants containing pELH2 were unable to grow on minimal glucose plates, suggesting that POQ activity is not fully restored in these strains. It is possible that this is due to differences in expression from the M. extorquens DNA or because of a low degree of identity between the proteins in the respective organisms. To determine which of the two pqq genes in pELH2 was responsible for complementation, pELH2::TphoA-E1 was introduced into KA216. This plasmid contains an insertion in pqqE but expresses functional pqqF (14). Transconjugants containing pELH2::TphoA-E1 were white on EMB-glucose plates, indicating that M. extorquens pqqE is the gene that is functionally equivalent to K. pneumoniae pqqF.

**Nucleotide sequence of pqqEF and surrounding region.** The sequence of the pELH2 insert revealed three intact open reading frames and one partial open reading frame in the direction shown from left to right in Fig. 1. The first open reading frame begins 171 bp from the leftmost HindIII site and encodes a putative product of 219 amino acids. This putative product of orf219 does not have significant identity to any known proteins.

The second open reading frame begins 972 bp into the sequence, 142 bp downstream of the end of orf219. This open reading frame encodes a putative product of 709 amino acids and extends across the PstI site, indicating that this is pqqE. The predicted M. extorquens AM1 PqqE shares identity with a superfamily of divergent cation-containing endopeptidases, showing 22% identity over 414 amino acids with the mitochondrial processing peptidase β-subunit (MPP) from *Neurospora crassa* (GenBank accession number M20928). The identity is highest in the N-terminal portion of the protein, including the conserved His-X-X-Glu-His sequence believed to be involved in metal binding, and a conserved Glu required for catalysis (16, 17). There is also extensive identity to a putative MPP-like peptidase of *Mycobacterium fortuitum* (29% over 399 amino acids; GenBank accession number L25634), to *E. coli* pitrilysin (25% over 227 amino acids; GenBank accession number M17095), and to several insulin-degrading endopeptidases (IDEs; GenBank accession numbers X67269, M58465, and M21188). M. extorquens AM1 PqqE shows 24% identity over 237 amino acids to *K. pneumoniae* PqqF, providing further evidence that *K. pneumoniae* PqqF and *M. extorquens* AM1 PqqE are homologs. Many members of this endopeptidase family are involved in processing small peptides (17). Phylogenetic analysis suggested that *M. extorquens* AM1 PqqE is related to the MPP subfamily, whereas *K. pneumoniae* PqqF is fairly distant from the known subfamilies but is most closely related to the pitrilysin/IDE subfamily (17) (Fig. 2). Members of the MPP subfamily form heterodimers with a second subunit, α, that shares a high degree of identity with the first

**FIG. 1. Restriction map and complementation analysis of the pqqEF region using derivatives of pELH2 as indicated. Small arrows indicate direction of the lacZ promoter on the pRK310 vector, where determined. The filled triangle represents the TphoA-E1 insertion. Complementation was scored as the ability of the clone to restore growth of the respective mutant on methanol; an r in the complementation table indicates recombinational rescue. The open reading frames are indicated at the bottom of the figure as large arrows with gene designations. Abbreviations: B = BamHI, H = HindIII, P = PstI.**
of the MPP and Pitrilysin/IDE subfamilies, open reading frame in a second subunit.

 accession numbers for these sequences are listed in the text, except for PQQF with the C-terminal portion of M. extorquens.

 mutants of K. pneumoniae pqqE is the functional equivalent of K. pneumoniae pqqF with respect to PQ biosynthesis. Sequence analysis indicates that M. extorquens AM1 pqqE encodes a member of a family of endopeptidases. K. pneumoniae PqqF is also a member of this family, although the M. extorquens AM1 and K. pneumoniae proteins are members of different subfamilies. These peptidases are likely to be involved in processing the template peptide during PQ biosynthesis. The putative product of M. extorquens AM1 pqqF shares some identity with two members of the endopeptidase family and may serve as a noncatalytic subunit associated with PqqE. These results suggest that M. extorquens AM1 and K. pneumoniae use divergent systems for processing the template peptide.

This work was supported by a grant from the Department of Energy (DEFG03-87ER13757).
We thank P. W. Postma for providing *K. pneumoniae* KA216 and for advice on the indicator medium.

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