GENETICS AND REGULATION OF PYRROLOQUINOLINE QUINONE (PQQ) BIOSYNTHESIS IN THE METHYLOTROPHIC BACTERIUM *METHYLOBACTERIUM EXTORQUENS* AM1

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Roopa Ramamoorthi

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Let us be up and doing
With a heart for any fate
Still achieving, still pursuing,
Learn to labor and to wait

ABSTRACT

Pyrroloquinoline quinone (PQQ) has potential applications in the treatment of Alzheimers disease and jaundice. PQQ is produced and secreted in large amounts by methylotrophic bacteria, and they have potential for commercial PQQ production.

PQQ is the prosthetic group in methanol dehydrogenase of the facultative methylotroph, *Methylobacterium extorquens* AM1. Previous work had shown that seven genes were required for PQQ production in *M.extorquens* AM1. Dr. Christina Morris in our lab had sequenced the first two genes, *pqqD* and *pqqG*. *pqqD* encoded a twenty-nine amino acid peptide containing tyrosine and glutamate separated by three amino acids. Tyrosine and glutamate had been shown to be the precursors of PQQ, and this peptide is believed to be the precursor of PQQ biosynthesis in vivo.

This thesis was initiated by mapping the transcriptional start site in front of *pqqD*. A high abundance 240 base pair transcript containing *pqqD* and a low abundance 1300 base pair transcript containing both *pqqD* and the next gene *pqqG* were detected using RNA-DNA blots. This was supported by data with Tn*5lac* insertions.

Studies were carried out to understand the regulation of PQQ biosynthesis in more detail. It was found that the products of the regulatory genes *moxM, moxD* and *moxB* were required for induction of *pqqD* on methanol plus methylamine compared to succinate, while the products of the regulatory genes *moxQ* and *moxE* were not required. The products of *moxM* and *moxD* were also required for high level transcription of *pqqD*. Measurements of PQQ

in the culture supernatants of wild-type and mutants indicated that transcription of *pqqD* was not the rate limiting step in PQQ biosynthesis, and in addition that regulation of PQQ biosynthesis occurs at more than one step in the process.

Using functional complementation it was found that *pqqE* of *M.extorquens* AM1 was equivalent to *pqqF* of *K.pneumoniae*. The middle portion of *pqqE* was sequenced and by aligning PqqE with PqqF of *K.pneumoniae*, the direction of transcription and size of *pqqE* was determined. PqqF shows similarity to proteases and this strongly suggests that a protease is involved in PQQ biosynthesis.

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Chapter 1

Introduction

Introduction

Methylotrophic bacteria are organisms which are capable of using carbon substrates with no carbon-carbon bonds as their sole source of carbon and energy. This includes bacteria capable of growing on methanol, methylamine, methylated glycines and methylated sulfur species. These bacteria are widespread in nature, are found in aquatic and terrestrial habitats and play an important role in carbon cycling in specific habitats. [22,23]

Most methylotrophs are gram negative but some gram positive methylotrophs also exist. Many of the known strains of methylotrophic bacteria are obligate methylotrophs and are not capable of growing on any compounds with carbon-carbon bonds. Among bacteria capable of growing on methanol there exist some that are facultative methylotrophs that are capable of growing on either one-carbon or multi-carbon compounds. [22]

Methylotrophs can be divided into functional groups depending on whether or not they can utilize methane (methanotrophs) and those capable of growth on methanol and other methylated compounds but not on methane. Some methylotrophic bacteria are capable of using dinitrogen as a nitrogen source and are called diazotrophs. Several methylotrophs also affect the nitrogen cycle by carrying out transformations of ammonia and nitrate. Some methylotrophs are also capable of using methylated sulfur species and play a role in sulfur cycling. [22] (Refer to Table I for detailed classification of methylotrophs.)

The ability to grow on reduced, one-carbon compounds requires the presence of special biochemical pathways for carbon and energy metabolism.

There are three main pathways for carbon assimilation, the serine cycle, the ribulose monophosphate (RuMP) pathway and the ribulose bisphosphate (RuBP) pathway (Calvin Benson cycle). Those bacteria using the serine cycle generally have a high GC content (65-69 mole%) while those that use the RuMP pathway have a GC content of 50-55 mole%. The methylotrophs that oxidize these substrates to CO_2 and then assimilate this by the classical Calvin Benson cycle are called autotrophic methylotrophs [22] (See Figure 1.)

The organisms that grow on one carbon compound as the source of carbon and energy convert this into formaldehyde, which is then either assimilated into the cell or is converted into CO_2 with the generation of ATP and $NADH_2$.The assimilation pathways are described above. There exist two possibilities for the oxidation of formaldehyde to CO_2 in methylotrophic bacteria. One of these is a linear pathway that involves the sequential action of the enzymes, formaldehyde dehydrogenase and formate dehydrogenase. The linear pathway of formaldehyde oxidation is used in methylotrophs that use the serine cycle for assimilation and also in many facultative methylotrophs containing the RuMP cycle. The second pathway is cyclic and involves the condensation of the C-1 compound with a five-carbon acceptor molecule, followed by oxidation of the resulting six-carbon compound. The enzymes carrying out these reactions are those of the ribulose monophosphate cycle for assimilation, with the exception of one novel enzyme, the 6-phosphogluconate dehydrogenase. The cyclic pathway of formaldehyde oxidation occurs mainly in the obligate methylotrophs containing the RuMP cycle. [22]

***Methylobacterium extorquens* AM1**

The methylotrophic bacterium used in this study is *Methylobacterium extorquens* AM1, which is a pink pigmented, gram negative facultative methylotroph belonging to the α–proteobacteria group of eubacteria. *M.*

extorquens AM1 is capable of growing on methanol, methylamine and methylated glycines as well as multi-carbon compounds such as succinate. It uses the serine cycle pathway for carbon assimilation [22,23] (See Figure 2 for details of the serine cycle pathway.) In the serine cycle pathway, 2 moles of formaldehyde and 1 mole of CO_2 are used and one mole of a 3 carbon compound is generated, which is assimilated by the cell.

Genetic Techniques in *M.extorquens* AM1

A number of genetic techniques have been developed for use in *M.extorquens* AM1. Broad host range vectors of the incompatibility group P1 replicate in this organism. These vectors have a copy number of about five to ten per cell. The common vectors used are

pVK100, which is a cosmid vector of size 23.0 kb with kanamycin and tetracycline resistance markers and having an IncP1 origin of replication. The useful cloning sites on this vector are *Hin*d III, *Sal* I and *Xho* I.[23]

pRK310 which is a common subcloning vector used. It has a size of 20.4 kb, a tetracycline resistance marker, and has an IncP1 origin of replication. The useful cloning sites are *Bam*H I, *Hin*d III and *Pst* I. It also has the *lacZ* gene, which can be used for screening colonies. [23]

Transformation does not work well in this organism and plasmids are introduced by conjugation using triparental matings. In triparental matings the strain containing the plasmid to be transferred, the mobilizer strain having a plasmid with the mob$^+$(mobilization genes) on it and an *M.extorquens* AM1 recipient are plated together on a filter or nutrient agar plate. The conjugation is allowed to proceed overnight and then the mated mixture is plated on medium with appropriate antibiotics to select for the plasmid and rifamycin to select for *M.extorquens* AM1. The common plasmid used for mobilization is pRK2073 which has a ColE1 origin of replication and the RK2 transfer system. [23]

Mutant Isolation-Over 200 mutants in the methanol oxidation pathway have been isolated in the Lidstrom laboratory using the allyl alcohol positive selection method. In this method allyl alcohol was added to the medium along with methanol, which was used to induce the methanol oxidation system, and either succinate or methylamine were used for growth of the bacteria. After mutagenesis of a pool of bacteria with ultraviolet light or EMS, the pool was plated on this medium. Organisms with a functional methanol oxidation system oxidized allyl alcohol to acrolein, which is a suicide substrate. Only those organisms which were impaired in one or more of the genes involved in methanol oxidation were able to grow. In this way twenty-one classes of methanol oxidation mutants were isolated. [21, 33, 34]

Methanol Dehydrogenase

In order to grow on methanol and methylamine, the methylotrophs require an enzyme for oxidizing methanol. The methanol dehydrogenase is a periplasmic enzyme with an $\alpha 2\ \beta 2$ structure, and it has pyrroloquinoline quinone (PQQ) as its prosthetic group. The size of the large subunit is 60 kilodaltons and the size of the small subunit is 10 kilodaltons. 2 moles of PQQ are present per tetramer and the enzyme also contains tightly bound calcium. The natural electron acceptor for methanol dehydrogenase is cytochrome c_L, a class of *c*-type cytochromes which is distinctive in having a low isoelectric point (pI=3-4) , large size (17kDa molecular mass) and little sequence similarity to other bacterial c-type cytochromes. [20, 32] (Refer to Figure 3.)

In *M.extorquens* AM1 the structural genes for the α and β subunits of methanol dehydrogenase, *moxF* and *moxI* are located in a single gene cluster along with two additional genes, *moxJ* and *moxG,* and are arranged in the order *moxFJGI. MoxG* encodes cytochrome c_L, the electron acceptor of methanol dehydrogenase. [20]

The *moxF* gene codes for the large (alpha) subunit of methanol dehydrogenase. It has been cloned from several methylotrophs, including *Methylobacterium* strains, *Paracoccus denitrificans* and some methanotrophs. The *moxF* gene appears to be highly conserved between these organisms. 96% similarity at the amino acid level was observed between the *moxF* sequences of *M. extorquens* AM1 and *Methylobacterium organophilum* XX. 82% similarity was found when these were compared to the *P. denitrificans moxF* sequence. Similarity at the amino acid level means identical residues or conserved substitutions. The *moxG* and *moxI* genes have also been cloned from *P. denitrificans.* The *moxI* gene codes for the small (beta) subunit of methanol dehydrogenase. [20]

The crystal structure of methanol dehydrogenase of *Methylophilus* W3A1 has recently been determined.[44] The structure showed that each large subunit consisted of a single disk-shaped barrel and each small subunit consisted of an extended N-terminal peptide segment and C-terminal helix which were wrapped around a portion of the outside surface of the large subunit. The PQQ cofactor was located in a central channel of the disk shaped protein and was sandwiched between a tryptophan side chain and a very unusual disulfide bridge. In the plane of the PQQ, a Ca^{++} ion was bridged between it and the protein molecule. The disulfide probably forms during PQQ incorporation and holds it in place. In the tetramer, a pair of $\alpha\beta$ subunits are placed so that the axes of symmetry are roughly perpendicular and the active sites are separated by about 45 Angstroms. [44]

Pyrroloquinoline Quinone (PQQ)

In 1967, Anthony and Zatman reported that the prosthetic group of alcohol dehydrogenase from *Psuedomonas* sp. M27 was unusual. Subsequently other methanol dehydrogenases were identified with unusual

cofactor characteristics. Forrest et al. [10] isolated an acid-stable adduct of the cofactor and used X-ray diffraction on the crystal to find the structure. They called it methoxatin and it is now most often referred to as pyrroloquinoline quinone (PQQ). PQQ is commercially available from Fluka. [10, 37] (For the structure of PQQ ref. toFigure 4.)

Physical Properties of PQQ -PQQ is a brick red compound that dissolves easily in water and the solution shows a green fluorescence, which is dependent on temperature. The reason is that PQQ in water-containing solutions is partly hydrated and two species (PQQ and PQQ-H_2O) exist. Lowering the temperature shifts the equilibrium in favor of the fluorescing species PQQ-H_2O. The molar absorption coefficient of a solution at pH7 at 342nm is 9,620 M-1 cm-1. [9]

Redox potential- The redox potential of the couple PQQ/ $PQQH_2$ at pH7.0 is +90 mV. [9]

Chemical reactivity-PQQ can be reduced by a large number of compounds to PQQH or $PQQH_2$. The C-5 carbonyl group of PQQ is very reactive towards nucleophilic agents. Reversible adduct formation takes place with H_2O, alcohols, urea and cyanide. [9]

Inhibitors-Organic cations like phenazine methosulfate (PMS) and iodonium compounds selectively inhibit the redox cycling of PQQ. The inhibition is released by thyroxine and Tiron (4,5-dihydroxy-1,3-benzene disodium sulfonate). [9]

Quinoproteins

Quinoproteins are a novel class of oxidoreductase enzymes which contain quinone cofactors or prosthetic groups. One class of these contains PQQ. PQQ has been demonstrated convincingly to be the prosthetic group in several bacterial enzymes, namely methanol dehydrogenase, ethanol

dehydrogenase, glucose dehydrogenase, glycerol dehydrogenase and polyethylene glycol dehydrogenase. [4, 5, 9, 12, 13, 25] Initially it was reported that PQQ was the covalently-bound cofactor in soybean lipoxygenase and bovine serum amine oxidase. [41] However, the identification was indirect and involved a procedure of derivatization with phenylhydrazine or hexanol, separation on an HPLC and detection by peak fluorescence. When direct analysis of the peptide containing the cofactor was carried out with Bovine Serum Amine Oxidase using mass spectrometry and proton nuclear magnetic resonance, it was found that the cofactor was 6-hydroxydopa and not PQQ. [18] Similarly, native soybean lipoxygenase samples failed to show formazan formation in the redox cycling assay using nitroblue tetrazolium /glycinate reagent, which detects PQQ in the oxidized as well as reduced form. [36, 43] A controversy exists about the existence of PQQ in biological tissues and fluids. When PQQ was isolated and analyzed using gas chromatography and mass spectrometry, only trace amounts of PQQ were detected. However, the level of PQQ could have been underestimated with this method, because is does not detect adducts between PQQ and amino acids. When the redox cycling assay was used with the addition of specific inhibitors of PQQ and agents which release this inhibition, it was found that PQQ in biological tissues was present in nanomolar concentrations. The redox cycling method detected both free PQQ and PQQ adducts. [11]

Some organisms like *M.extorquens* AM1, *Klebsiella pneumoniae* and *Acinetobacter calcoaceticus* are able to synthesize PQQ. *Escherichia coli* cannot normally synthesize PQQ, but it makes an apoenzyme glucose dehydrogenase which can be reconstituted into active holoenzyme in the presence of externally added PQQ. [15] There is evidence that *E.coli* contains some of the genes required for PQQ biosynthesis, as *Methylobacterium*

organophilum DSM760 *pqqE* and *pqqF* mutants can be complemented by *E.coli* chromosomal DNA. [39] Also an *E.coli* mutant has been isolated that synthesizes PQQ. [6] In the bacterial PQQ-linked quinoproteins the PQQ is tightly attached but is not covalently bound. [4, 5, 7, 12, 13, 24, 25, 26, 27]

Biosynthesis of PQQ

The amino acids glutamate and tyrosine have been shown to be the precursors of PQQ biosynthesis. *M. extorquens* AM1 was grown on [1-^{13}C] or [2-^{13}C] ethanol and the resulting ^{13}C enrichments in PQQ were compared to the labeling patterns in the amino acids using ^{13}C NMR. [16] These data indicated that PQQ was synthesized from one molecule of glutamate and either one molecule of tyrosine or phenylalanine. Direct incorporation of tyrosine was then observed using [^{13}C] tyrosine labeled in the phenol side chain and at the methylene position to confirm that tyrosine was the precursor. [16] *M.extorquens* AM1, *K.pneumoniae* and *A. calcoaceticus* synthesize small peptides of 24 to 29 amino acids which contain tyrosine and glutamate at conserved positions separated by three amino acids. It has been proposed that these peptides could be the precursors of PQQ biosynthesis. Mutants that are defective in the genes encoding these peptides, do not synthesize PQQ. [13, 14, 26, 27] (Ref. to Figure 4.)

Methods of PQQ Production. Chemically, PQQ was synthesized by Corey and Tramontano using 2-methoxy-5-nitroaniline as the starting material, and an overall yield of 20% was obtained in a ten step procedure. [9, 37]

PQQ can be produced using methylotrophic bacteria, which synthesize and secrete large amounts of PQQ to the medium. The production of PQQ by *Hyphomicrobium* X (a methylotrophic bacterium) is 3000 to 6000 nanomoles per liter and that of another methylotroph, *M. organophilum* XX is 2000 to 8000 nanomoles per liter when grown on methanol. In contrast, the PQQ production

by *K. pneumoniae* when grown on glucose is less than 2 nanomoles per liter and that of *A. calcoaceticus* is 50 to 200 nanomoles per liter when grown under inducing conditions. [42]

Factors Affecting PQQ Production by Bacteria-When PQQ production was investigated using a strain of *Hyphomicrobium* , it was found that PQQ levels in the culture broth were increased by decreasing the amount of iron added to the medium. [40] It was found that there was no feedback inhibition of PQQ biosynthesis when PQQ was added to the medium. This was observed in the case of both *Hyphomicrobium* X and *A.calcoaceticus.* When amino acids were present in the culture medium (e.g., from peptone or yeast extract) they formed adducts with PQQ, rendering it biologically inactive. [40, 42]

Commercial Applications of PQQ

Chemical Reactivity- PQQ reacts readily with amines and amino acids.[17] The ability of PQQ to add to nucleophiles is in part related to the location of the pyridine nitrogen that is peri to C-5 carbonyl in PQQ. The redox capabilities of the dicarbonyl functional center and the ability to react with primary amines provide PQQ with properties that are analogous to combining some of the best chemical features of riboflavin, ascorbic acid and pyridoxal cofactors into one molecule. [37] PQQ is able to carry out redox cycling so that picomole amounts of PQQ are capable of generating micromolar amounts of product. [37] It was shown that PQQ in the absence of an enzyme can foster many oxidation reactions such as the catalytic oxidation of amines. [35] In vitro, PQQ also catalyzes the oxidative decarboxylation of α-amino acids to give the corresponding aldehydes under aerobic conditions. PQQ is gradually deactivated by conversion into oxazolopyrroloquinoline derivatives. [35] In the reaction with the β-hydroxy amino acids tyrosine and tryptophan, oxidative

dealdolation proceeds effectively(Cα-Cβ fission). PQQ has substantial potential as a therapeutic agent and hence understanding the reactivity of PQQ towards amines and amino acids is of great importance.

Pharmaceutical Applications-The effect of PQQ and PQQ oxazole (PQQ-glycine adduct) on DNA synthesis of cultured human fibroblasts was investigated using ^{3}H thymidine incorporation as a measure of DNA synthesis. It was found that DNA synthesis was enhanced in the presence of 0.003 to 30 μM PQQ and was slightly enhanced at concentrations of 15-750μM PQQ-oxazole. The potency of PQQ in stimulating proliferation of fibroblasts was comparable to that of epidermal growth factor and was much higher than that of fibroblast growth factor or insulin growth factor. [28] PQQ could be used in cell culture to enhance cell proliferation and perhaps might replace epidermal growth factor in certain applications.

When PQQ is omitted from chemically defined diets, growth impairment is observed in mice and rodents, if the diet is introduced prior to neonatal development. A normal growth response is observed when PQQ is added to the deficient diet, suggesting that PQQ may be an essential nutrient required for prenatal growth. [37]

Administration of PQQ to fertile chicken eggs has been shown to protect against the formation of lens cataract in developing embryos treated with hydrocortisone. Hydrocortisone causes decreased levels of reduced glutathione which allows oxidative damage to occur in the lens. PQQ protects against this phenomenon. [37]

When hydrocortisone hemisuccinate sodium was administered to fertilized hens eggs it caused accumulation of biliverdin in the embryonic liver. This accumulation was effectively prevented by PQQ, possibly through the enhancement of biliverdin excretion from the liver to the gall bladder. This PQQ

action was due to its preventive effect against the decrease of glutathione in the liver caused by glucocorticoid, since glutathione is suggested to play a role in the elimination of bile components from the liver. The understanding of bile movement in the liver and finding agents for treating dysfunctions of bile excretion from the liver are extremely important in the treatment of jaundiced patients. [30] PQQ may be useful in the treatment of jaundiced patients by enhancement of liver function.

PQQ has been shown to be able to interact with the redox modulatory site of N-methyl-D-aspartate (NMDA) receptor in cortical neuron cells in vitro. PQQ oxidizes the NMDA redox receptor site and is able to reduce its activity. Pathological activation of NMDA receptors have been implicated in various central nervous system disorders. Hence PQQ may have use as a therapeutic agent in these cases by decreasing NMDA receptor function via its redox modulatory site. [1]

PQQ was found to increase the production of nerve growth factor in vitro in an L_M cell line that was known to be a nerve growth factor (NGF) producing cell line. It was observed that oxazopyrroloquinoline quinone (OPQ) increased the NGF content in rat brains in vivo, probably by its conversion to active PQQ. PQQ itself stimulated peripheral nerve growth in vivo but did not increase NGF content in rat brains, possibly because PQQ exists complexed with proteins and cannot go through the blood-brain barrier. NGF functions as a neurotrophic molecule for the magnocellular cholinergic neurons in basal forebrain nuclei, which are specifically lost during Alzheimer's disease and are supposed to be involved in memory and learning processes. Hence OPQ could possibly serve as an anti-dementia drug. [45]

Biosensor Applications -Quinoprotein dehydrogenases like aldose and fructose dehydrogenases have been used for the detection of glucose, xylose or fructose in biosensor applications. [3, 19, 38]

Applications of Methylotrophic Bacteria

Methanol Utilizers- The biosynthesis of compounds labeled with the stable isotope ^{13}C is an example in which methylotrophic metabolism is superior to that of heterotrophic organisms. The natural abundance of ^{13}C is approximately 1%. Cryogenic distillation of ^{13}C-carbon monoxide is the method of choice for the enrichment of ^{13}C. ^{13}C labeled carbon monoxide is converted to $^{13}CH_3OH$ chemically, and is then fed to methylotrophs. ^{13}C labeled methanol can then be easily converted by methylotrophs to ^{13}C labeled products such as ^{13}C sugars, amino acids, etc. These materials are of clinical value in magnetic resonance spectroscopy and ^{13}C carbon dioxide based diagnostic breath tests. [23]

Methanol utilizing bacteria are found associated with the leaves of green plants. Recently it was shown that foliar sprays of aqueous methanol (10-50%) increased growth and development of C_3 plants that were maintained under direct sunlight. C_3 plants under direct sunlight photorespire and produce glycolate, which is toxic to the plants. It is possible that the methylotrophic bacteria might utilize this glycolate although there is no proof that this actually happens. [8,31]

Commercially patented processes exist for the production of single cell protein (SCP) using methylotrophs but it did not compare favorably economically with other methods for the production of single cell protein. [22] However there has been an interest in this of late as it was found that when bacterial single cell protein was fed to poultry, the meat obtained was lower in fat compared to when conventional sources of single cell protein were used.

Conclusions-PQQ appears to be an interesting cofactor with novel properties and potential pharmaceutical and biosensor applications. It is likely that there will be a larger market for PQQ in the future and in that case methylotrophic bacteria would become the organisms of choice to produce PQQ, as they excrete large amounts of PQQ into the medium. Hence it becomes important to study the genetics and regulation of PQQ production in methylotrophs so that we are able to manipulate these organisms to maximize PQQ production. *M. extorquens* AM1 was used in this project to study PQQ genetics and regulation, as mutants in PQQ production genes existed and genetic techniques have been developed in this organism.

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Table I Characteristics of aerobic methylotrophs

(this table is taken from Ref. 22)

Note: [a]-some of the methylotrophs grow on this substrate, RuMP-ribulose monophosphate, RuBP-ribulose bis phosphate

Group	Growth Substrate	Major assimilation pathway	N_2 fixing	GC content mol%	Gram reaction
Obligate	**methylo-trophs**				
Type I	methano-trophs				
Methylo-monas	CH_4, CH_3OH[a]	RuMP	No	50-54	Negative
Methylob-acter	CH_4, CH_3OH[a]	RuMP	No	50-54	Negative
Methylo-coccus	CH_4, CH_3OH[a]	RuMP	Yes	62-64	Negative
Type II	methano-trophs				
Methylo-sinus	CH_4, CH_3OH[a]	Serine	Yes	62.5	Negative
Methylo-cystis	CH_4, CH_3OH[a]	Serine	Yes	62.5	Negative
Methanol	Utilizers				
Methylo-philus	CH_3OH, CH_3NH_2	RuMP	No	50-55	Negative
Methylo-bacillus	CH_3OH, CH_3NH_2	RuMP	No	50-55	Negative
facultative	methylo-trophs				
Methylo-bacterium-	CH_3OH, CH_3NH_2, multi carbon compounds	Serine	No	65-68	Negative
Hypho-microbium	CH_3OH, CH_3NH_2, DMSO, DMS, some 2-C and 4-C compounds denitrificat-ion	Serine	No	65-68	Negative
Acetoba-cter	CH_3OH[a], multi carbon compounds	RuMP	No data	No data	Negative

Group	Growth Substrate	Major assimilation pathway	N2 fixing	GC content mol%	Gram reaction
facultative	methylo-trophs				
Xantho-bacter	CH_3OH, multi carbon compounds	RuBP	Yes	67-69	Negative
Microcyclus	CH_3OH, multi carbon compounds	RuBP	Yes	65-67	Negative
Thio-bacillus	CH_3OH[a], CH_3NH_2[a], H_2S, S_2O_3, multi carbon compounds	RuBP	No	65-69	Negative
Paracoc-cus	CH_3OH, CH_3NH_2, denitrifica-tion	RuBP	No	66	Negative
Rhodopse-udomonas	CH_3OH[a], multi carbon compounds photosyn-thesis	RuBP	No	62-72	Negative
Mycobact-erium	CH_4[a], CH_3OH[a], multi carbon compounds	RuMP	No	65-69	Positive
Nocardia	CH_3OH[a], multi carbon compounds	RuMP	No data	No data	Positive
Arthrobact-er	CH_3NH_2[a], multi carbon compounds	RuMP	No data	No data	Positive
Bacillus	CH_3OH[a], CH_3NH_2 multi carbon compounds	RuMP	No data	No data	Positive

Figure Legends

1. Organisms capable of growing on methanol have methanol dehydrogenase, which oxidizes methanol to formaldehyde. Formaldehyde is then either converted into CO_2 to generate ATP and $NADH_2$ or is assimilated into cell material. Enzymes: 1, methane monooxygenase; 2, methanol dehydrogenase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase; 5, methylated amine oxidases; 6, methylated amine dehydrogenase; 7, methylated sulfur dehydrogenase or oxidase. The methane monooxygenase to convert methane to methanol is present only in the methanotrophs. (Taken from ref. 22.)

2. The serine cycle for formaldehyde assimilation. (Taken from ref. 22.)

3. Methanol oxidation in *M. extorquens* AM1 occurs in the periplasm via the enzyme, methanol dehydrogenase (MDH). MDH is a PQQ (pyrroloquinoline quinone) linked enzyme that is coupled to a specific cytochrome c.

4. This figure shows the parts of PQQ which come from tyrosine and the portions which come from glutamate. I: glutamate, II : PQQ, III : tyrosine. (Taken from ref. 16.)

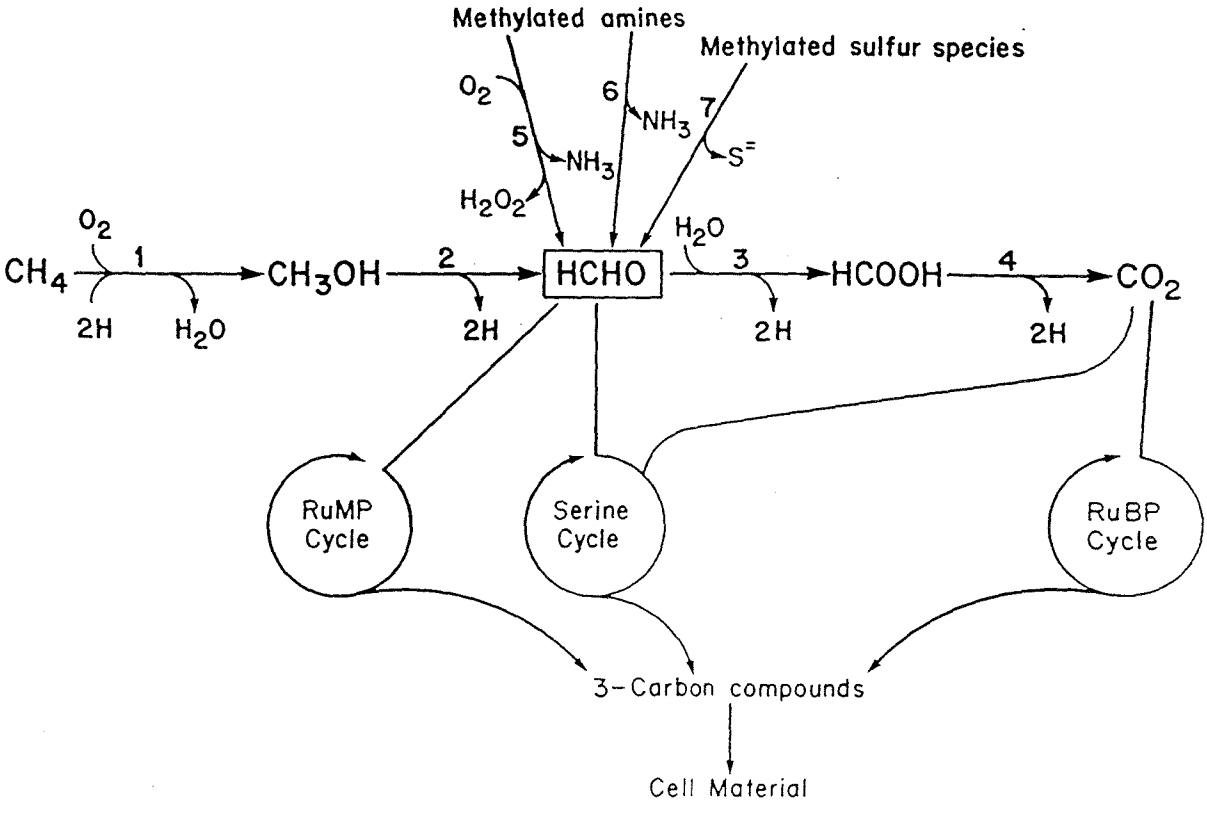
Methylated amines
Methylated sulfur species
O_2
6
NH_3
7
$S^=$
5
NH_3
H_2O_2
O_2
CH_4
1
2H
H_2O
CH_3OH
2
2H
HCHO
H_2O
3
2H
HCOOH
4
2H
CO_2
RuMP Cycle
Serine Cycle
RuBP Cycle
3-Carbon compounds
Cell Material

FIGURE 1

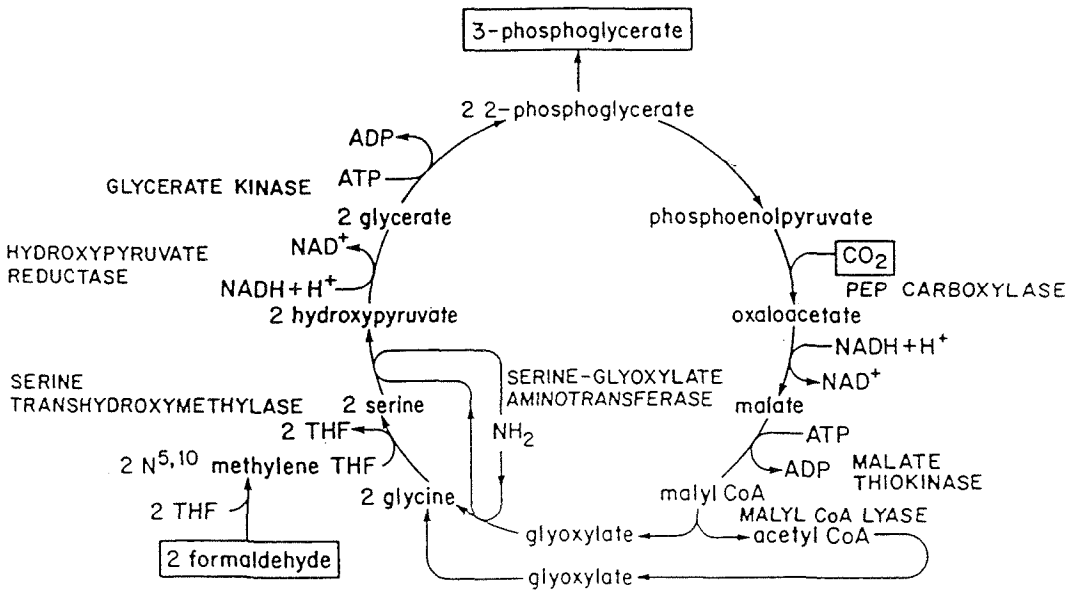
3-phosphoglycerate
2 2-phosphoglycerate
ADP
ATP
GLYCERATE KINASE
2 glycerate
phosphoenolpyruvate
HYDROXYPYRUVATE REDUCTASE
NAD+
NADH+H+
CO2
PEP CARBOXYLASE
2 hydroxypyruvate
oxaloacetate
NADH+H+
NAD+
SERINE TRANSHYDROXYMETHYLASE
2 serine
SERINE-GLYOXYLATE AMINOTRANSFERASE
malate
2 THF
NH2
ATP
2 N5,10 methylene THF
ADP
MALATE THIOKINASE
malyl CoA
2 THF
2 glycine
MALYL CoA LYASE
glyoxylate
acetyl CoA
2 formaldehyde
glyoxylate

FIGURE 2

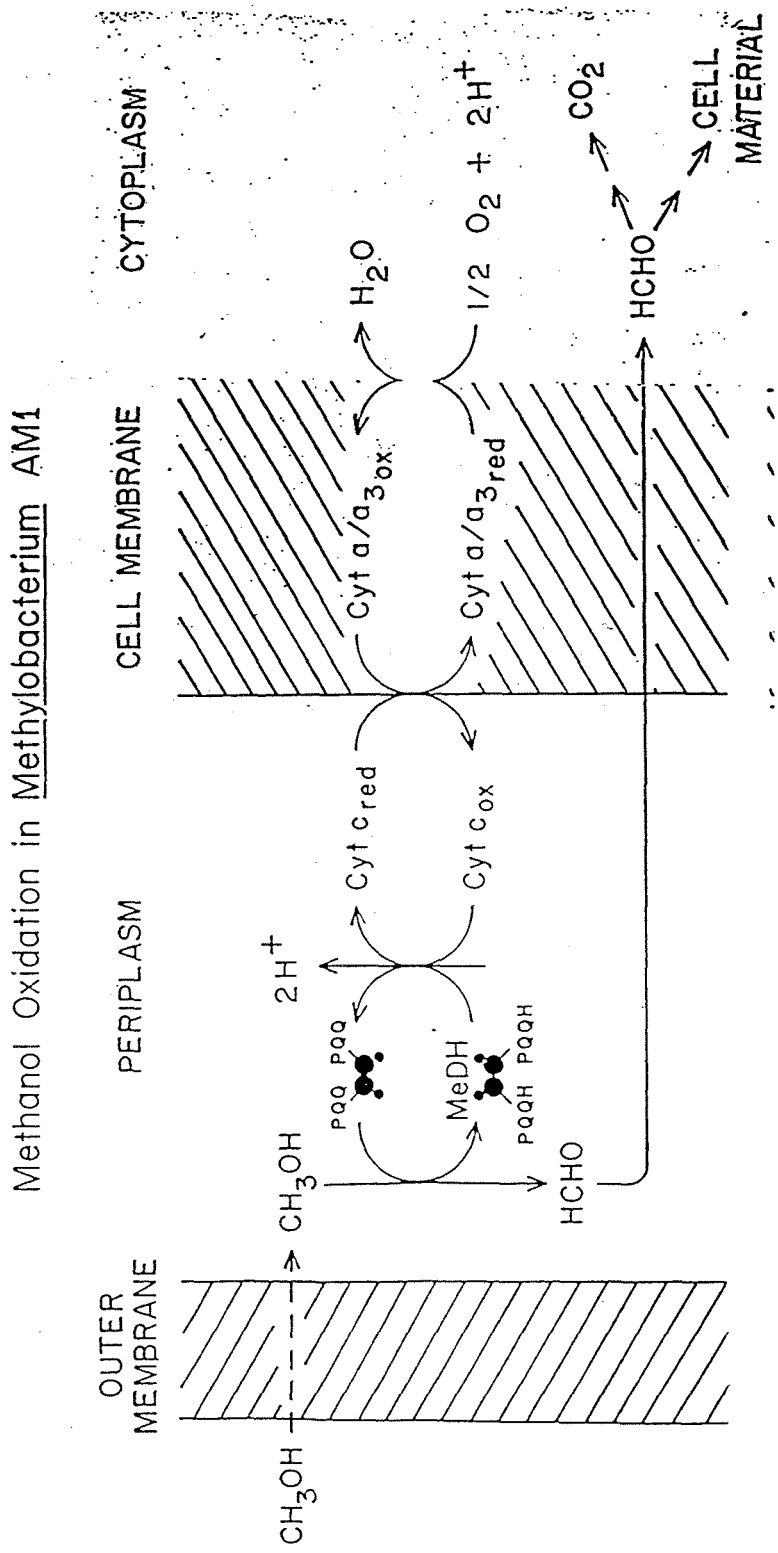
Methanol Oxidation in Methylobacterium AM1
OUTER MEMBRANE
PERIPLASM
CELL MEMBRANE
CYTOPLASM
CH_3OH
CH_3OH
PQQ PQQ
MeDH
PQQH PQQH
HCHO
HCHO
$2H^+$
Cyt c_{red}
Cyt c_{ox}
Cyt $a/a_{3_{ox}}$
Cyt $a/a_{3_{red}}$
H_2O
$1/2\ O_2 + 2H^+$
CO_2
CELL MATERIAL

FIGURE 3

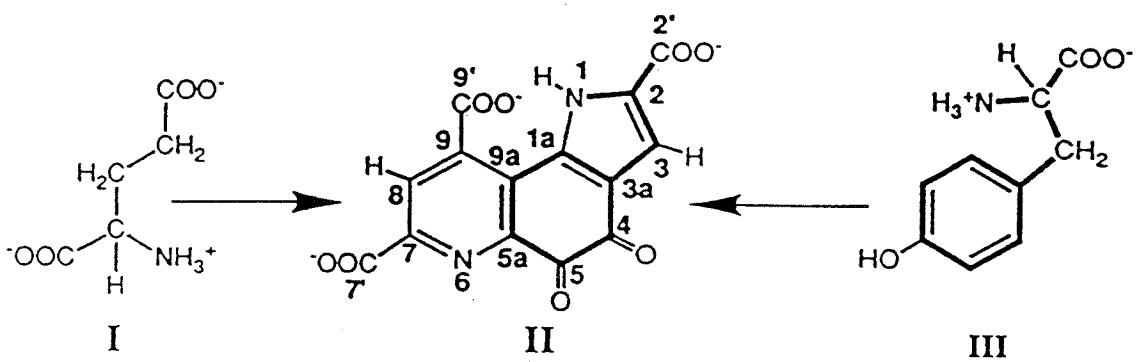
COO⁻
CH₂
H₂C
⁻OOC
C
NH₃⁺
H
I
2'
COO⁻
9'
COO⁻
H
N
1
2
9
1a
9a
H
3
H
8
3a
4
⁻OOC
7'
7
N
6
5a
5
O
O
II
H
COO⁻
H₃⁺N
C
CH₂
HO
III

FIGURE 4

Chapter 2

Sequence of *pqqD, pqqG* and *pqqC'* from *Methylobacterium extorquens* AM1 and transcriptional analysis of *pqqD*.

This chapter describes a collaborative project between Dr. Christina Morris and myself. Christina Morris did the sequencing of *pqqD*, *pqqG* and part of *pqqC* while I did the transcriptional start site mapping, northern analysis, sequencing of the transposon insertion sites and β–galactosidase assays.

ABSTRACT

The nucleotide sequence of a 2 kb *Methylobacterium extorquens* AM1 DNA fragment known to complement MoxO PQQ biosynthesis mutants has been determined. Two complete open reading frames and the N-terminal sequence of a third were identified and designated *pqqDGC*. The predicted gene products of all three show substantial similarity to the gene products of corresponding *pqq* genes in *Acinetobacter calcoaceticus* and *Klebsiella pneumoniae*. A unique transcription start site was mapped to a guanidine nucleotide 95 bp upstream of the *pqqD* initiator codon. RNA blot analysis identified two transcripts, a major one of 240 bases and a minor one of 1300 bases. The larger transcript is predicted to encode *pqqD* and *pqqG* and terminate between *pqqG* and *pqqC*. The smaller transcript is likely to be terminated at a stem-loop structure within the second gene, *pqqG*, and encodes only *pqqD*. *pqqD* encodes a 29 amino acid peptide which contains a tyrosine and a glutamate residue that are conserved in the equivalent peptides of *K. pneumoniae*, PqqA (23 amino acids) and *A. calcoaceticus*, PqqIV (24 amino acids), and are thought to be the precursors for PQQ biosynthesis. The organization of a cluster of five PQQ biosynthesis genes appears to be similar in four different bacteria (*M. extorquens* AM1, *Methylobacterium organophilum* DSM 760, *K. pneumoniae* and *A. calcoaceticus*). Five complementation groups, previously referred to as Mox (methanol oxidation), but containing PQQ biosynthesis mutants, have been re-designated Pqq, and a total of seven *pqq* genes have been identified in *M. extorquens* AM1.

INTRODUCTION

Methylobacterium extorquens AM1 is a pink-pigmented facultative methylotroph capable of growth on single carbon compounds such as methanol and methylamine as its sole source of carbon and energy [27]. Oxidation of methanol to formaldehyde is the initial step of the biochemical pathway and further assimilation of carbon into the cell is achieved through the serine cycle [4]. Investigation of the methanol oxidation system of *M. extorquens* AM1 has revealed a large number of genes involved at the initial step [26], which is catalyzed by methanol dehydrogenase (MeDH) [4]. *moxF* and *moxI* encode structural polypeptides for the periplasmic MDH heterodimer ($\alpha_2\beta_2$) and *moxG* encodes the electron acceptor for MDH, cytochrome c_L [2, 26, 38, 39, 40, 41]. MDH requires a non-covalently linked pyrroloquinoline quinone (PQQ) cofactor and Ca^{2+} ions for activity [25, 43]. Mox genes have been identified that are involved in facilitating insertion of the Ca^{2+} into MDH [43], transcription regulation [26, 28, 35, 36, 41, 49], and PQQ biosynthesis [6].

PQQ is an important redox cofactor in bacterial dehydrogenases [12]. It is associated with glucose dehydrogenases in *Acinetobacter calcoaceticus* and *Klebsiella pneumoniae* [18, 37], alcohol dehydrogenases in *Pseudomonas sp.* [19] and methanol dehydrogenases in methylotrophs [11]. Other organisms, such as *Escherichia coli* and *Acinetobacter lwoffii*, are known to synthesize apoproteins requiring PQQ for activity, yet are unable to synthesize the cofactor and must be provided with an exogenous supply [22, 46].

Mutants of *Methylobacterium organophilum* DSM 760 [7, 30], *A. calcoaceticus* [15] and *K. pneumoniae* [31, 32] unable to synthesize PQQ have been used to identify PQQ biosynthesis genes through complementation [7, 15,

32] and expression in *E. coli* [8, 16, 32]. Six genes have been identified and sequenced from *K. pneumoniae* (*pqqABCDEF*) [33] and six have been identified in *M. organophilum* (*pqqEDCBA* and *pqqF*) [7]. Sequence analysis of the PQQ genes for *A. calcoaceticus* has identified five genes in an operon (*pqqIV*, *pqqV*, *pqqI*, *pqqII* and *pqqIII*), only four of which are essential for PQQ biosynthesis (*pqqIV*, *pqqI*, *pqqII* and *pqqIII*) [16].

pqqA of *K. pneumoniae* and *pqqIV* of *A. calcoaceticus* encode small peptides of 23 amino acids and 24 amino acids respectively [17, 33]. Both peptides contain the known amino acid precursors of PQQ, glutamate and tyrosine, as conserved residues in their sequence. It has been proposed that these small peptides play a role in PQQ biosynthesis as templates providing the precursor substrates on a protein matrix where ring closure, hydroxylation and oxidation would occur before the release of PQQ [33]. This proposal would account for the inability to detect PQQ biosynthesis intermediates. In *K. pneumoniae*, PQQ biosynthesis requires a functional PqqF, which has sequence similarity with a family of proteases [33]. PqqF could therefore be involved in cleavage of (a) peptide bond(s) resulting in the release of PQQ. Two other genes, located close to the *pqq* genes in *K. pneumoniae* (*orfX*) and in *A. calcoaceticus* (*orfR*), encode polypeptides with sequence similarity with a human dipeptidase [16, 33]. These polypeptides may play a role in formation of PQQ on the protein matrix. The functions of the products of the remaining *pqq* genes are unknown.

Eighty-four *M. extorquens* AM1 mutants unable to grow on methanol have been isolated and shown to be defective in PQQ biosynthesis [6]. Cross complementation analysis between *M. extorquens* AM1 and *M. organophilum* DSM 760 clones and *M. organophilum* XX, *M. extorquens* AM1 and *M. organophilum* DSM 760 *pqq* mutants has suggested six complementation groups

are present in *M. extorquens* AM1 and *M. organophilum* DSM 760, designated MoxC, MoxH, MoxO, MoxT, MoxU and MoxV in *M. extorquens* AM1 [6]. We have begun to identify the individual genes in these complementation groups through sequence analysis and comparison to the corresponding genes sequenced in *K. pneumoniae* and *A. calcoaceticus*. In this study we report the sequence of a 2049 bp *Bgl*II-*Hin*dIII DNA fragment which complemented mutants in the *M. extorquens* AM1 MoxO complementation group [6], the identification of three *pqq* genes from this sequence (*pqqDGC*), and a preliminary study of transcription of the first of these genes (*pqqD*).

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table I.

Media and growth conditions. *M. extorquens* AM1 strains were grown at 30°C on the ammonium-mineral salts medium described by Harder *et al.* [20] supplemented with a vitamin solution [45]. Succinate was added to 0.2% (wt/vol), and methanol was added to 0.5% (vol/vol). For growth on methylamine, medium was supplemented with both methylamine at 0.2% (wt/vol), and methanol at 0.2% (vol/vol). *E. coli* strains were grown at 37°C in Luria broth [29]. Agar was added to 1.5% (wt/vol) for plates. Antibiotics were added to sterile media in the following concentrations: rifamycin, 20 μg/ml; tetracycline, 10 μg/ml; kanamycin, 30 μg/ml (*M. extorquens* AM1) or 50 μg/ml (*E. coli*); ampicillin, 100 μg/ml; streptomycin, 10 μg/ml.

Bacterial matings. Tri-parental matings with *M. extorquens* AM1 were performed as described previously [14]. Mating mixtures were plated on both succinate minimal medium and methanol minimal medium, supplemented with tetracycline and rifamycin, for complementation analysis.

DNA manipulations. Restriction enzyme digestions, ligations, plasmid isolations, and transformations of DNA into *E. coli* DH5α were carried out as described in Maniatis *et al.* [29]. DNA sequencing was done according to the dideoxy chain-termination method of Sanger *et al.* (44), using Sequenase™ (US Biochemical Corp., Cleveland OH), or by the UCLA Sequencing Facility on an Applied Biosystems Model 373A automated sequencer.

RNA isolation. Total bacterial RNA was isolated from *M. extorquens* AM1 cells growing exponentially on methanol. A total of 50 ml cell culture was quick chilled on dry ice, centrifuged at 4°C and the pellet of cells resuspended in 15 ml of diethylpyrocarbonate treated water. The remaining steps in the RNA isolation procedure were as described in Waechter-Brulla *et al.* [47].

Primer extension analysis. Two oligonucleotides, 5'-GACTTTATGGAACGCCGGAACCGCGCG-3' (R1) and 5'-ACTTCATGGTGTCCTCTCCTCGACTTTATGG-3' (R3), complementary to nucleotides -14 to -40 and +6 to -23, respectively, with respect to the translation start site of *pqqD*, were synthesized by the Caltech Microchemical Facility. Superscript™ reverse transcriptase (GIBCO-BRL) was used for primer extension according to the manufacturers instructions except that each reaction mixture contained 7.5 μg of RNA and a pulse-chase label was used. Initially a pulse label of 1 μl mixed dNTP stock (10 mM each dATP, dGTP and dTTP) and 1 μl of α-

^{32}P dCTP was added and the reaction was incubated for 10 minutes. A chase of 1 µl 10 mM dCTP was added and the incubation was continued for an additional 40 minutes. The products of transcription were then prepared as described previously [47] and subjected to electrophoresis on 6% (wt/vol) polyacrylamide gels simultaneously with a sequencing ladder generated with the same primer.

RNA blot analysis. *M. extorquens* AM1 RNA (10 µg) was subjected to electrophoresis in 1% (vol/vol) formaldehyde-agarose gels with 1X buffer (20 mM MOPS, pH 7.0, 8 mM sodium acetate, 1 mM EDTA, pH 8.0) as described in Maniatis *et al.* [29]. Transfer to a Zeta-Probe membrane (Bio-Rad Lab., Richmond CA) was accomplished using a dry electroblot apparatus, 0.17X buffer (conductivity 320 mmho) and the transfer was allowed to continue for 4 h at 50 mA. Hybridization was as described in Maniatis *et al.* [29] using a γ-^{32}P ATP 5' end-labeled oligonucleotide, 5'-CAGATCTCGGAAACGATGGGGGCAGCCCACTTC-3' (R2), complementary to nucleotides +2 to +34 with respect to the translation start site of *pqqD*, as the radioactive probe.

Preparations of cell free extracts. Cell free extracts of *M. extorquens* AM1 strains were prepared as described previously [36].

Protein determination. Total protein was assayed using the Bio-Rad protein assay (Bio-Rad Lab., Richmond CA). Stock solutions of bovine serum albumin (BSA) were used as standards.

β-galactosidase assay. β–galactosidase activity was assayed as described previously by Miller [34].

Nucleotide sequence accession number. The nucleotide sequence of the *pqqDGC* region reported here has been assigned GenBank accession number (submitted).

RESULTS

Nucleotide sequence of *pqqDGC'*. The nucleotide sequence of the large *Bgl* II-*Hin*d III fragment, that was known to complement *M. extorquens* AM1 mutants in the MoxO complementation group, was determined on both strands using a series of plasmids including pCM187, pCM188, pCM198 and various subclones of these plasmids. This fragment was found to be 2049 bp, and contained two complete open reading frames and the N-terminus of a third (Figs. 1, 2). The first open reading frame, which traverses the internal *Bgl* II site, encodes a peptide of 29 amino acids in length. Genes that encode similar peptides have been reported from *A. calcoaceticus* (*pqqIV* 24 amino acids) and *K. pneumoniae* (*pqqA* 23 amino acids) [16, 33]. Seven amino acids are conserved in all three peptides, including the two amino acids thought to be important in providing the structural building blocks for PQQ, glutamate (E16) and tyrosine (Y20), (Fig. 3). These residues are separated by 3 amino acids in each case. The *M. organophilum* DSM 760 gene that has been suggested to be equivalent to *pqqIV* of *A. calcoaceticus* has been called *pqqD* [7], and we propose to designate this gene *pqqD* in *M. extorquens* AM1. The second open reading frame lies 47 bp downstream of *pqqD* and encodes a polypeptide of 299 amino acids. It shows substantial amino acid sequence identity to the open reading frames encoded by the second gene in the *pqq* clusters of *A. calcoaceticus* (*pqqV*, 36%) and *K. pneumoniae* (*pqqB*, 36%). This open reading frame has been designated *pqqG*,

since no equivalent gene has been reported for *M. organophilum* DSM 760. The third open reading frame follows *pqqG* at a distance of 167 bp. Only the first 111 amino acids are known since the open reading frame is truncated at the internal *Hin*d III site. The 111 amino acids determined show 50% and 43% identity with the third gene in the *pqq* clusters of *K. pneumoniae* (*pqqC*) and *A. calcoaceticus* (*pqqI*), respectively. The gene encoding this open reading frame corresponds to the *M. extorquens* AM1 complementation group , MoxT[6]. The equivalent gene in *M. organophilum* DSM 760 has been termed *pqqC* [6, 7], and we propose to designate this gene *pqqC* in *M. extorquens* AM1. The direction of transcription is from *pqqD* through *pqqG* and *pqqC*, therefore primer extension analysis concentrated on identifying the transcription start site in the region upstream of *pqqD*.

Transcription start site analysis. Two oligonucleotide primers complementary to regions within *pqqD* were used to map the transcription start site for the *M. extorquens* AM1 *pqq* operon. Figure 4 shows a major extension product produced with primer R1 (see Materials and Methods) that indicates the 5' end of the *pqq* mRNA lies 95 bp upstream of the *pqqD* translation start site, initiating at a guanidine nucleotide at position 419 in the presented sequence (Fig. 1). The primer extension product produced with a second primer R3 confirmed this nucleotide as the transcription start site (data not shown). Some minor extension products were also seen but these varied from experiment to experiment and were not confirmed by both primers. Levels of transcription observed in cells grown on succinate were equivalent to those seen in cells grown on methanol and were initiated at the same *pqqD* transcription start site (Fig. 4).

RNA blot analysis. RNA blot analysis with *M. extorquens* AM1 RNA from methanol grown cells was carried out using an oligonucleotide probe complementary to the beginning of *pqqD* (R2, see Materials and Methods). Two transcripts were detected (Fig. 5), a major transcription product of approximately 240 bases and a second, less abundant transcript of approximately 1300 bases. The same pattern and relative amounts of transcription products were also observed for RNA isolated from *M. extorquens* AM1 cells grown on succinate. The primer extension analysis suggests that both transcripts have the same start site. If so, the smaller one would terminate within *pqqG* just upstream of a region containing a potential stem-loop structure. Within *pqqG* at nucleotides 685 to 694, and 700 to 710, two 10 bp inverted repeats exist with a single mismatched base (T, 705). These could potentially form a 10 bp stem topped by a 5 base loop with a free energy value of $DG^{o'}$=-22.7 kcal. The second transcript would terminate between *pqqG* and *pqqC*.

Tn*5lac* insertions. Two Tn*5lac* insertions (Tn*5*-136 and Tn*5*-19) have been reported that abolish complementation of MoxO mutants [6]. Tn*5lac* is constructed in such a way that in the correct orientation, a transcriptional fusion to *lacZ* is generated [24]. The sites of insertion of these two transposons were determined by sequencing to lie within *pqqG* between nucleotides 701 and 702, and 1295 and 1296, respectively (Figs. 1, 2). The insertion site of Tn*5*-136 disrupts the possible stem-loop structure described above. Both Tn*5*-136 and Tn*5*-19 are in the orientation such that their internal β-galactosidase genes would be transcribed in the same direction as *pqqDGC*. Therefore, these transposon insertions were used as indicators of transcription levels from the *pqqD* promoter. β-galactosidase activities were measured in cell free extracts of methanol-grown *M. extorquens* AM1 containing plasmids inserted with the two different

transposons. Extracts of methanol-grown cells containing p1130D::Tn*5*-136 had 10-fold greater β-galactosidase activity (192 nmoles min^{-1}mg $protein^{-1}$) than extracts of cells containing p1130D::Tn*5*-19 (19 nmoles min^{-1}mg $protein^{-1}$). β-galactosidase activities were similar in extracts of succinate-grown cells (p1130D::Tn*5*-136, 146 nmoles min^{-1}mg $protein^{-1}$; p1130D::Tn*5*-19, 23 nmoles min^{-1}mg $protein^{-1}$). The difference in β-galactosidase activity levels correlates with the levels of the two transcripts observed (Fig. 5). The insertion site of p1130D::Tn*5*-136 could lie within the major (smaller) transcript while p1130D::Tn*5*-19 should be located within the minor (larger) transcript.

DISCUSSION

The results presented here show that several *pqq* genes of *M. extorquens* AM1 are equivalent to and are clustered in the same gene order as *pqq* genes in other bacteria (Fig. 6). A *Bgl*II-*Hin*dIII DNA fragment that complements *M. extorquens* AM1 PQQ biosynthesis mutants of the MoxO complementation group [6], encodes two complete open reading frames and the N-terminus of a third. The first open reading frame of 29 amino acids showed sequence identity with the small peptides encoded by *pqqA* and *pqqIV* in *K. pneumoniae* [33] and *A. calcoaceticus* [16, 17] respectively. Conservation of the glutamate and tyrosine residues and their relative positions within the polypeptide is maintained in all three polypeptides sequenced to date (Fig. 3). This first gene has been named *pqqD* in keeping with the designations [6] previously proposed for *M. organophilum* DSM 760 [30]. Although the equivalent gene has been named *pqqA* in *K. pneumoniae* [33] and *pqqIV* in *A. calcoaceticus* [16] (Fig. 6), we have followed the convention of naming genes in the manner reported first [7]. The

second complete open reading frame in the *M. extorquens* AM1 DNA showed amino acid sequence similarity with *pqqB* in *K. pneumoniae* and *pqqV* in *A. calcoaceticus*. This gene was named *pqqG* because no equivalent gene was identified in *M. organophilum* DSM 760 by the initial mutant complementation analysis [7]. However, sufficient space (approximately 1 kb) does exist between *pqqD* and *pqqC* of *M. organophilum* DSM 760 for a gene of similar size. In addition, *M. organophilum* DSM 760 clones containing this region complement the *M. extorquens* AM1 mutants defective in *pqqG* [6], indicating that *pqqG* is present in *M. organophilum* DSM 760, as shown in Fig. 6. The *A. calcoaceticus pqqV* gene was shown to be non-essential for PQQ biosynthesis [16]. It may well be that a similar case exists in *M. organophilum* DSM 760. A third open reading frame located downstream of *pqqG* is truncated by the *Hin*dIII site after 111 amino acids. The N-terminal sequence showed similarity with *K. pneumoniae pqqC* and *A. calcoaceticus pqqI* and has been designated *pqqC* in *M. extorquens* AM1, again with reference to the equivalent *M. organophilum* DSM 760 *pqqC*. The *K. pneumoniae* PqqC and *A. calcoaceticus* PqqI polypeptides are 251 amino acids and 252 amino acids respectively. Therefore, approximately another 140 residues of the *M. extorquens* AM1 PqqC are expected to be encoded by the adjacent DNA fragment [6], which would place the 3' end of *pqqC* approximately 0.4 kb beyond this *Hin*dIII site (Fig. 2).

The data presented here show that *M. extorquens* AM1 mutants of the MoxO complementation group [6] are defective in *pqqG*. The two Tn*5lac* insertions that abolish MoxO complementation (Tn*5*-19 and Tn*5*-136) were shown by sequencing to be located within *pqqG*. In addition, the smallest subclone that complemented MoxO mutants (p1130D-HBg) [6] contains the complete *pqqG*, but truncates *pqqD*. This shows that, as in *K. pneumoniae pqqB* but unlike *A. calcoaceticus pqqV*, the *M. extorquens* AM1 *pqqG* gene product

appears to be essential for PQQ biosynthesis. These data also suggest that of the 84 *M. extorquens* AM1 Pqq mutants studied [6], none are defective in *pqqD*. This is not surprising given the small size of this gene.

It is not clear at this time whether the *M. organophilum* DSM 760 PqqD mutant 71 is defective in *pqqD* or *pqqG*. Although the initial complementation data mapped this mutation to a 0.1 kb DNA region, suggesting it was in *pqqD* (7), it showed the same complementation pattern with *M. extorquens* AM1 clones and Tn*5lac* insertions as the *M. extorquens* AM1 MoxO (*pqqG*) mutants [6]. Further studies would be necessary to resolve this discrepancy.

Although the studies described here did not involve the last two genes of the *pqq* cluster in *M. extorquens* AM1, the sequencing data and the comparison to *K. pneumoniae* and *A. calcoaceticus pqq* clusters provide information concerning their probable size and location. Complementation studies had identified two complementation groups in this region equivalent to those groups that defined *pqqA* and *pqqB* of *M. organophilum* DSM 760 [6]. The data indicated that these genes were located between *pqqC* and the Tn*5lac* insertion Tn*5*-6 (Fig. 2) in the order *pqqA-pqqB-pqqC* [6]. The sequencing data presented here provide an approximate location for the 3' end of *pqqC*, and therefore define the *pqqA-pqqB* region more precisely. If *pqqA* and *pqqB* of *M. extorquens* AM1 are equivalent in size and function to *pqqE* and *pqqD* of *K. pneumoniae* and *pqqIII* and *pqqII* of *A. calcoaceticus*, as has been suggested for *M. organophilum* DSM 760 [7], then they would fit well in the known region for the corresponding complementation groups (Fig. 6). Further sequencing will be required to determine whether *pqqA* and *pqqB* of *M. extorquens* AM1 are equivalent to the corresponding genes in *K. pneumoniae* and *A. calcoaceticus*.

Two other *pqq* genes have been identified by complementation studies in *M. organophilum* DSM 760 and *M. extorquens* AM1 [6, 7]. These have been

designated *pqqE* and *pqqF* in *M. organophilum* DSM 760 [7] and we propose to use the same designations in *M. extorquens* AM1 (Fig. 6). It is not known whether equivalent genes are present in *K. pneumoniae* or *A. calcoaceticus*.

As shown in Fig. 6, a total of at least 7 genes have now been shown to be present in *M. extorquens* AM1 and *M. organophilum* DSM 760 (*pqqDGCBA* and *pqqEF*). A unique transcription start site was identified 95 bp upstream of *pqqD*. The sequences immediately upstream of the transcription start site have some similarities to the -10 and -35 sequences seen for *E. coli* and *K. pneumoniae*. The -10 sequence CGATAT has 3 of 6 bases identical to the *E. coli* σ^{70} -10 consensus sequence TATAAT [21] and 5 of 6 bases identical to the -10 sequence of *K. pneumoniae pqqABCDEF* CAATAT [33]. The -35 sequence TTGCAG has 3 of 6 bases identical to the *E. coli* σ^{70} -35 consensus sequence TTGACA [21], and the *K. pneumoniae pqqABCDEF* -35 sequence TTGATC [33; Fig. 1]. The putative methylotrophic consensus promoter found upstream of *moxF* in *M. extorquens* AM1 and *M. organophilum* XX (AAAGACA-18 bp-TAGAAA-4 to 5 bp-+1) [28] is not present 5' to *pqqD*, although the sequence AAGAAA is present at -55 to -50 with respect to the transcription start site (Fig. 1). Xu *et al.* [49] determined that a septanucleotide AGAAATG was associated with methanol-regulated promoters in *M. organophilum* XX. Although this precise sequence is not found upstream of the *pqqD* transcription start site, the sequence AGAAACG is present at bases -54 to -48. The *M. extorquens* AM1 *pqqD* promoter does not however appear to be regulated by methanol (see below).

The data presented here suggest that the five genes, *pqqDGCBA* may not be cotranscribed. Two transcripts were detected that were apparently initiated at the *pqqD* transcription start site. The smaller, more abundant, transcript (240 bases) would be expected to encode *pqqD* alone while the larger product (1300 bases) would encode both *pqqD* and *pqqG*. No larger transcripts were detected.

A potential stem-loop structure lies 265 bases downstream of the transcription start site between *pqqD* and *pqqG*. This may act as a transcription terminator or a processing site, resulting in the higher levels of the smaller transcript. This is consistent with the expectation that the small peptide, PqqD, would be required in greater amounts than the PQQ biosynthesis enzymes if it acts as a precursor of PQQ. The β-galactosidase levels measured in extracts of cells containing the plasmids p1130D::Tn*5*-19 and p1130D::Tn*5*-136 also indicate that the stem-loop structure is important. Insertion of Tn*5*-136 within the inverted repeat would disrupt the putative stem-loop formation, which would be expected to result in high level transcription of *lacZ* in Tn*5*-136. Alternatively, since Tn*5*-19 is inserted downstream of the stem-loop structure within *pqqG*, transcription of *lacZ* in Tn*5*-19 should be lower. In fact, the measured activity of β-galactosidase in extracts of cells containing p1130D::Tn*5*-136 was 10 fold greater than in those containing p1130D::Tn*5*-19. Further studies are necessary to determine the precise role of this stem-loop structure in transcription of these genes. It should be noted that the ribosome binding sequence for *pqqD* is much stronger than for *pqqG* and *pqqC*, and so it is possible that a further increase of the ratio of peptide to biosynthetic enzymes could be accomplished at the level of translation.

The transcription of the other *pqq* genes is uncertain. Although no transcripts longer than 1300 bases were detected using the oligonucleotide complementary to DNA near the start of *pqqD*, it is possible that larger transcripts are made that are too unstable to detect. The PqqB mutant of *M. organophilum* DSM 760 was not complemented unless the entire *pqqC* gene of *M. extorquens* AM1 was present [6], suggesting that transcription in these genes is coupled. Further work will be needed to map possible transcripts and transcription start sites for the remainder of the gene cluster.

It has been shown that methanol induces transcription from the *M. extorquens* AM1 *moxF* promoter [1, 3, 36] and three other *mox* promoters including *moxF* in *M. organophilum* XX [49]. This increase in MDH production requires sufficient PQQ for an increase in enzyme activity. Greater amounts of PQQ are synthesized by *M. extorquens* AM1 cells grown on methanol than by cells grown on succinate [42]. However, our data suggest this is not due to an increase in transcription of *pqqD* or *pqqG*. No significant difference was observed in the levels of either transcript isolated from *M. extorquens* AM1 grown on succinate or methanol as measured with both the primer extension and RNA blot analyses. In addition, β-galactosidase activities measured in extracts of cells containing p1130D::Tn*5*-136 or p1130D::Tn*5*-19 were equivalent regardless of the growth substrate. Further study will be necessary to determine the mechanism whereby PQQ biosynthesis is regulated by methanol.

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TABLE 1. Bacterial strains and plasmids used in this study

Strains		Relevant traits	Source or reference
E. coli			
	DH5α	r^- m^+ *recA1 lacZYA* φ80d*lac*Δ(*lacZ*)*M15*	BRL, Inc.
	HB101	r^- m^- *recA13*	(9)
	MM294	*recA*$^+$	(5)
M. extorquens AM1			
	AM1rif	Rif^r derivative	(40)
Plasmids			
	pRK2013	mobilizing "helper" plasmid, Km^r	(13)
	pRK2073	mobilizing "helper" plasmid, Sm^r	(13)
	pVK100	Tc^r, Km^r, IncP cosmid	(23)
	pRK310	Tc^r, *lacPOZ'*, IncP plasmid	(10)
	pUC18/19	Ap^r, *lacZ'*, multiple cloning site	(48)
Plasmids containing *M. extorquens* AM1 DNA			
	p1130D	Tc^r, *Hin*dIII-*Pst*1 subclone from pEL41 in pRK310, complements MoxO mutants	(6)
	p1130D-HBg2.1	Tc^r, *Hin*dIII-*Bgl*II subclone from p1130D in pRK310 (partial, 2.1 kb fragment), complements MoxO mutants	Eun Lee
	p1130D-HBg	Tc^r, *Hin*dIII-*Bgl*II subclone from p1130D in pRK310 (1.7 kb fragment), complements MoxO mutants	(6)

p1130D::Tn*5*-19	Tc^r, Km^r, Tn*5lac* insertion in p1130D, no complementation of Pqq^- mutants	(6)
p1130D::Tn*5*-136	Tc^r, Km^r, Tn*5lac* insertion in p1130D, no complementation of Pqq^- mutants	(6)
pCM187	Ap^r, 2.1 kb *Hin*dIII-*Eco*RI subclone from p1130D-HBg2.1 in pUC18	This study
pCM188	Ap^r, 1.7 kb *Hin*dIII-*Eco*RI subclone from p1130D-HBg in pUC18	This study
pCM198	Ap^r, 1.7 kb *Hin*dIII-*Eco*RI subclone from p1130D-HBg in pUC19	This study

FIGURE LEGENDS

Figure 1. Nucleotide sequence of *M. extorquens* AM1 *pqqDGC'*. The 2049 bp *Bgl*II-*Hin*dIII fragment encodes *pqqD*, *pqqG* and the N-terminus of *pqqC*. The relevant deduced amino acid residues are indicated below the nucleotide sequence. Termination codons are indicated (---). Sequence complementary to the primers used to determine the transcription start site is marked by a lines above (R1) or below (R3) the bases. The transcription start site is indicated in bold and labeled +1. Bases in common with *K. pneumoniae* -10 and -35 sequences are indicated by dots (·) above the nucleotides. Asterisks (*) mark the AAGAAA sequence similar to the proposed methylotrophic consensus promoter. Sites of insertion for Tn*5*-136 and Tn*5*-19 are indicated (V). Arrows above the nucleotide sequence identify inverted repeats that could form a 10 base pair stem-5 base loop structure in the mRNA.

Figure 2. The *pqqDGCBA* region of *M. extorquens* AM1 (map from Biville *et al.*, 1993 [6]). Shaded boxes denote sequenced region, open boxes denote assumed gene sizes in comparison to those from *K. pneumoniae* [33] and *A. calcoaceticus* [16]. Tn*5lac* insertions (▼) 19 and 136 abolish complementation of MoxO mutants (*pqqG*); insertion 82 abolishes complementation of MoxC mutants (*pqqA*); insertion 6 does not abolish complementation of MoxC mutants. Transcription for *pqqD*, *pqqG* and *pqqC* is right to left, as shown. Size marker for 1 kb is shown. P, *Pst*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII.

Figure 3. Alignment of the peptides encoded by *K. pneumoniae pqqA*, *M. extorquens* AM1 *pqqD* and *A. calcoaceticus pqqIV*. The peptides are aligned to maximize identity between them, indicated by *. Peptide length (amino acid

residues) is indicated at right. The conserved glutamate (E) and tyrosine (Y) residues are boxed.

Figure 4. Transcription start site analysis. ^{35}S-αdATP labeled sequencing reactions, synthesized from primer R1 and subjected to electrophoresis through a 6% polyacrylamide sequencing gel, are labeled G, A, T or C. Adjacent lanes indicate extension products synthesized from primer R1 and labeled with ^{32}P-αdCTP, using 7.5 µg total RNA isolated from methanol grown cells (M) or succinate grown cells (S) as the template. A 1 in 10 dilution of the extension reaction was loaded per lane. The nucleotide sequence including the transcription start site (■) is indicated.

Figure 5. RNA blot analysis. Electrophoresis of 10 µg total RNA isolated from methanol grown cells (M) and succinate grown cells (S) through a 1% agarose-formaldehyde gel. Hybridization was with ^{32}P-γATP labeled oligonucleotide R2. Sizes marked were determined using an 0.24 - 9.5 kb RNA ladder (BRL., Inc.)

Figure 6. Alignment of *pqq* gene clusters from *K. pneumoniae*, *A. calcoaceticus*, *M. extorquens* AM1 and *M. organophilum* DSM 760. Shaded boxes indicate regions that have been sequenced. Open boxes represent deduced open reading frames and gene designations are shown below. Solid lines indicate DNA fragments known to encode the indicated genes through complementation analysis. Vertical dashed lines indicate equivalent genes. Naming of *M. extorquens* AM1 *pqq* genes is based upon equivalent *M. organophilum* DSM 760 genes complemented by *M. extorquens* AM1 clones [6].

Alignment is based on the genes encoding the small peptides (*pqqA*, *pqqIV* or *pqqD*). Size marker for 1 kb is shown.

```
AGATCTTCGACCGGCTCTGGGCGAGCCAGGGCGGCCTCTCGCTGAACGTGGTCGATGTCT   60
ATGTCGGCTACCTGCGCTCGAAGCTCTCGGATTTCGTGCGGCTGGGCGGCCCGGTAATCG  120
TCACGGTGCGCGGCAAGGGCTTCATGCTCGATCTGCGCGGCCAGGATTTCCGTCACTGAC  180
CCCCGCGGCATTTCGCTCCGAGCGCCGTATCCGACCGGATGGCATCAGGCCGGCGTCTCC  240
CGGTCTTTGCTTGAACGCGCTTTCTTCCGCCGAACCGGCGTCCACCTCGTCGGAAAGCGC  300
TCTCGGACCAAAGTTTAACCCGCGTCTCACGCGAAGTTATGCCGATCTCAGCGCGAACTT  360
   ******          ...                . ....      +1
GGGAAGAAACGGCAAGCTGGCTCTTGCAGGCGGGGCCGGAATTGGCGATATACCTCCGGC  420
                                                  R1_____
GTGCGAAAACGAGTGCGTCGCCAGTATCTTACGATCCTCTTAATCAGTGGGATCGCGCGG  480
_____
TTCCGGCGTTCCATAAAGTCGAGGAGAGACACCATGAAGTGGGCTGCCCCCATCGTTTCC  540
          R3                pqqD    M  K  W  A  A  P  I  V  S
GAGATCTGCGTCGGCATGGAAGTCACGAGCTACGAGTCGGCCGAGATCGACACCTTCAAC  600
 E  I  C  V  G  M  E  V  T  S  Y  E  S  A  E  I  D  T  F  N
TAAGGTGATTTGAGCCGGGTTGGGGTTGCAGGCATCAGCGGGTTTTCACCATGCATGTCG  660
---                                          pqqG    M  H  V
                                    Tn5-136
                                      \/
TAATCCTGGGCTCGGCTGCGGGCGGCGGCGTTCCTCAATGGAACTGCCGCTGCTCCATCT  720
V  I  L  G  S  A  A  G  G  G  V  P  Q  W  N  C  R  C  S  I
GCTCCCTGGCCTGGGCGGGCGATTCCCGCGTCAGGCCGCGCACGCAGTCGAGCATCGCAG  780
C  S  L  A  W  A  G  D  S  R  V  R  P  R  T  Q  S  S  I  A
TCTCTCCTGACGGGGAACGCTGGCTCCTGCTGAACGCCTCTCCCGATATCCGTCAGCAGA  840
V  S  P  D  G  E  R  W  L  L  L  N  A  S  P  D  I  R  Q  Q
TCCAGGCCAATCCGCAGATGCATCCGCGCGAGGGCCTGCGCCACTCGCCGATCCACGCGG  900
I  Q  A  N  P  Q  M  H  P  R  E  G  L  R  H  S  P  I  H  A
TGCTGCTGACGAACGGCGACGTCGATCACGTTGCGGGCCTGCTGACCCTGCGCGAGGGCC  960
V  L  L  T  N  G  D  V  D  H  V  A  G  L  L  T  L  R  E  G
AGCCCTTCACGCTCTACGCGACACCCGGCATCCTGGCCTCCGTCTCCGACAACCGCGTCT 1020
Q  P  F  T  L  Y  A  T  P  G  I  L  A  S  V  S  D  N  R  V
TCGACGTGATGGCCGCCGACGTGGTGAAGCGGCAGACGATCGCCCTCAACGAGACCTTCG 1080
F  D  V  M  A  A  D  V  V  K  R  Q  T  I  A  L  N  E  T  F
AGCCGGTGCCCGGCCTCTCGGTGACGCTGTTCTCCGTCCCCGGCAAGGTGCCGCTCTGGC 1140
E  P  V  P  G  L  S  V  T  L  F  S  V  P  G  K  V  P  L  W
TGGAAGACGCCTCGATGGAGATCGGGGCGGAGACCGAAACCACGGTCGGCACGATGATCG 1200
L  E  D  A  S  M  E  I  G  A  E  T  E  T  T  V  G  T  M  I
AGGCCGGGGGCAAGCGCCTCGCCTACATCCCCGGCTGCGCCCGGGTGACGGAGGATCTCA 1260
E  A  G  G  K  R  L  A  Y  I  P  G  C  A  R  V  T  E  D  L
                                    Tn5-19
                                      \/
AAGCCCGCATCGCCGGCGCAGACGCGCTCCTGTTCGACGGCACGGTGCTGGAGGACGACG 1320
K  A  R  I  A  G  A  D  A  L  L  F  D  G  T  V  L  E  D  D
```

```
ACATGATCCGCGCCGGTGTCGGCACCAAGACCGGCTGGCGCATGGGCCATATCCAGATGA 1380
D  M  I  R  A  G  V  G  T  K  T  G  W  R  M  G  H  I  Q  M

ACGGCGAGACCGGCTCGATCGCGTCTCTCGCCGATATCGAGATCGGCCGACGGGTCTTCG 1440
N  G  E  T  G  S  I  A  S  L  A  D  I  E  I  G  R  R  V  F

TTCACATCAACAACACCAATCCGGTCCTGATCGAGGATTCGTACGAGCGCGCGAGCGTCG 1500
V  H  I  N  N  T  N  P  V  L  I  E  D  S  Y  E  R  A  S  V

AGGCGCGCGGCTGGACCGTCGCCCATGACGGCCTGACCCTCGATCTCTGATCAGGCTGAT 1560
E  A  R  G  W  T  V  A  H  D  G  L  T  L  D  L  ---

GTCTTGGGAAGAGCCCGGTCTGGAAATTTAGTGCCGGACTGAATATGTTTTGCACGATCC 1620

AATCGTGCGGCAGCGGCCCTGCCCCGATCGGTACCGGGCCCCATTTAAAAATAAATCCAG 1680

GAAACGCGACTCGAAGCTCGGGGGAAACCGAACGCCATGACCGCCCAATTCCCGCCGCCC 1740
                                   pqqC   M  T  A  Q  F  P  P  P

GTCCCGGACACCGAGCAACGCCTGCTGAGCCACGAGGAGCTTGAGGCGGCGCTCCGCGAT 1800
 V  P  D  T  E  Q  R  L  L  S  H  E  E  L  E  A  A  L  R  D

ATCGGTGCACGGCGCTACCACAACCTCCACCCGTTCCACCGGCTGCTGCACGACGGCAAG 1860
 I  G  A  R  R  Y  H  N  L  H  P  F  H  R  L  L  H  D  G  K

CTGTCGAAGGATCAGGTCCGGGCCTGGGCGCTCAACCGCTACTATTATCAGGCGATGATT 1920
 L  S  K  D  Q  V  R  A  W  A  L  N  R  Y  Y  Y  Q  A  M  I

CCGGTGAAGGATGCAGCGCTGCTGGCTCGCCTGCCGGATGCGCAGCTTCGCCGAATCTGG 1980
 P  V  K  D  A  A  L  L  A  R  L  P  D  A  Q  L  R  R  I  W

CGCCAGCGCATCGTCGATCACGACGGCGACCATGAGGGCGACGGCGGCATCGAGCGTTGG 2040
 R  Q  R  I  V  D  H  D  G  D  H  E  G  D  G  G  I  E  R  W

CTCAAGCTT                                                      2049
 L  K  L
```

FIGURE 1

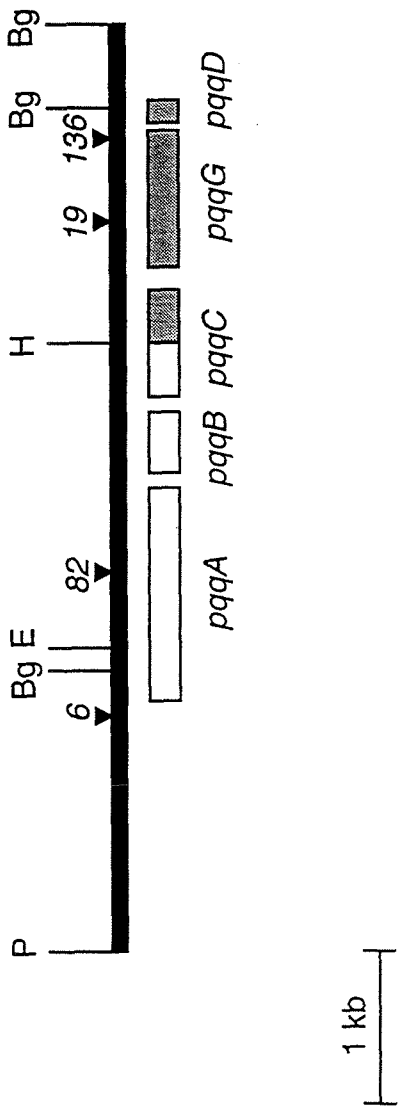

FIGURE 2

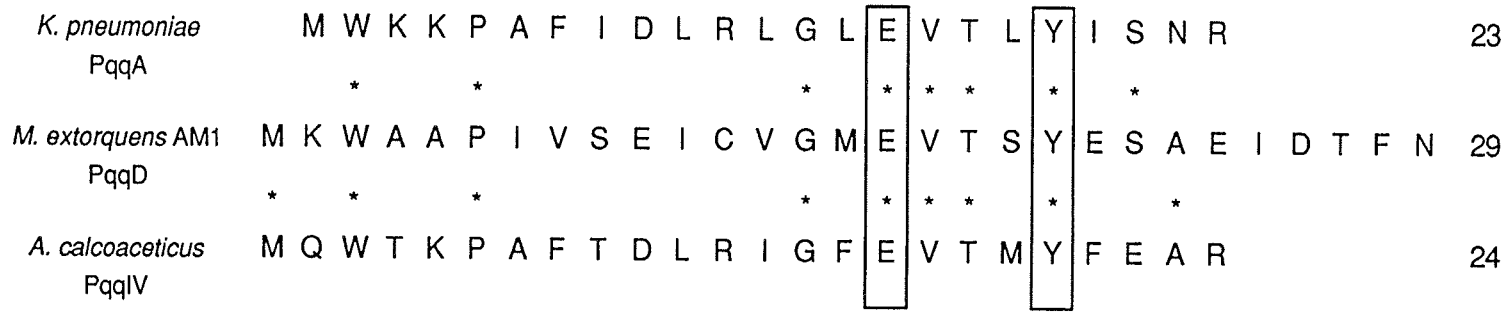
K. pneumoniae PqqA
M W K K P A F I D L R L G L E V T L Y I S N R 23
M. extorquens AM1 PqqD
M K W A A P I V S E I C V G M E V T S Y E S A E I D T F N 29
A. calcoaceticus PqqIV
M Q W T K P A F T D L R I G F E V T M Y F E A R 24

FIGURE 3

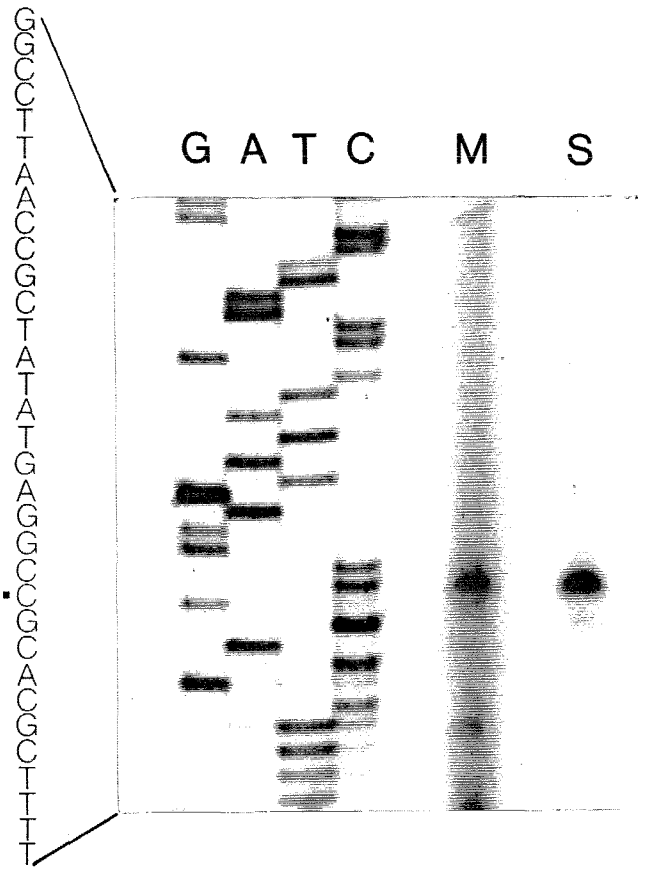

FIGURE 4

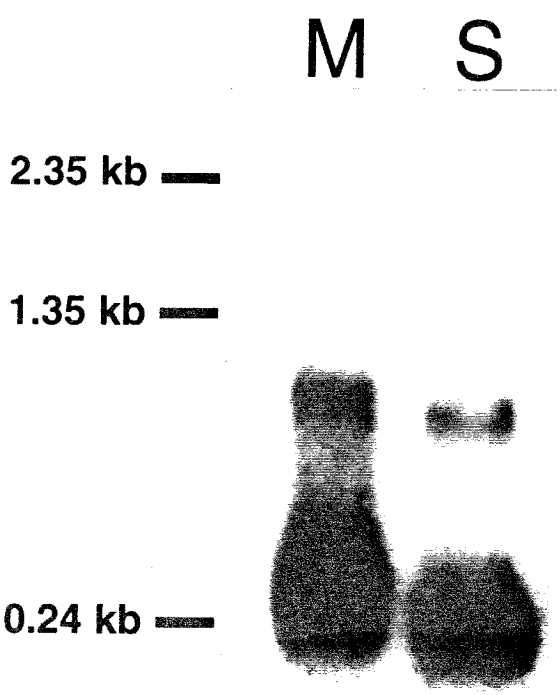

FIGURE 5

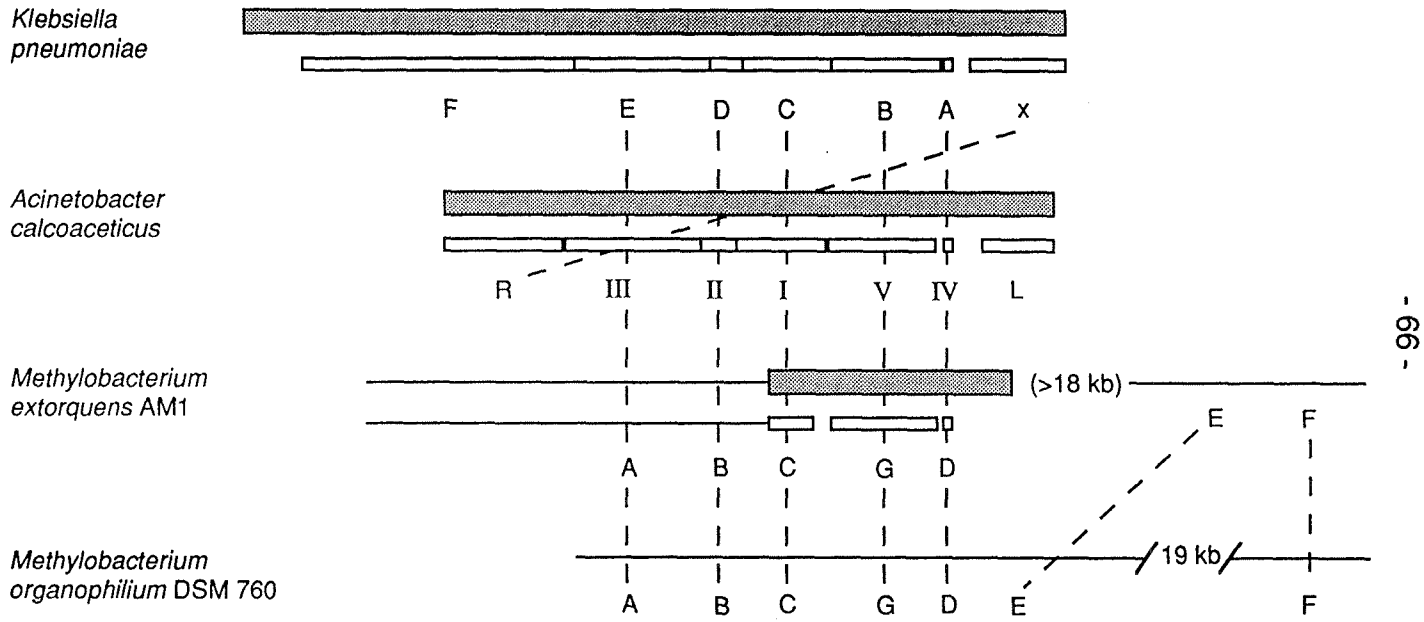
Klebsiella pneumoniae
F
E
D
C
B
A
x
Acinetobacter calcoaceticus
R
III
II
I
V
IV
L
Methylobacterium extorquens AM1
(>18 kb)
A
B
C
G
D
E
F
Methylobacterium organophilium DSM 760
19 kb
A
B
C
G
D
E
F
1 kb

FIGURE 6

Chapter 3

Study of the regulation of pyrroloquinoline quinone (PQQ) biosynthesis in the facultative methylotroph *Methylobacterium extorquens* AM1

Abstract

Methanol dehydrogenase, the enzyme that oxidizes methanol to formaldehyde in gram negative methylotrophs contains the prosthetic group, pyrroloquinoline quinone(PQQ). The regulation of PQQ production was studied in strains of *Methylobacterium extorquens* having mutations in *moxQ*, *moxE*, *moxB*, *moxM* and *moxD.* These five genes are required for transcription of genes involved in methanol oxidation. *PqqD* encodes a twenty-nine amino acid polypeptide that is believed to be the substrate for PQQ biosynthesis in vivo. Hence the transcriptional regulation of the *pqqD* promoter was studied in these five classes of regulatory mutants. A 500 base pair DNA fragment containing the entire *pqqD* promoter and part of the region coding for the peptide was cloned in front of a promoterless *xylE* gene to create a transcriptional fusion in pHX200, a low copy number, broad host range vector. The activity of the enzyme encoded by *xylE*, catechol dioxygenase, was measured in wild-type and different mutant strains containing this construct. It was observed that in the wild-type and *moxQ* and *moxE* mutants catechol dioxygenase activity was two to three fold higher in cells grown on methanol plus methylamine compared to growth on succinate. The catechol dioxygenase activity for the *moxQ* mutant was comparable to that for wild-type, while in the *moxE* mutant it was about half the wild-type level. The catechol dioxygenase activity in the *moxM* and *moxD* mutants was five to ten fold lower than in the *moxQ* mutant and also did not show induction in cells grown on methanol plus methylamine compared to growth on succinate. The catechol dioxygenase activity in the *moxB* mutant was at an intermediate level and also did not show induction in cells grown on

methanol plus methylamine compared to growth on succinate. Therefore these results indicate that the *moxM, moxD* and *moxB* gene products are all required for induction of *pqqD*, while the *moxQ* and *moxE* gene products are not. In addition, the *moxM* and *moxD* gene products seem to be required for high level transcription of *pqqD*.

In order to compare the results for transcription of *pqqD* to the levels of PQQ produced by the cells, PQQ was measured in culture supernatants. It was found that the level of PQQ in the *moxM* and *moxD* mutants was lower than in the *moxQ* and *moxE* mutants, but in all the cases PQQ was four to six fold higher when the bacteria were grown on methanol plus methylamine compared to when the bacteria were grown on succinate alone. These data suggest that transcription of *pqqD* is not the rate limiting step in PQQ biosynthesis, and in addition, that regulation of PQQ biosynthesis occurs at more than one step in the process.

Introduction

Methylobacterium extorquens AM1 is a facultative methylotrophic bacterium capable of growing on single carbon compounds like methanol and methylamine as well as multicarbon compounds like succinate. Methanol is oxidized to formaldehyde by the periplasmic enzyme methanol dehydrogenase and then the formaldehyde is either assimilated into the cell or is oxidized to CO_2 with the generation of energy. The methanol dehydrogenase contains pyrroloquinoline quinone (PQQ) as the prosthetic group. [12, 13]

A complex array of genes are involved in methanol oxidation (*mox* genes) in *M.extorquens* AM1 and functions have been determined for a number of them.(Figure 1.) *moxF* encodes the large subunit of methanol dehydrogenase, *moxI* encodes the methanol dehydrogenase small subunit , and *moxG* encodes the cytochrome c_L structural polypeptide.[1, 18, 19] *moxA*, *moxK* and *moxL* are involved in the insertion of calcium in the active site of methanol dehydrogenase . [18, 19,20] *MoxM* , *moxD*, *moxQ*, *moxE* and *moxB* are genes involved in transcriptional regulation . [11, 18, 19]

Seven genes are involved in PQQ biosynthesis, *pqqD*, *pqqG*, *pqqC*, *pqqB*, *pqqA*, *pqqE* and *pqqF*.[16, 18 ,19] *pqqD* encodes a small polypeptide of 29 amino acids containing conserved tyrosine and glutamate residues separated by three amino acids. [16, previous chapter.] Tyrosine and glutamate have been shown to be the precursors of PQQ biosynthesis and it is believed that the peptide serves as the substrate for PQQ biosynthesis in the bacteria. [6, 10]

The transcriptional regulation of the *moxF* promoter in *M.extorquens* AM1 regulatory mutants has already been studied and it was found that in the wild-type strain there was a six-fold induction of *moxF* transcription in cells grown on methanol compared to succinate. In strains defective in *moxQ*, *moxE*, *moxM*, *moxD* or *moxB* the transcription from the *moxF* promoter was negligible both on succinate and on medium containing methanol and methylamine. [17, Morris and Lidstrom, unpublished observations]

We wanted to observe how PQQ biosynthesis was regulated compared to how the production of the structural polypeptides for methanol dehydrogenase were regulated, and in this way also to gain a greater understanding of the role of the different regulatory genes involved in the methanol oxidation process.

In order to study the regulation of PQQ in *M.extorquens* AM1, PQQ levels in the medium were measured in the various regulatory mutants and the wild-type. The promoter fragment containing the entire *pqqD* promoter was cloned in a broad host range vector pHX200 to create a transcriptional fusion with the *xylE* gene, and this construction was conjugated into the various regulatory mutant strains of *M.extorquens* AM1 to determine how the transcription of the *pqqD* promoter was affected in these regulatory mutants.

Materials and Methods

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table I. Plasmid pHX200 was provided by Prof. R.S. Hanson, University of Minnesota.

Media and growth conditions. *M. extorquens* AM1 strains were grown at 30°C on the ammonium-mineral salts medium described by Harder et al. [8] supplemented with a vitamin solution. [22] Succinate was added to 0.27% (wt/vol.),and methanol was added to 0.5% (wt/vol.) For growth of the *M.extorquens* AM1 mutants and wild-type strain on methanol plus methylamine medium, methanol was added to 0.2% (vol./vol.) and methylamine was added to 0.2% (wt/vol.). *E.coli* strains were grown at 37°C in Luria broth. *Pseudomonas testosteroni* was grown on the same mineral salts medium used for *M.extorquens* AM1 and 0.3% (vol./vol.) ethanol was added as the carbon source. Agar was added to 1.5%(wt/vol.) for plates. Antibiotics were added to sterile medium in the following concentrations: rifamycin, 20μg/ml; tetracycline, 10μg/ml, ampicillin, 100μg/ml .

Bacterial matings. Tri-parental matings were performed as described previously. [5] The *M.extorquens* AM1 wild-type or mutant which was the recipient, the *E.coli* strain containing pRR8, and the *E.coli* HB101 strain containing the mobilizer plasmid pRK2073 were spotted on nutrient agar (Difco, Michigan) plates with no antibiotic in the ratio of about 5:1 of *M.extorquens* AM1 to *E.coli.* The conjugation was allowed to proceed overnight and then the mated mixture was plated on succinate minimal medium with appropriate antibiotics. Rifamycin was used to select for *M.extorquens* AM1 strains and tetracycline was used to select for the plasmid pRR8.

DNA manipulations. Plasmid DNA from *E.coli* strains was prepared according to the protocol by Gilles Morelle. [15] It worked efficiently for plasmids up to 40 kb. Restriction enzyme digestions, ligations and

transformations of DNA into *E.coli* DH5α were carried out as described in Maniatis et al. [14] Agarose gel electrophoresis was carried out in 0.8% gels with tris borate or tris acetate as the running buffer in a horizontal electrophoresis apparatus.

Preparation of cell free extracts *M.extorquens* AM1 strains were grown in liquid culture with appropriate additions of succinate, methanol, methylamine, vitamins and antibiotics. Cells were harvested, washed once with the ammonium-mineral salts medium, recentrifuged, and 150 mls of original culture was resuspended in 2mls of the same medium. Cells in the suspension were then broken by three passes through a French pressure cell at 20,000psi. Cell suspensions were kept on ice and the French pressure cell was chilled to 4°C. The cell extracts were centrifuged at 30,000g for 30min, and the supernatants were decanted and stored at -20°C. [17] In cases in which the catechol dioxygenase assay was done, a small amount of the lysate (about 200µl) was immediately spun at 14,000 rpm for 4 minutes in eppendorf tubes and the supernatant was assayed immediately for activity, as this enzyme was unstable.

Methanol dehydrogenase assay. Methanol dehydrogenase(MeDH) activity was assayed by the phenazine methosulfate(PMS)-dichlorophenol indophenol (DCPIP) dye linked method described previously. [17]

Catechol 2,3-dioxygenase activity (XylE assay) Catechol 2,3-dioxygenase (expressed from the *xylE* gene) transforms catechol into a yellow product, 2-hydroxymuconic semialdehyde. The enzyme reaction was conducted in a cuvette in a total volume of 1ml containing 960 µl of 50mM phosphate buffer

pH 7.5, 20 µl of cell-free protein lysate or dilutions and 20 µl of (10mg/ml) catechol. A kinetic assay was conducted at 376 nm using a Hewlett Packard model 8452A diode array UV-vis spectrphotometer. [25]

Protein determination. Protein was assayed using the Bio-Rad protein assay (Bio-Rad Lab., Richmond CA). Stock solutions of bovine serum albumin(BSA) were used as standards.

PQQ assay. The *Pseudomonas testosteroni* alcohol dehydrogenase apoenzyme was partially purified as described by Groen *et al.* [7] except that the cells were grown on ethanol, and a Tris-acryl column was used rather than a DEAE Sephacel column. No further purification of the apoenzyme was carried out. PQQ was measured in the following assay (F. Biville, personal communication). Apoenzyme (specific activity 0.7) was diluted 1 to 5 in 0.1 M potassium phosphate buffer, pH 7.0. 20 µl of diluted apoenzyme was then mixed with 170 µl 100 mM Tris-OH, pH 7.0, 3 mM $CaCl_2$ containing pure PQQ (1 to 5 picomoles) or various volumes of culture supernatant. The culture supernatant was obtained when the cells were harvested by centrifugation (as described in preparation of cell free extracts) KCN (10 µl) was added to a final concentration of 5 mM. The mixture was incubated at room temperature for 5 min. To this volume was added 800 µl containing 0.2 mM butanol, 55 mM Tris-OH, pH 7.0, 1.66 mM $CaCl_2$, 1.65 mM phenazine methosulfate (PMS), 0.1 mM dichlorophenol indophenol (DCPIP) and the kinetics followed for 2 min. at 610 nm. PQQ amounts were determined by comparison with a PQQ calibration curve.

Results

Construction of a *pqqD-xylE* transcriptional fusion.

The transcriptional start site of *pqqD* had been mapped and the direction of transcription was known (see previous chapter) so a 500 base pair fragment containing the *pqqD* promoter and part of the *pqqD* peptide coding region was linked to a promoterless *xylE* reporter gene to create a transcriptional fusion in pHX200, a low copy number plasmid with broad host range (see Figure 2). This plasmid was called pRR8. The 500 base pair fragment should contain the entire *pqqD* promoter, as *moxM* is located just upstream of *pqqD*, is transcribed in the same direction as *pqqD* and terminates in the 500 base pair fragment. [21]

Expression of *xylE* from the *pqqD* promoter. Plasmid pRR8 was conjugated into *M.extorquens* AM1 and *moxM*, *moxD*, *moxQ*, *moxE* and *moxB* mutant strains using triparental matings as described in Materials and Methods. Transconjugants were isolated and grown in liquid culture with either succinate or methanol plus methylamine as the carbon sources. Cells were harvested in exponential phase and cell-free extracts were prepared. Catechol 2,3-dioxygenase activity was then measured which gave an indication of the level of transcription from the *pqqD* promoter in the various mutant strains(TableII). Methanol dehydrogenase activity was also measured to confirm that the mutants had not reverted during growth. Methanol dehydrogenase activity of the mutant strains was always below detectable levels. In the wild-type and the *moxQ* mutant, the level of catechol dioxygenase activity in cells grown on methanol plus methylamine was two to three fold higher than the level on succinate, and in the *moxQ* mutant were comparable to that in the wild-type.

The catechol dioxygenase activity in the *moxM* and *moxD* mutants was about five to ten fold less than that in the *moxQ* mutant or wild-type, and also did not show induction in cells grown on methanol plus methylamine when compared to succinate. The catechol dioxygenase activity in the *moxB* mutant was intermediate between that in the *moxM* and *moxD* mutants and that in the wild-type strain, and it also did not show any induction in cells grown on methanol plus methylamine compared to succinate[Table II]. When pRR9, which contains the *pqqD* promoter in the opposite direction from the *xylE* gene was conjugated into wild-type and catechol dioxygenase activity was measured, it was found that the activity was negligible (less than 0.1 μmol min^{-1} mg $protein^{-1}$) both on methanol and on succinate.

PQQ assays. The catechol dioxygenase measurements gave an indication of how the transcription from the *pqqD* promoter was affected in the various regulatory mutants. As seven gene products are involved in PQQ biosynthesis, we also measured the levels of PQQ production in the mutant and wild-type strains grown on methanol plus methylamine or grown on succinate. The cells were harvested in exponential phase and the PQQ level was measured in both culture supernatants and cell-free extracts. All the detectable PQQ was excreted to the medium in both the mutants and wild-type strains, and a negligible amount of PQQ was accumulated within the cell as measured in cell-free extracts. Therefore only the PQQ level in the culture supernatant is reported in Table III. The levels of PQQ in *M.extorquens* AM1 wild-type grown on methanol alone and on betaine (trimethyl-glycine) were also measured. It was found that in the wild-type the level of PQQ was maximum during growth on methanol and was lowest during growth on succinate. The levels of PQQ production on methanol plus methylamine and on betaine were intermediate.

The levels of PQQ in the *moxQ* and *moxE* mutants were comparable to each other, while the PQQ levels in the *moxM*, *moxD* and *moxB* mutants were lower. In all the mutants the level of PQQ on methanol plus methylamine was four to six fold higher than on succinate, which is the same amount of induction observed with *moxF* transcription in wild-type cells grown on methanol compared to growth on succinate[17, Table III].

Discussion

The promoter probe vector pHX200 was used to study transcription from the *pqqD* promoter. XylE-promoter fusions using this vector had previously been shown to be correctly regulated in methylotrophic bacteria [25]. Background levels of catechol dioxygenase in cells containing the vector alone were negligible(less than 0.1 μmol min^{-1} mg $protein^{-1}$) (Morris and Lidstrom, unpublished observations).

The catechol dioxygenase measurements in the various regulatory mutants containing pRR8 led us to conclude that *moxM* and *moxD* were involved in the transcription and induction of *pqqD*, acting as positive regulators. In both mutants activities were low and non-inducible [Table II]. *MoxB* was required for induction of *pqqD*, but intermediate activity was observed in this mutant, suggesting that it had a moderate effect in transcription. *MoxE* and *moxQ* did not appear to be required for transcription or for induction, although the activity in the *moxE* mutant was about half that in the *moxQ* mutant. *MoxQ*, *moxE*, *moxM*, *moxD* and *moxB* gene products all act as positive regulators of transcription of the *moxF* promoter [17, 25, Morris and Lidstrom

unpublished observations] while the data presented show that only a subset of these are involved in transcription of *pqqD*.

It had previously been shown in *Methylobacterium organophilum* XX, a methylotroph closely related to *M.extorquens* AM1 that *moxM* and *moxD* acted as master regulators and were involved in the transcriptional regulation of two other methanol inducible promoters besides *moxF,* while *moxQ*, *moxE* and *moxB* appeared to be involved only in the regulation of *moxF* transcription. [25] Furthermore, preliminary sequence data suggest that *moxM* and *moxD* belong to the family of two component regulatory systems, with *moxD* acting as the sensor and *moxM* acting as the regulator. [21] *MoxM* and *moxD* are located adjacent to each other on the chromosome in *M.extorquens* AM1 (Fig.1). Similarly, *moxQ* and *moxE* are also located adjacent to each other on the *M.extorquens* AM1 chromosome and it is possible that they also form a two component regulatory system, although no sequence data are available for these genes as yet.

It is not yet known whether these gene products act together to simultaneously regulate the genes they affect, whether they act in a sequential, linear pathway, or a sequential, branched pathway. However our data clearly show that *pqqD* is regulated differently than *moxF*.

If the transcription of *pqqD* is the rate-limiting step for PQQ biosynthesis, we would expect that the PQQ levels measured would reflect the transcription patterns measured with the *pqqD-xylE* fusions. However, these correlations were not observed. Although the PQQ levels in the *moxM* and *moxD* mutants were lower than in the *moxQ* and *moxE* mutants, there was still considerable production of PQQ in the *moxM* and *moxD* mutants, to a level of about one-third that in the *moxQ* mutant. This was surprising, since the catechol dioxygenase

activity in the *moxM* and *moxD* mutants was 10-20 fold lower than in the *moxQ* mutant grown under similar conditions (Tables II, III). Therefore, transcription from the *pqqD* promoter does not appear to be the rate limiting step in PQQ production. Furthermore, even in *moxM* and *moxD* mutants there is induction of PQQ production when the bacteria are grown on methanol plus methylamine compared to when they are grown on succinate, and no such induction occurs for the *pqqD* promoter in these mutants. This indicates that transcription of *pqqD* is not the only level of regulation in PQQ production by *M.extorquens* AM1. It is not yet known at which point this regulation occurs. The data from the previous chapter show that the steady-state level of the *pqqD* transcript is similar in methanol and succinate grown cells, supporting the *pqqD-xylE* fusion data and suggesting that the regulation does not occur at the level of *pqqD* transcript stability. Instead, it must occur either via transcriptional regulation of other *pqq* genes, or via post-transcriptional regulation.

The level of PQQ production during growth on methanol plus methylamine for wild-type *M.extorquens* AM1 is lower than that for the *moxQ* or *moxE* mutants grown under similar conditions. This could be because under these growth conditions the wild-type strain uses both methanol plus methylamine for growth while the *moxQ* and *moxE* mutants are unable to use methanol as a carbon source. In these strains methanol serves only as an inducer, and the bacteria use methylamine for growth. Although it is not clear why this occurs, it probably reflects yet another facet of the complexities of PQQ regulation in *M.extorquens* AM1.

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Table I.

Bacterial strains and plasmids used in this study

Strains	Relevant traits	Source or reference
E.coli		
DH5α	r^-m^+ *recA*1 *lacZYA* φ80d*lac*Δ(*lac*Z)M15	BRL,Inc.
HB101	*recA* St^r	2
Pseudomonas testosteroni	ATCC 15667 (wild type)	7
M.extorquens AM1		
AM1 rif	Rif^r derivative	18
UV25rif	*moxB* mutant of AM1 rif	18
UV9rif	*moxD* mutant of AM1 rif	18
EMS7-20rif	*moxM* mutant of AM1 rif	11
EMS7.10	*moxQ* mutant of AM1 rif	11
AA31	*moxE* mutant of AM1 rif	18
Plasmid		
pRK2073	Sm^r mobilizing helper	4
pUC18/19	Ap^r,*lacZ*, multiple cloning site	26
pBluescript	Ap^r, *lacZ*, large multiple cloning site	Pharmacia

Plasmid	Relevant characteristics	Source or reference
pRK310	Tc^r, IncP1	3
pHX200	Tc^r, IncP1, promoterless *xylE* transcriptional fusion vector	25
pCM187	Ap^r, 2.1 kb *Hin*d III-*Eco*R I subclone from p1130D-HBg2.1 in pUC18	16
pRR7	500 base pair *Bgl* II *Eco*R I fragment from pCM187 is cloned between the *Bam*H I and *Eco*R I sites of pBluescript. It contains the entire *pqqD* promoter as well as a part of the protein coding region	this study
pRR8	500 base pair *Hin*d III *Xba* I fragment from pRR7 cloned into pHX200. Here the *pqqD* promoter is cloned in the correct orientation with respect to the *xylE* gene to create a transcriptional fusion	this study
pRR9	500 base pair *Xba* I *Kpn*I fragment cloned from pRR7 into pHX200. Here the *pqqD* promoter is cloned in the opposite orientation with respect to the *xylE* gene.	this study
p1130D-HBg2.1	pRK310 containing a 2.1 kb *Hin*d III-*Bgl* II fragment(Mox O) inserted between the *Hin*d III and *Bam*H I sites of pRK310	

Table II
Catechol dioxygenase assays in cells containing plasmid pRR8

All determinations were carried out at least twice and values agreed within +/- 20% except those indicated by * which agreed within +/- 40%.

Strain	Defective gene	Catechol dioxygenase (μmol.min^{-1} mg protein^{-1}) cells grown on		
		Succinate	Methanol + Methylamine	Methanol
AM1 rif		2.9*	5.7	2.3
EMS7-20	*moxQ*	2.3*	6.8	
AA31	*moxE*	1.1	3.3	
EMS7-20 rif	*moxM*	0.5	0.6	
UV9 rif	*moxD*	0.3	0.3	
UV25 rif	*moxB*	1.6	1.6	

Table III
PQQ assays

PQQ levels are reported in nmoles mg protein^{-1}

All determinations were carried out at least twice and values agreed within +/- 20% except those indicated by * which agreed within +/- 40%.

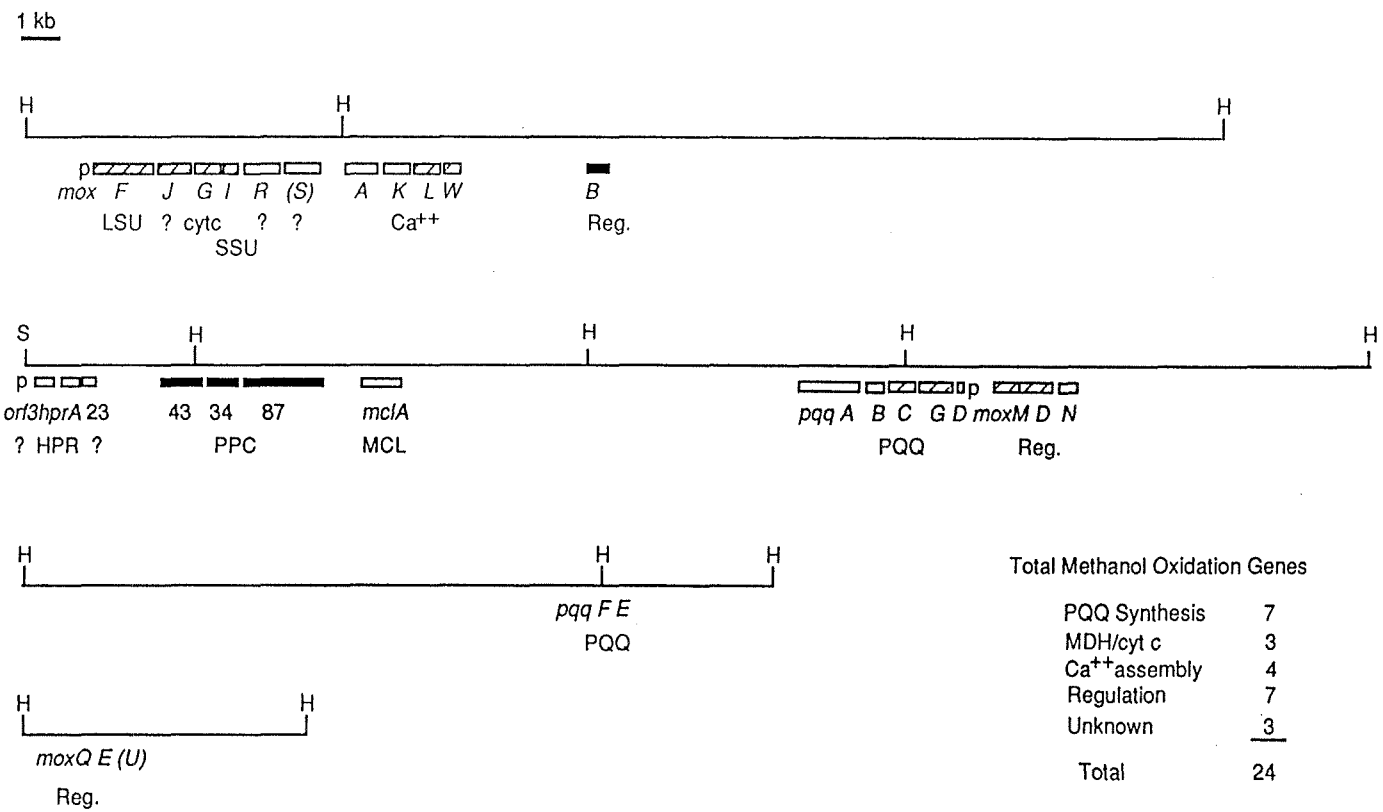
Strain	Defective gene	PQQ in cells grown on succinate	methanol + methyl-amine	methanol	betaine
AM1rif		12	32	59	32
EMS7.10	*moxQ*	11	64		
AA31	*moxE*	13	49		
EMS7-20 rif	*moxM*	2.5	19		
UV9	*moxD*	3.5	19		
UV4	*moxB*	7*	31*		

Figure Legends

Figure 1. Organization of methylotrophy genes in *Methylobacterium extorquens* AM1. Abbreviations: Reg- regulation, LSU- large subunit, SSU- small subunit, HPR- hydroxypyruvate reductase, MCL- malyl coA lyase, PPC- phosphoenolpyruvate carboxylase. Restriction enzyme abbreviations as follows H-*Hin*d III, S-*Sma* I. The HPR, PPC and MCL protein products are involved in the formaldehyde assimilation pathway (the serine cycle) while the rest of the genes shown here are involved in the oxidation of methanol to formaldehyde.

Figure 2. Plasmid map of pHX200 showing the multiple cloning site and the direction of transcription of the *xylE* gene. In pRR8 the *pqqD* promoter was cloned between the *Hin*d III and *Xba* I sites to generate a transcriptional fusion to *xylE* (figure taken from ref. 25) P stands for *Pst* I

Methylotrophy Genes in *Methylobacterium extorquens* AM1



Total Methanol Oxidation Genes

PQQ Synthesis	7
MDH/cyt c	3
Ca^{++}assembly	4
Regulation	7
Unknown	3
Total	24

FIGURE1

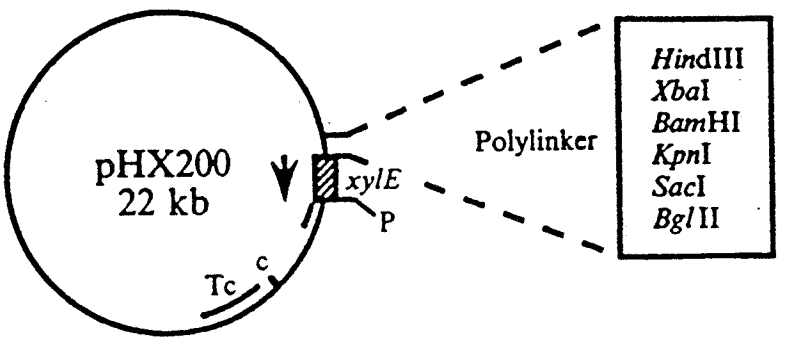

pHX200
22 kb
xylE
P
Tc
c
Polylinker
HindIII
XbaI
BamHI
KpnI
SacI
BglII

FIGURE 2

Chapter 4

Functional complementation of the putative protease PqqF of *Klebsiella pneumoniae* by PqqE of *Methylobacterium extorquens* AM1 and sequencing of a portion of *pqqE*

Abstract

Pyrroloquinoline quinone (PQQ) is the prosthetic group in several enzymes, including methanol dehydrogenase and glucose dehydrogenase. In *Klebsiella pneumoniae*, *Acinetobacter calcoaceticus* and *Methylobacterium extorquens* AM1, one of the genes required for PQQ biosynthesis encodes a peptide of 23 to 29 amino acids which contains conserved tyrosine and glutamate residues separated by three amino acids. This peptide is believed to be the precursor of PQQ biosynthesis in vivo.

In *K. pneumoniae* it was found that one of the genes required for PQQ biosynthesis encoded a polypeptide with similarity to proteases. It was proposed that this putative protease could be involved in PQQ biosynthesis by cleaving the peptide or a biosynthetic intermediate derived from the peptide. A *K. pneumoniae* mutant defective in the gene encoding the putative protease was complemented by a *M.extorquens* AM1 DNA fragment containing two *pqq* genes, and further complementation experiments showed that the mutant was complemented by *pqqE.* These data suggested that *pqqE* of *M.extorquens* AM1 encoded a protease. A portion of the region containing *pqqE* was sequenced, and an open reading frame was identified which showed 28% identity over 90 amino acids with the putative protease in *K.pneumoniae* (PqqF). These data provided a location and direction of transcription of *pqqE*, and suggested that *pqqF* was located upstream of *pqqE*.

Introduction

Pyrroloquinoline quinone (PQQ) is the prosthetic group of a number of bacterial dehydrogenase enzymes including methanol dehydrogenase, the enzyme that oxidizes methanol to formaldehyde in gram-negative methylotrophic bacteria[16,17], and glucose dehydrogenase, which oxidizes glucose to gluconate in several bacteria including *Klebsiella pneumoniae* and *Acinetobacter calcoaceticus* .[8, 9, 21, 22]

In *K.pneumoniae* six genes have been shown to be required for PQQ biosynthesis, namely *pqqA*, *pqqB*, *pqqC*, *pqqD*, *pqqE* and *pqqF*.[21, 22] In *A.calcoaceticus* four genes have been shown to be involved in PQQ biosynthesis, *pqq* genes (I, II, III, IV) in the order, IV-I-II-III. Between *pqq*I and *pqq*IV an additional open reading frame (*pqq*V), not required for PQQ biosynthesis is present.[8, 9, 10] In *M. extorquens* AM1 seven genes, *pqqA*, *pqqB*, *pqqC*, *pqqD*, *pqqE pqqF* and *pqqG* have been identified to be involved in PQQ biosynthesis. *PqqE* and *pqqF* are located together while the other five genes are present in a separate gene cluster in the order (D-G-C-B-A).[24, Chapter 2] Six PQQ complementation groups were also identified in *M.organophilum* DSM760 and correspond to the *M. extorquens* AM1 genes *pqqA*, *pqqB*, *pqqC*, *pqqD*, *pqqE* and *pqqF*, as determined by complementation of DSM760 *pqq* mutants with *M. extorquens* AM1 DNA encoding the *pqq* genes. [2, 24]

Tyrosine and glutamate have been shown to be the precursors of PQQ biosynthesis. When C^{13} labeled ethanol was fed to *M. extorquens* AM1 the level of C^{13} enrichment in PQQ was found to be identical to the level of C^{13} enrichment found in the amino acids tyrosine and glutamate.[12] It has previously

been reported that *pqqD* of *M. extorquens* AM1 encodes a 29 amino acid peptide, which contains a tyrosine and glutamate residue that are conserved in the equivalent peptides of *K.pneumoniae*, PqqA (23 amino acids) and *A.calcoaceticus*, PqqIV (24 amino acids), and are thought to be the precursors of PQQ biosynthesis in vivo [10, 22, Chapter 2].

It has been proposed that these small peptides play a role in PQQ biosynthesis as templates, providing the precursor substrates on a protein matrix where ring closure, hydroxylation and oxidation would occur before the release of PQQ.[22] This proposal would account for the inability of researchers to detect PQQ biosynthesis intermediates. In *K. pneumoniae*, PQQ biosynthesis requires a functional PqqF, which has sequence similarity with proteases from a variety of organisms.[22] PqqF could therefore be involved in cleavage of (a) peptide bond(s) resulting in the release of PQQ. Two other genes, located close to the *pqq* genes in *K. pneumoniae* (*orfX*) and in *A. calcoaceticus* (*orfR*), have sequence similarity with dipeptidases. [9,22] These polypeptides may play a role in formation of PQQ on the protein matrix, but in *K.pneumoniae, orfX* was not required for PQQ biosynthesis. The functions of the remaining *pqq* genes are unknown.

A *pqq* gene coding for a protein which shows similarity to proteases has only been found so far in *K.pneumoniae,* but if the protease function was necessary for PQQ biosynthesis it would be expected that this gene would be conserved in the other organisms that synthesize PQQ. Likewise, the putative dipeptidase might be expected to be conserved. This study was undertaken to determine whether any of the *pqq* genes in *M.extorquens* AM1 was equivalent to *pqqF* or *orfX* of *K.pneumoniae*.

Materials and Methods

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table I.

Media and growth conditions. *E. coli* strains were grown at 37°C in Luria broth. [18] *K. pneumoniae* KA216 was grown at 30°C in minimal medium A [28] with vitamins [29] and supplemented with 0.2% gluconate. Agar was added to 1.5% (wt/vol) for plates. Antibiotics were added to sterile media in the following concentrations: ampicillin, 100μg/ml, tetracycline, 10μg/ml, kanamycin, 50μg/ml, streptomycin, 10μg/ml.

DNA manipulations. Plasmid DNA from *E.coli* strains was prepared according to the protocol by Gilles Morelle. [23] It worked efficiently for plasmids up to 40 kb. Restriction enzyme digestions, ligations and transformations of DNA into *E.coli* DH5α were carried out as described in Maniatis et al. [18] Agarose gel electrophoresis was carried out in 0.8% gels with tris borate or tris acetate as the running buffer in a horizontal electrophoresis apparatus. DNA sequencing was done by the UCLA Sequencing Facility on an Applied Biosystems Model 373A automated sequencer.

DNA-DNA hybridizations. Digested plasmid DNA samples were subjected to electrophoresis in Tris-borate agarose gels, 0.8% (wt/vol) .[18] Gels were denatured, neutralized and then dried.[19] DNA fragments for radioactive probes were isolated from pBCP325 (Table I) using the Geneclean II ^{TM} kit (Bio 101, Inc., La Jolla, CA). Hybridization of gels with ^{32}P-αdCTP random prime labeled DNA (Boehringer Mannheim GmbH, Mannheim, FRG) was performed for 12 h at 45°C using 7X SSC (1X SSC is 0.05M NaCl plus 0.015M sodium citrate), 0.5%

(wt/vol) SDS, 11 mM Na-EDTA pH 8.0, and 0.5% (wt/vol) powdered milk. Washes were done at 45°C with 0.5X SSC and 0.1% (wt/vol) SDS.

Bacterial Matings. Matings were performed as described previously with the following modifications. [7] Tri-parental matings with *K. pneumoniae* were spotted onto Peptone plates (Peptone, 10 g/l; K_2HPO_4, 2 g/l) and incubated overnight at 30°C. Transconjugants were then plated onto glucose(0.2%wt/vol) minimal medium A, gluconate(0.2%wt/vol) minimal medium A and glucose Eosin-methylene blue (EMB) medium [13] each supplemented with appropriate antibiotics, tetracycline to select for the plasmid and kanamycin or ampicillin to select for the *K. pneumoniae* strain. *E.coli* HB101 with plasmid pRK2073 was used as the mobilizer.

Analysis of DNA and protein sequences. Protein and DNA sequences were analyzed using the Genetics computer group (GCG) sequence analysis software package .

Results

Similarity with *K. pneumoniae pqqF and orfX*. In order to assess whether any of the *M. extorquens* AM1 *pqq* genes identified were equivalent to *pqqF* (coding for a putative protease)or *orfX* (coding for a putative dipeptidase) of *K.pneumoniae*, hybridization and complementation experiments were carried out. [9, 22, 24] For hybridization, plasmid pBCP325 (Table I) was used as a source for DNA probes containing *K. pneumoniae pqqB*, *pqqF* and *orfX* .[22] A

1.3 kb *Eco*R I-*Bam*H I fragment was used for *pqqB*, and also contained 0.2 kb of the 5' end of *pqqC*. A 2.8 kb *Sma* I-*Bam*H I fragment was used for *pqqF*. It contains the entire *pqqF* and 0.6 kb of DNA 3' to *pqqF*. A 0.9 kb *Bam*H I-*Eco*R I fragment was used for *orfX*, which contains the C-terminal portion of *orfX*, and all of *pqqA*. All probes were purified from other plasmid sequences as described in Materials and Methods. The *K. pneumoniae pqqF* and *orfX* probes were hybridized with the DNA of *Hin*d III-digested pDN102 containing *M. extorquens* AM1 *pqqCBA* and 6.5 kb of DNA 3' to *pqqA* [24], and pELH2, containing *M. extorquens* AM1 *pqqE* and *pqqF*. The *pqqB* probe was used as a positive control, and was hybridized with *Hin*d III-*Eco*R I digested pCM181, containing *M. extorquens* AM1 *pqqG*, the equivalent to *K. pneumoniae pqqB* (data not shown). Under conditions that allowed clear hybridization of *K. pneumoniae pqqB* to *M. extorquens* AM1 *pqqG*, no hybridization was observed for either the *pqqF* or the *orfX* probes (data not shown).

Complementation analysis. In order to test whether any of the plasmids containing known *M. extorquens* AM1 *pqq* genes and adjoining DNA regions could restore PQQ synthesis in a PqqF mutant of *K. pneumoniae*, plasmids pDN102, pEL41 and pELH2 were conjugated into the *K. pneumoniae pqqF* mutant KA216 (Table I), selecting for the Tc^r marker on the plasmids and the Kan^r of KA216. Transconjugants were tested for color change on EMB-glucose medium, an indicator of PQQ-linked glucose dehydrogenase activity. pDN202 was used as a negative control. Transconjugants containing pELH2 turned purple on EMB plates indicating they had regained ability to ferment glucose (Table II). Under the same conditions the colonies with plasmid pDN202 or pDN102 remained white. These data indicated that the ability to ferment glucose in the cells containing pELH2 was not due to a reversion of the *pts*I mutation in KA216, and that pELH2 contained a gene that was functionally equivalent to *pqqF* of

K.pneumoniae. However growth of KA216 containing pELH2 was not obtained on minimal glucose plates containing kanamycin and tetracycline, suggesting a low level of complementation. This was presumably due to poor expression or poor functional complementation. To investigate which of the two *pqq* genes in pELH2 was equivalent to *pqqF* of *K.pneumoniae*, pELH2::TnphoA-E1 was used for complementation. pELH2::TnphoA-E1 contains an insertion that destroys complementation of *pqqE* mutants in *M.extorquens* AM1 but still complements *pqqF* mutants(ref. Table I). When pELH2::TnphoA-E1 was used in the complementation analysis, ampicillin was used to select for KA216 (it contains plasmid pAMH62 which is Ap^r) while pELH2::TnphoA-E1 was selected using tetracycline. When this plasmid was conjugated into KA216 the colonies were white, indicating that PqqF function was not restored. This indicates that in *M.extorquens* AM1 *pqqE* is functionally equivalent to *pqqF* in *K.pneumoniae.*

DNA sequence and analysis. The 4kb *Hin*d III fragment of pELH2 containing *pqqE* and *pqqF* of *M.extorquens* AM1 was subcloned as a 1.8 kb *Hin*d III *Pst* I fragment in pUC19 (pRR1) and a 2.2 kb *Hin*d III-*Pst* I fragment in pUC19 (pRR2) (ref. Table I, Figure1). These were sequenced on an automated sequencer as described in Materials and Methods. The sequence which was obtained was analyzed using the GCG software package. The universal primer was used for sequencing from the *Pst* I end and the reverse primer was used for sequencing from the *Hin*d III end of each subclone. The sequence obtained from the *Pst* I end of pRR2 and the sequence obtained from the *Pst* I end of pRR1 were linked and it was translated into amino acid sequence in all six open reading frames. Of these only one open reading frame did not show a stop codon in this region. When the amino acid translations in the six frames were run against the PqqF protein sequence of *K. pneumoniae* , maximum identity was found to the open reading frame with no stop codons. The identity was 28% over ninety amino

acids and this portion of PqqE corresponded to the middle portion of the *K.pneumoniae* PqqF (ref. Figure 3). From alignment of the amino acid sequences it was found that the *pqqE* of *M.extorquens* AM1 was transcribed across the *Pst* I site going from the 2.2 kb *Hin*d III-*Pst* I fragment to the 1.8 kb *Pst* I-*Hin*d III fragment. *pqqF* in *K. pneumoniae* is 2.8 kb.[22] If as expected *pqqE* of *M.extorquens* AM1 is of similar size, then the start of the open reading frame of *pqqE* should be 0.6 kb upstream of the *Pst* I site and *pqqE* should encode an open reading frame of about 2.4 kb in size[Fig.1, Fig.3].

The sequences from the *Hin*d III ends of pRR1 and pRR2 did not show significant homology to any entries in the data base, at either the DNA of amino acid levels, nor did they show significant similarity to the putative dipeptidase genes (*orfR* and *orfX*) from *K.pneumoniae* and *A.calcoaceticus* (Figs. 4 and 5).

Discussion

It has been proposed that in vivo the precursor for PQQ biosynthesis is a short peptide of twenty-three to twenty-nine amino acids containing conserved tyrosine and glutamate residues separated by three amino acids. It had been shown previously that one of the *K.pneumoniae pqq* biosynthesis genes *pqqF* shows similarity to a group of proteases, and it was speculated that this putative protease was involved in cleavage of one or more peptide bonds, perhaps to release an intermediate in PQQ biosynthesis. [24, 22]

Here we have confirmed the existence of a gene functionally equivalent to *pqqF* of *K.pneumoniae* in *M.extorquens* AM1, by functional complementation of the *K.pneumoniae pqqF* mutant strain KA216 with *pqqE* of *M.extorquens* AM1 (ref. Table I and Table II). DNA-DNA blot hybridizations failed to detect the protease gene in *M.extorquens* AM1 when the probe used was random prime

labeled *pqqF* of *K. pneumoniae*. This probably reflects the low level of amino acid similarity and the difference in the GC contents of the two organisms (52 to 59% for *K. pneumoniae* and 65 to 68% for*M.extorquens* AM1).

The *pqqE* gene of *M.extorquens* AM1 is equivalent to *pqqE* in *M.organophilum* DSM760.[24] Hence, both strains contain this putative protease gene.

It has been reported that the *pqqF* gene product of *K.pneumoniae* (761 amino acids) showed similarity to a family of proteases sequenced previously. Similarity was observed with protease III of *E. coli* (949 amino acids, 23% identical amino acids) and a human insulin degrading enzyme (952 amino acids, 21% identical amino acids). [22] When a search with the protein product of *pqq*F of *K. pneumoniae* was run on the motifs program of the GCG package, we found that it belonged to a class of metalloendopeptidases which include human insulin degrading enzyme and protease III of *E.coli* (ref. figure 6). These proteases are large (the genes are about 2.5 to 3 kb in size) and they act specifically on small peptides (about 7 kilodaltons or less i.e., eighty amino acids or less). That is interesting in view of the fact that the peptide containing tyrosine and glutamate is small (about 23 to 29 amino acids) and the protease could be expected to have specificity for this peptide. This family of proteases also show high conservation mainly at the N-terminal domain which contains the active site and the metal binding site. [1, 4] Since the sequence of *pqqE* reported here was from the middle portion and not the N-terminal end, it is not surprising that low similarity to PqqF was observed.

Our finding that two methylotrophs, *M.extorquens* AM1 and *M.organophilum* DSM760 contain the functional equivalents of the putative protease gene, pqqF, and that this is required for PQQ synthesis in these strains, provides strong support for the hypothesis that PQQ is synthesized from a

peptide precursor. Why do the bacteria first synthesize the peptide and then use the tyrosine and glutamate on the peptide as precursors for PQQ biosynthesis instead of using the free tyrosine and glutamate pool present in the cell? One possibility could be that the peptide serves as a kind of template, holding the tyrosine and glutamate close to each other so that specific reactions can occur. It is also possible that the peptide bonds to the tyrosine protect the NH_2-functional group while initial enzyme reactions take place, and then this could be released by cleavage with the protease for further reactions. The exact mechanism of PQQ biosynthesis will need further study.

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Table I.

Bacterial strains and plasmids used in this study

Strains	Relevant traits	Source or reference
E.coli		
DH5α	r⁻m⁺ *rec*A1 l*acZYA* ϕ80d*lac*Δ(*lac*Z)M15	BRL,Inc.
HB101	*rec*A St^r	3
K. pneumoniae		
KA216	*ptsI103*, *pqqF17*::Tn*10*, Km^r, contains plasmid pAMH62	P.W. Postma
Plasmids		
pRK2073	mobilizing "helper" plasmid, Sm^r	6
pVK100	Tc^r, Km^r, IncP cosmid	14
pRK310	Tc^r, *lacPOZ'*, IncP plasmid	5
pUC18/19	Ap^r, *lacZ'*, multiple cloning site	30
pBCP325	Tc^r, *K.pneumoniae pqqBCDEF*, non polar mutation in *pqqA* present	22
pAMH62	*lamB*, colE1 replicon, Ap^r	11

Plasmid	Relevant traits	Source or reference
Plasmids containing *M. extorquens* AM1 DNA		
pEL41	pVK100 containing a 24kb *Hind* III fragment (*pqq DGCBA*), tet^r	24
pDN102	pVK100 containing a 8kb *Hind* III fragment (*pqqBA* and part of *pqqC*), tet^r	24
pDN202	pVK100 containing a 7.5 kb *Hind* III fragment coding for *moxQ* and *moxE*, tet^r	15
p1130C	pRK310 containing a 3.4 kb *Pst* I-*Hind* III fragment (*pqqAB* and part of *pqqC*)	24
p1130D	pRK310 containing a 6.8 kb *Hind* III-*Pst* I fragment (*pqqDG* and part of *pqqC*)	24
pELH2	pRK310 containing a 4kb *Hind* III fragment (*pqqEF*)	24
pELH2::TnphoA-E1	pELH2 containing Tn*PhoA* in *pqqE*, Kan^r	24
pRR1	1.8 kb *Hind* III-*Pst* I fragment from pELH2 cloned into pUC19.	this study
pRR2	2.2 kb *Hind* III-*Pst* I fragment from pELH2 cloned into pUC19.	this study
pCM181	Ap^r, 7 kb *Hind* III-*Eco*R I subclone from p1130D in pUC18	24

Table II

Complementation of *K.pneumoniae* KA216 with *M.extorquens* AM1 DNA

The *K.pneumoniae* strain KA216 (see Table I) was used as the recipient for the complementation analysis. KA216 was selected using kanamycin or ampicillin while the donor plasmid was selected using tetracycline. Note + indicates growth while - indicates poor growth. For EMB plates purple color indicates ability to ferment glucose. For details of the plasmids refer to Table I.

Plasmid	Genes	EMB glucose	Minimum gluconate	Minimum glucose
pDN202	*moxQE*	white	+	-
pELH2	*pqqEF*	purple	+	-
pELH2:: TnPhoA-E1	*pqqF*	white	+	-
pDN102	*pqqAB* and part of *pqqC*	white	+	-
pEL41	*pqqDGCBA*	white	+	-

Figure Legends

1. Map of the *Hind* III fragment of pELH2 containing *pqqE* and *pqqF*. A *Pst* I site is located 1.8kb from one end and 2.2 kb from the other end. The 1.8 kb *Hind* III-*Pst* I fragment was subcloned into pUC19 to give pRR1 while the 2.2 kb *Hind* III-*Pst* I fragment was subcloned into pUC19 to give pRR2. The arrow indicates the direction of transcription of *pqqE* in *M.extorquens* AM1, the arrow plus box indicates the deduced *pqqE* and the hatched box indicates the region sequenced. Abbreviations: P-*Pst* I, H-*Hind* III. For more description of plasmids see Table I.

2. The DNA sequence across the *Pst* I site shown in Fig.1. The DNA sequence from the universal primer (*Pst* I end) of pRR2 was inverted and complemented and linked to the sequence from the universal primer (*Pst* I end) of pRR1. The direction of the DNA sequence is the same as that shown by the arrow in the previous figure. One base could not be read clearly and is marked as n standing for nucleotide. The DNA sequence of the *Pst* I site is underlined. The sequence from 1 to 127 is from pRR2 and the rest is from pRR1.

3. The translated amino acid sequence of a portion of *pqqE*, compared to PqqF of *K.pneumoniae*. The DNA sequence in Figure 2 was translated into amino acids in the six open reading frames and each was run against PqqF of *K. pneumoniae* using the FASTA program of the Genetics computer group (GCG) sequence analysis software package. The figure shows the alignment between the open reading frame which showed the maximum identity to PqqF of *K.pneumoniae* and the PqqF region to which this corresponds. There was 28% identity over ninety amino acids. note : pqqE stands for PqqE of *M.extorquens*

AM1. kpqqF stands for the PqqF of *K. pneumoniae*. The letters are standard one letter designations for amino acids. Vertical lines denote identical amino acids while dots denote conserved substitutions.

4. The DNA sequence going from the reverse primer (*Hin*d III end) of pRR1. This DNA sequence or the amino acid translation in any of the six open reading frames did not show any significant similarity with any sequence in the data base when the FASTA program was run using the Genetics computer group (GCG) sequence analysis software package.

5. The DNA sequence going from the reverse primer (*Hin*d III end) of pRR2. This DNA sequence or the amino acid translation in any of the six open reading frames did not show any significant similarity with any sequence in the data base when the FASTA program was run using the Genetics computer group (GCG) sequence analysis software package.

6. The signature sequence or consensus pattern present in all metalloendopeptidases of the insulinase family to which the PqqF of *K.pneumoniae* belongs. The signature sequence was found by running the PqqF protein on the motifs program of the Genetics computer group (GCG) sequence analysis software package and the match of PqqF with this signature sequence was 100%. [27] The capital letters are the one letter designations for the amino acids and X stands for any amino acid. This sequence is found in the N-terminal region of these proteases, and is not included in the sequence shown in Fig. 3. Note: groups of amino acids inside brackets denote that any of the amino acids within the brackets may be found at that position.

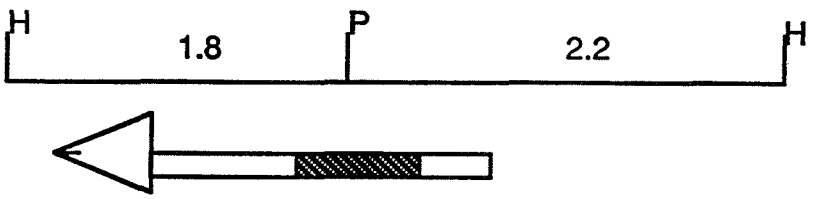

FIGURE 1

1 CCATCTCCAG CACCAGCTTG CGGTAGAGGA ACGAGGTCGA GCCGCCGCCG

51 ACGACCTCGG CGAGCAGNTC TCAGGCGTAG CCCTCGCCGT CGCGGGCGGT

101 CATGCAGGAG GGGGTGAGGT AGAGGCGCTG CAGGGTGGTC TGGCTCGACC

151 TTCGGGTCGG CCACCGCGAT CCGACGCATC GCCCGCGGCT CCGGCTCGCG

201 CGGGCGAGTG CGCAGGGCCG CGCGCCCTGC GGCGTCACCC GGCCGTAGTA

251 TCCTCGGCCA GACGCCGCAC CTCGTCCGGC GTCACGTCGC CGGACCACCA

301 CGAGGA

FIGURE 2.

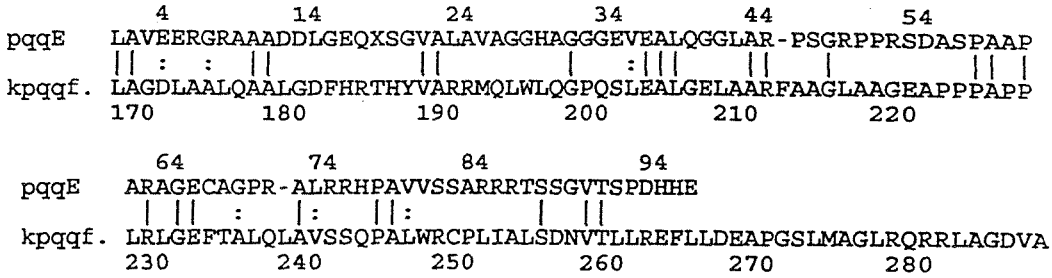
4 14 24 34 44 54
pqqE LAVEERGRAAADDLGEQXSGVALAVAGGHAGGGEVEALQGGLAR-PSGRPPRSDASPAAP
kpqqf. LAGDLAALQAALGDFHRTHYVARRMQLWLQGPQSLEALGELAARFAAGLAAGEAPPPAPP
170 180 190 200 210 220
64 74 84 94
pqqE ARAGECAGPR-ALRRHPAVVSSARRRTSSGVTSPDHHE
kpqqf. LRLGEFTALQLAVSSQPALWRCPLIALSDNVTLLREFLLDEAPGSLMAGLRQRRLAGDVA
230 240 250 260 270 280

FIGURE 3

1 GAGGTCAGGC GCGCCCCAGC CGCGTCAAGG TGCCATACCT TCGCCTGAAA

51 CCCGGCTCAC CTCGGTTTGC GTGGGCTCGA CGCGCTTCAC GATGAAGTGA

101 TGTCCGGCTG ACCGTCCCGG ATCAAAAGCG GGCGGGGCCG GTCCGTAGCC

151 TTGGGGAAAT CGGCATGAAC GGGCGGCGCA ACGTCTCGCA GGCTTTCGGA

201 TCG

FIGURE 4.

1 GCCGCTCCGC CGACGCGCAG AGAGAATGCG CGAGATGCTG TCGGTGCCGC

51 GCCGCTCGGC GNGCGCCGGC CAGTCGAAGA AGGTGATGTC GGTGCCGGGC

101 GAGGCGCGGC CGTCGGCGTA GAACAGGTGA TAGGCCGAGA CATCGTCCTG

151 ATTGACGGTC TTCTTCACGA GCCGGAGCCC CAGCACGC

FIGURE 5

G-X(9)-G-X-[STA]-H-[LIVMFY]-X-[DE]-[HRK]-[LIVMFY]-X-[LIVMFY]-X-[GST]

FIGURE 6

Chapter 5

Conclusions and directions for future work

Conclusions and directions for future work

This thesis focused on the genetics and regulation of pyrroloquinoline quinone (PQQ) biosynthesis in the pink pigmented facultative methylotroph, *Methylobacterium extorquens* AM1. PQQ is the prosthetic group of the enzyme methanol dehydrogenase, which is responsible for the oxidation of methanol to formaldehyde. It had previously been shown that seven genes, *pqqA*, *pqqB*, *pqqC*, *pqqD*, *pqqE*, *pqqF* and *pqqG* were involved in the biosynthesis of PQQ in *M.extorquens* AM1. Of these genes *pqqE* and *pqqF* are located together and separate from the other five genes which are clustered together in the order (*pqqD*, *pqqG*, *pqqC*, *pqqB*, *pqqA*).

The sequencing of *pqqD*, *pqqG* and part of *pqqC* was done by Dr. Christina Morris. It was found that *pqqD* encoded a twenty-nine amino acid peptide with conserved tyrosine and glutamate residues separated by three amino acids. The equivalent peptide-encoding genes are also found among the PQQ biosynthesis genes in *Acinetobacter calcoaceticus* and *Klebsiella pneumoniae*. Tyrosine and glutamate have been shown to be the precursors of PQQ biosynthesis and hence it is believed that this peptide acts as the precursor of PQQ biosynthesis in vivo. The transcriptional start site upstream of *pqqD* was mapped. A unique transcription start site was mapped to a guanidine nucleotide 95 bp upstream of the *pqqD* initiator codon. RNA blot analysis identified two transcripts, a major one of 240 bases and a minor one of 1300 bases. The larger transcript is predicted to encode *pqqD* and *pqqG* and terminate between *pqqG* and *pqqC*. The smaller transcript is likely to be terminated at a stem-loop structure within the second gene, *pqqG*, and encodes only *pqqD*. These conclusions were supported by data with transposon insertions. Two Tn*5lac* insertions were present in a plasmid containing the *pqq*

biosynthesis genes *pqqD* and *pqqG*. These transposons were in the correct orientation with respect to the direction of transcription of *pqqD* and *pqqG* to create a transcriptional fusion of the β-galactosidase gene with this region. Sequencing showed that both transposons were inserted in *pqqG,* but one of the transposons gave a ten-fold higher level of β–galactosidase than the other. This could be because this transposon interrupted a stem and loop structure which perhaps served as a terminator and so its transcription was linked to that of *pqqD* , while the β–galactosidase activity from the other transposon gave a measure of the level of transcription of *pqqG.*

The regulation of PQQ production was studied in strains of *M.extorquens* AM1 having mutations in *moxQ, moxE, moxB, moxM* and *moxD.* These five genes are required for transcription of genes involved in methanol oxidation. PqqD is believed to be the substrate for PQQ biosynthesis in vivo. Hence the transcriptional regulation of the *pqqD* promoter was studied in these five classes of regulatory mutants. A 500 base pair DNA fragment containing the entire *pqqD* promoter and part of the region coding for the peptide was cloned in front of a promoterless *xylE* gene to create a transcriptional fusion in pHX200, a low copy number, broad host range vector. The activity of the enzyme encoded by *xylE*, catechol dioxygenase was measured in wild-type and different mutants containing this construct. It was observed that in the wild-type and the *moxQ* and *moxE* mutants, catechol dioxygenase activity was two to three fold higher in cells grown on methanol plus methylamine compared to growth on succinate. The catechol dioxygenase activity for the *moxQ* mutant was comparable to that for wild-type while in the *moxE* mutant it was about half the wild-type level. The catechol dioxygenase activity in the *moxM* and *moxD* mutants was five to ten fold lower than in the *moxQ* mutant and also did not show any induction in cells grown on methanol plus methylamine compared to

growth on succinate. The catechol dioxygenase activity in the *moxB* mutant was at an intermediate level and also did not show any induction in cells grown on methanol plus methylamine compared to growth on succinate. These results indicated that the *moxM, moxD* and *moxB* gene products are all required for induction of *pqqD*, while the *moxQ* and *moxE* gene products are not. In addition, the *moxM* and *moxD* gene products seem to be required for high level transcription of *pqqD*. In order to compare the results for transcription of *pqqD* to the levels of PQQ produced by the cells, PQQ was measured in culture supernatants. It was found that the level of PQQ in the *moxM* and *moxD* mutants was lower than in the *moxQ* and *moxE* mutants, but in all the cases PQQ production was four to six fold higher when bacteria were grown on methanol plus methylamine compared to when the bacteria were grown on succinate alone. These data suggest that transcription of *pqqD* is not the rate limiting step in PQQ biosynthesis, and in addition that regulation of PQQ biosynthesis occurs at more than one step in the process.

It had been previously shown that one of the *pqq* genes, *pqqF* in *K.pneumoniae* encoded a protein which had identity with the insulinase family of proteases and it was speculated that this putative protease might be involved in the cleaving of one or more peptide bonds to release PQQ or some intermediate structure. By functional complementation of a *K.pneumoniae pqqF* mutant strain with a plasmid containing the *M.extorquens* AM1 genes *pqqE* and *pqqF,* it was shown that one of these genes in *M.extorquens* AM1 was equivalent to *pqqF* of *K.pneumoniae.* Further complementation experiments with a plasmid containing intact *pqqF* of *M.extorquens* AM1 but containing a Tn*phoA* insertion in *pqqE,* showed that *pqqE* in *M.extorquens* AM1 was the gene equivalent to *pqqE* in *K.pneumoniae.* Preliminary sequencing was also carried out in this region, which showed that the identity between the putative

protease genes of *M.extorquens* AM1 and *K.pneumoniae* was 28% over ninety amino acids. By aligning the two sequences, the direction of *pqqE* transcription in *M.extorquens* AM1 was also determined. Another interesting feature noted was that proteases belonging to the insulinase family specifically cleave small peptides of 7 kilodaltons or less. This is significant as this protease could then perhaps act specifically on the putative PQQ peptide precursor.

Much interest currently exists in solving the three-dimensional structure of proteins, and one of the commonly used methods for small proteins is solution NMR. 2D NMR using deuterium (^{2}H) and sometimes also ^{13}C NMR is used to resolve the structure of proteins. However the natural abundance of C^{13} is extremely low and if *Escherichia coli* is the organism used to express the protein whose structure is to be determined, then *E.coli* has to be fed C^{13}-labeled glucose. C^{13}-glucose is expensive to synthesize, while the cost of synthesizing C^{13}-methanol is 5-10% that of C^{13}-glucose. Hence if methylotrophic bacteria such as *M. extorquens* AM1, which can grow on methanol instead of glucose are used, the cost of doing C^{13} NMR of proteins would decrease ten to twenty fold. For this to be feasible it is necessary to find promoters which are active in *M.extorquens*. The *pqqD* promoter has been shown to be a strong constitutive promoter in *M.extorquens* AM1 and hence it was used in a model system. A transcriptional fusion between the *pqqD* promoter and the troponin C gene was created in the IncP1 vector pHX200. Attempts to conjugate this plasmid into *M.extorquens* AM1 failed and one of the reasons that this might have happened is that the troponin C expressed at high levels could be toxic to *M.extorquens* AM1. Troponin C is a calcium binding protein and it could be toxic to the cell by sequestering the available calcium, which might be needed by other calcium binding proteins in the cell.

Directions for Future Work. The transcriptional start site in front of *pqqD* was mapped and a transcript that included both *pqqD* and *pqqG* was found. It is not known whether a larger transcript exists covering *pqqD, pqqG, pqqC, pqqB* and *pqqA* which could not be detected with the RNA blots, or if there are internal start sites that would result in smaller transcripts. It would be interesting to look for transcriptional start sites between *pqqG* and *pqqC* .

From studies on the regulation of PQQ biosynthesis in the various regulatory mutants it appears as if *moxM* and *moxD* are master regulatory genes. It would be interesting to create transcriptional fusions of the other regulatory genes *moxQ, moxE* and *moxB* with a reporter gene (*xylE*) and measure the catechol dioxygenase activity in the *moxM* and *moxD* mutants and in this way find out if the transcription of the other regulatory genes are themselves regulated by the *moxM* and *moxD* gene products.

The *pqqD* promoter was used to attempt to obtain expression of troponin C for doing C^{13} NMR but this was unsuccessful. (reasons are discussed above) However the *pqqD* promoter is still a candidate for obtaining high level expression of proteins in *M. extorquens* AM1. This should be tested using other small proteins.

pqqE in *M.extorquens* AM1 was found to have similarity to a family of proteases. Preliminary sequencing has been done in this region, however the sequencing should be completed. It appears more and more likely that in vivo the peptide encoded by *pqqD* serves as the substrate for PQQ biosynthesis, and the putative protease encoded by *pqqE* would probably cleave one or more of the peptide bonds for the release of the final product PQQ or some intermediate. If antibodies were generated against the peptide, it would be possible to use these for immunoprecipitations in the *pqqE* mutant strains of

M.extorquens AM1. If cleavage by the putative protease is one of the final stages, it should be possible to precipitate intermediates in the PQQ biosynthesis pathway. This immunoprecipitation technique could also be used in the strains having mutations in the other PQQ genes, and then the immunoprecipitated products could be analyzed by mass spectrometry and NMR. By determining the extent of modifications, it may be possible to resolve the functions of the individual proteins in the PQQ biosynthesis pathway.

PQQ has a great deal of potential in pharmaceutical applications such as treatment of jaundiced patients and of patients with Alzheimers disease. In the future the market for PQQ may be much larger and then methylotrophs such as *M.extorquens* AM1 would be the organisms of choice for the production of PQQ as methylotrophs produce and excrete a large amount of PQQ to the culture supernatant. Another interesting direction for future work would be to vary the growth conditions, medium trace element composition etc. to optimize the production of PQQ by *M.extorquens* AM1.

Appendix 1.

Application of methylotrophs for C^{13} NMR of proteins

Introduction

The three-dimensional structure of proteins is currently an area of intense research. The three-dimensional structure of proteins provides information about how the protein folds and where various residues are present on the folded protein. It is also the three-dimensional structure of the protein which confers on proteins their power of recognition , selectivity and catalysis. Hence if the three-dimensional structure of the protein is known one can rationally change amino acid residues to give different perhaps more desirable properties to the protein. With present innovations in sequencing strategy such as the use of automated DNA sequencers the amino acid sequences for a large number of proteins are available, but the three-dimensional structures of only a very small subset of these proteins have been solved. [5] The two common methods used to determine the three-dimensional structure of proteins are Xray crystallography and solution NMR.

In order to use Xray crystallography to solve the structure of the protein, the protein has to be crystallized and some proteins are very recalcitrant to crystallization. Solution NMR is becoming more popular to solve the structure of proteins, since it does not require crystallization, and since it provides structural information for the protein in a more natural state. Solution NMR has some major limitations however. So far proteins of only small size (usually less than 20 kilodaltons) can be solved using solution NMR. To solve the protein structure, 2D NMR is used, mainly deuterium (H^2) NMR and sometimes to get more resolution of the protein structure (C^{13})NMR is also used. However the natural abundance of C^{13} is extremely low and if *Escherichia coli* is the organism used to express the protein whose structure is to be determined, then *E.coli* has to be fed C^{13}-labeled glucose. C^{13}-glucose is expensive and the cost

of the isotope alone can make solution NMR experiments prohibitively expensive. However, C^{13}-methanol can be synthesized for 5-10% of the cost of C^{13}-glucose. Hence if methylotrophic bacteria such as *Methylobacterium extorquens* AM1, which can grow on methanol instead of glucose are used for producing the protein the cost of carrying out C^{13} NMR of proteins would decrease ten-to twenty-fold. [6] Methylotrophs are naturally capable of producing proteins in large amounts, as methanol and methylamine dehydrogenases can both be expressed to levels as high as 20% of the total soluble protein, and so the potential exists to use methylotrophs as production strains.

In order to use *M.extorquens* AM1 to do C^{13} NMR of heterologous proteins, expression vectors are needed which can be used in this organism. The broad host range vectors with IncP1 origin of replication can be used in these organisms. However, well characterized promoters are needed which are active, as *E. coli* promoters are not always useful in these organisms.

The *pqqD* promoter has been shown to be a strong constitutive promoter in *M.extorquens* AM1(Chapter 3). The *pqqD* gene codes for a peptide containing conserved tyrosine and glutamate residues which is involved in PQQ biosynthesis in *M.extorquens* AM1. As the substrate peptide must be synthesized at high levels, the *pqqD* promoter is very strong [9; Chapters 2 and 3].

As a model system we attempted to express troponin C,(TnC) which is a calcium binding protein, under the control of the *pqqD* promoter in *M.extorquens* AM1. In muscle, TnC is present in the thin filament in a complex with troponinI and troponinT which serve to transmit information concerning the free calcium ion concentration from TnC to tropomyosin, actin and ultimately myosin, thereby regulating the contractile event. The crystal structure of

troponinC has already been solved and so it was considered a good model system to use for solution NMR. The protein is also of a small size of 18 kilodaltons, encoded by a gene of 500 base pairs. [13]

Materials and Methods

Strains and plasmids. The strains and plasmids used are listed in Table I.

Growth conditions. *E. coli* was grown at 37°C in Luria broth. *M. extorquens* AM1 strains were grown at 30°C on the ammonium-mineral salts medium described by Harder et al. [4] supplemented with a vitamin solution. [12] Succinate was added to 0.27% (wt/vol.), and methanol was added to 0.5% (wt/vol.) for growth of *M.extorquens* AM1. Agar was added to 1.5%(wt/vol.) for plates. Antibiotics were added to sterile medium in the following concentrations: rifamycin, 20μg/ml; tetracycline, 10μg/ml, ampicillin, 100μg/ml .

Bacterial matings. Tri-parental matings were performed as described previously. [3] The *M.extorquens* AM1 wild-type or mutant which was the recipient, the *E.coli* strain containing pRR10 or pRR8 and the *E.coli* HB101 strain containing the mobilizer plasmid pRK2073 were spotted on nutrient agar (Difco, Michigan) plates with no antibiotic in the ratio of about 5:1 *M.extorquens* AM1 to *E.coli.* The conjugation was allowed to proceed overnight and then the mated mixture was plated on succinate minimal medium with appropriate

antibiotics. Rifamycin was used to select for *M.extorquens* AM1 strains and tetracycline was used to select for the plasmid pRR10 or pRR8.

DNA manipulations. Plasmid DNA from *E.coli* strains was prepared according to the protocol by Gilles Morelle. [8] It worked efficiently for plasmids up to 40 kb. Restriction enzyme digestions, ligations and transformations of DNA into *E.coli* DH5α were carried out as described in Maniatis et al. [7] Agarose gel electrophoresis was carried out in 0.8% gels with tris borate or tris acetate as the running buffer in a horizontal electrophoresis apparatus.

Results and Discussion

Subcloning of the Troponin C gene in the broad host range vector pHX200. The troponin C gene from pGak11 (which contains the troponin gene cloned in pUC19) was cut with *Xba* I and *Sac* I and subcloned between the *Xba* I and *Sac* I sites of pRR8 so that a transcriptional fusion was created between the *pqqD* promoter and the troponin gene. In this construction a promoterless troponin gene with its own ribosome binding site was inserted between the *pqqD* promoter and the *xylE* gene of pRR8 to create plasmid pRR10 [Fig.1].

Attempts were made to conjugate plasmid pRR10 into *M.extorquens* AM1 according to the procedure described in Materials and Methods. No transconjugants were obtained under conditions in which pRR8 was transferred at high frequency. This suggested that the troponin protein was perhaps toxic to *M.extorquens* AM1, especially when it was expressed at high levels, which

would be the case when it was under the control of the *pqqD* promoter. Troponin C could be toxic to *M.extorquens* AM1 due to its function as a calcium binding protein. It might sequester calcium available in the cell, and inhibit growth. It has previously been shown in *Escherichia coli* that addition of calcium chelators such as EDTA leads to premature segregation of nucleoids. In addition the autophosphorylation of DnaK, a heat shock protein involved in initiation of chromosome replication in *E.coli* has been augmented ten-fold in vitro by the addition of calcium. [10]

Attempts to provide extra calcium to the cell were not successful, due to precipitation in the medium, and the project was not taken further. It would appear that *M.extorquens* AM1 may not be an appropriate system for testing this approach. However, another member of the laboratory, Yevgeny Gak, has been successful in expressing troponin C in a different class of methylotroph, *Methylobacillus flagellatum* KT, and the project will be pursued in that strain.

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TABLE I.

Bacterial strains and plasmids used in this study.

Strains	Relevant traits	Source or reference
E.coli		
DH5α	r^-m^+ *recA*1 l*acZYA* ϕ80d*lac*Δ(*lac*Z)M15	BRL,Inc.
HB101	*recA* St^r	1
M.extorquens AM1		
AM1 rif	Rif^r derivative	11
Plasmid		
pRK2073	Sm^r mobilizing helper	2
pUC18/19	Ap^r,*lacZ*, multiple cloning site	15
pHX200	Tc^r, IncP1, promoterless *xyl E* transcriptional fusion vector	14
pRR8	500 base pair *Hind* III *Xba* I fragment from pRR7 cloned into pHX200. Here the *pqqD* promoter is cloned in the correct orientation with respect to the *xylE* gene to create a transcriptional fusion	Chapter 3
pRR10	500 base pair *Xba* I *Sac* I fragment from pGak11 containing the troponin gene cloned into pRR8 so that there is a transcriptional fusion between the troponin gene and the *pqqD* promoter.	this study
pGAK11	The troponin gene was subcloned into pUC19	Yevgeny Gak, 13

FIGURE LEGEND

1. This figure gives a partial map of pRR10 indicating the region where the troponin gene was subcloned. Here a transcriptional fusion is created between the *pqqD* promoter and the troponin gene which is inserted between the *pqqD* promoter and the *xylE* gene. For more details refer to Table I. Abbreviations: H: *Hin*d III, X: *Xba* I, S: *Sac* I, B: *Bgl* II, P: *Pst* I.

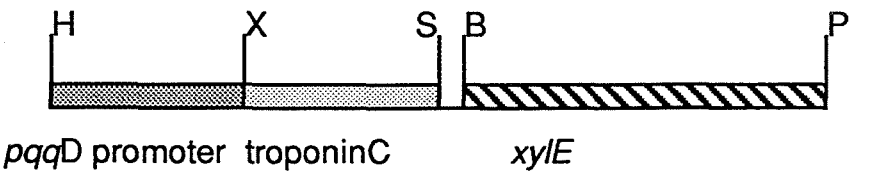

FIGURE 1