MOLECULAR DISSECTION OF MINERAL PHOSPHATE SOLUBILIZATION IN *Acetobacter diazotrophicus* Pal5

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CERTIFICATE

This is to certify that the thesis entitled "**MOLECULAR DISSECTION OF MINERAL PHOSPHATE SOLUBILIZATION IN *Acetobacter diazotrophicus* Pal5** " submitted by **Ms. JYOTHI S.** for the degree of **MASTER OF SCIENCE (AGRICULTURE)** in **AGRICULTURAL MICROBIOLOGY** to the University of Agricultural Sciences, Dharwad is a record of research work done by her during the period of her study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any other degree, diploma, associateship, fellowship or other similar titles.

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(***Jyothi, S.***)

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Introduction

I. INTRODUCTION

Phosphorus, a major nutrient for crop plants is involved in many essential processes including cell division, photosynthesis, break down of sugar, energy transfer and nutrient transfer within the plant. (Tandon, 1987). Plants obtain phosphorous from the soil in the form HPO_4^{2-} and $H_2PO_4^-$. However, major portion of the applied phosphatic fertilizer gets fixed into unavailable forms in the soil, resulting in only 10 percent becoming available to plants. The concentration of total P in soil ranges from 0.02 to 0.5 percent and averages approximately 0.05 percent (Barber, 1984). The concentration of P in the soil solution, which is the major sink of available P for plant uptake is 0.05 mg/l and seldom exceeds 0.3 mg/l (Ozanne, 1980).

The importance of microorganisms in soil nutrient cycling and their role in plant nutrition has been realized for a long time. Microorganisms are known to recycle phosphates in soil. Many rhizosphere (Katznelson and Bose, 1959) and endorhizosphere bacteria (Krishnaraj and Gowda *et al.,* 1990) are capable of releasing P_i in the readily available form as $H_2PO_4^-$ and HPO_4^{2-} from insoluble fixed forms by a phenomenon called mineral phosphate solubilization. Phosphate solubilizing organisms have been reported to solubilize insoluble forms of P by excreting organic acids that directly dissolve phosphatic mineral forms and chelate the cationic partners of the P ion (Sperber, 1958; Katznelson and Bose, 1959).

Although inoculation of MPS bacteria has resulted in increased P_i uptake and economic yield in crop plants, further genetic improvement of MPS microbes to achieve stable inheritable characteristics towards enhanced efficiency of the MPS activity is a desirable step. (Kucey *et al.*, 1989; Gaur 1990). Bacterial genes involved in mineral phosphate solubilization have been

proposed (Goldstein, 1986). A fragment from *Erwinia herbicola* necessary for gluconic acid production in *E. coli* has been shown to express enhanced dissolution of hydroxiapatite (Liu *et al.*, 1992).

The mechanism of MPS activity has been reported due to the production of organic acids (Sperber, 1957, Gaur, 1990), production of chelating substances (Tinker, 1980) and direct acidification (Illmer and Schinner, 1992). Organisms like *Pseudomonas, Erwinia, Burkholderia* are known to produce gluconic acid via glucose dehydrogenase (GDH), a quino protein requiring pyrroloquinoline quinone (PQQ) as a co-factor.

Acetobacter diazotrophicus, a promising endophytic MPS bacteria (Mahesh *et al.*, 1999) has been shown to possess *pqq* linked *gdh* (Galar, 1995). However, genetic characterization of MPS loci through isolation and analysis of MPS mutants is lacking. In view of insufficient information on genetic basis of MPS, the present study was initiated with the following objectives;

1. Development and collection of random MPS defective mutants of *A. diazotrophicus.*

2. Cloning of *gcd, gnd* and *pqq* gene from *A. diazotrophicus.*

3. Transgenic expression of *mps* genes in *Azospirillum.*

Review of Literature

II. REVIEW OF LITERATURE

Phosphorus is an essential element for plant growth. Although the agricultural fields contain good phosphorus reserves as a result of regular application of fertilizers, their availability for plants is less due to the chemical processes that "fix" soluble phosphorus of fertilizers to insoluble form not available for plant nutrition. Hence, the development of strategies that facilitate the acquisition of phosphate by plant receives great attention.The soil contains microorganisms that release Pi from fixed phosphates and allow the plants to absorb the released phosphates. The phenomenon is better termed as Mineral Phosphate Solubilization (MPS). An understanding of the mechanism of the Pi release from fixed phosphates and the regulation of the activity would lead to directed improvement of such traits in microbes to enhance their utility in bioameliorating Pi. Hence, the present study focuses on the genetic characterization of *Acetobacter diazotrophicus* Pal5, identified to possess the mineral phosphate solubilization phenotype (MaheshKumar *et al.*, 1999). The literature generated by the research in this field is reviewed here.

2.1 PHOSPHOROUS AS A NUTRIENT

Phosphorus, a major nutrient for crop plants is involved in many essential processes including cell division, photosynthesis, breakdown of sugar, energy transfer and nutrient transfer within the plant (Tandon, 1987; Armstrong, 1988). Commensurate 'P' nutrient benefits the plants by producing deeper and abundant roots, enabling uniform maturity in time, providing protection against biotic and abiotic stress and results in high yield of superior quality produce (Tandon, 1987).

2.2 PHOSPHORUS FLUX IN SOIL

Soils are often rich in insoluble organic and inorganic phosphates but deficient in available orthophosphate (P_i). Nearly 90% of the applied phosphate fertilizer is fixed in the soil, rendering these fertilizers unavailable to plant (Cosgrove, 1977; Kucey *et al.,* 1989). The soil P can be generally categorized as soil solution P, insoluble inorganic P or insoluble organic P. The immediate source of P for plants is a small amount that is in the soil solution. In soil, phosphate is bound to Ca^{+2}, Al^{+3} and Fe^{+3}. The calcium complexes of phosphate get solubilized when pH drops down below 5.0 (Gyaneshwar *et al.,* 1998).

The concentration of P in the soil solution is about 0.05 mg/l and seldom exceeds 0.3 mg/l in unfertilized soils (Ozanne,1980). Both organic and inorganic phosphates exist in soil. The organic phosphates are derived from plants and microorganisms and are composed of nucleic acids, phospholipids and phytin (Salisbury and Ross, 1992). Of the P forms taken up by the cell, the major ionic forms are $H_2PO_4^-$ or HPO_4^{-2}.

2.3 MINERAL PHOSPHATE SOLUBILIZATION BY BACTERIA

Soil microorganisms are involved in a range of processes that affect P transformation and influences the subsequent availability of phosphate to plant roots (Richardson, 2001). Certain microorganisms including fungi, actinomycetes, yeast and bacteria have the capacity to convert inorganic unavailable 'P' form *viz.,* varisite [$AL(OH)_2H_2PO_4$], stringite [$Fe(OH)_2H_2PO_4$], flourapatite [$Ca_{10}(PO_4)7_2$], hydroxyapatite [$Ca_5(PO_4)_3OH$] and tricalcium phosphate [$Ca_3(PO_4)_2$] to primary orthophosphate (H_2PO_4) and secondary orthophosphates (HPO_4^{-2}) (Cosgrove, 1977). The microbial biophores

solubilize inorganic phosphorus compounds and increase the availability of phosphate in the rhizosphere (Gerretsen, 1948; Sundara Rao, 1963; Gaur 1990; Jackson *et al.,* 1973; Bardiya and Gaur, 1972; Arora and Gaur, 1979; Santi, 1998; Deepa, 2000), which can be taken up by plants. The involvement of microorganisms in the solubilization of insoluble phosphate was first shown by Stalstorm in 1903.

The biological process of conversion of unavailable fixed form of phosphorus into primary orthophosphate ($H_2PO^-_4$) and secondary orthophosphate (HPO_4^{-2}) has been termed as mineral phosphate solubilization (MPS) (Goldstein, 1995). The phenomenon is a characteristic trait of many rhizosphere and endorhizosphere bacteria (Katznelson, 1959; Krishnaraj, 1990).

Phosphate solubilizing microorganisms, in addition to directly helping plant uptake of solubilized P have other beneficial roles including production of plant growth promoting substances, antibiotics *etc.*, Solubilization of the precipitated calcium phosphate in agar medium has been used as the initial criterion for isolation and enumeration of MPS microorganisms (Sperber, 1958a; Katznelson and Bose, 1959). The identification of MPS phenotype in microorganisms led to the isolation of different genera of microorganisms from different locations. A partial list of the different genera of microbes isolated is given in Table 1.

2.4 MECHANISM OF MINERAL PHOSPHATE SOLUBILIZATION

The genetic and biochemical mechanisms for the solubilization are as varied as the spectrum of P containing soil compounds (Goldstein, 1987). One adaptive strategy could involve specific association between plant roots

Table 1: List of diverse groups of MPS microbes isolated by various workers

Organism	Niche	Reference
Actinomycetes		
Streptomyces sp.	Gangetic aluvival poil	Banik and Dey (1982)
Streptomyces sp.	Rhizosphere of rye, wheat	Molla *et al.* (1984)
Bacteria		
Bacillus megatherium	Soil	Bardiya and Gaur (1972)
Bacilus polymyxa	Soil	Gaur and Bardya (1972)
Serratia sp.	Rhizosphere of rye, wheat	Molla *et al.* (1984)
Pseudomonas sp.	Rhizosphere of rye, wheat	Molla *et al.* (1984)
Pseudomonas sp.	Forest soil	Illmer and Schinner (1992)
Micrococcus sp.	Gangetic aluvival soil	Banik and Dey (1982)
Arthrobacter sp.	Gangetic alluvival soil	Banik and Dey (1982)
Acetobacter diazotrophicus	Sugarcane rhizosphere	Cavalcante and Dobereiner (1988)
Cynobacteria	Not known	Roychoudhury and Kaushik (1989)
Westillopsis prolifica *Calothrix branii*		
Fungi		
Fusarium solani	Rice rhizosphere	Barthakur (1978)
Penicillum sp.	Cultivated and Virgin	Kucey (1983)
Aspergillus niger	Alberta soil Roots of mangroove	Vazquez *et al.* (2000)
Yeast		
Schwanniomyces occidentalis	Rhizosphere of cowpea	Taha *et al.* (1969)
Saccharomyces cervisiae	Soil	Narsian and Patel (1995)

and MPS bacteria. Phosphate solubilizing organism have been reported to solubilize inorganic forms of P by excreting organic acids that directly dissolve phosphatic materials and/or chelate cationic partners of the 'P' ion (Sperber, 1958; Katznelson and Bose, 1959). Analysis of culture filtrates of pure isolates of these organisms has revealed a number of organic acid products, (Table 2), all of which have chelating properties and could serve as active components of P solubilization (Sperber, 1958; Duff *et al.,* 1989; Taha *et al.,* 1969; Banik and Dey, 1981, 1982). The most effective MPS phenotype in gram-negative bacteria results from the extra cellular oxidation of glucose to gluconic acid via the activity of quinoprotein, glucose dehydrogenase (Goldstein 1987; Goldstein, 1993). Duff *et al.,* 1989 observed that 2-ketogluconic acid produced by several MPS bacteria and fungi affected the release in solution of numerous phosphate and silicate materials. Moghimi and Tate (1978) concluded that the main action of 2-ketogluconic acid was to act as a source of hydrogen ion in the dissolution of calcium phosphates. Direct correlations of the pH drop and increase in available 'P' has been observed in certain cases (Sperber, 1957; Agnihotri, 1970; Lin *et al.,* 1992). Largely, however, the correlation doesn't seem to be there (Mehta and Bhide, 1970; Wani *et al.,* 1979; Krishnaraj, 1987; Asea *et al.,* 1988). Other postulated mechanisms involve reduction of Fe in anaerobic condition and flooded soils (Alexander, 1979), liberation of H_2S which react with ferric phosphate to yield ferrous sulphide and liberate PO_4 (Sperber, 1957).

2.5 MOLECULAR BIOLOGY OF MPS

The MPS phenotype has historically been of interest to agricultural microbiologists. (Goldstein, 1994). Goldstein in 1993 has shown that the strong MPS phenotype exhibited by *Erwinia herbicola* and *Pseudomonas*

Table 2: **Principle organic acids produced by phosphate solubilizing microorganisms**

Sl. No.	Organism	Organic acid produced	References
1	*Acetobacter diazotrophicus*	Gluconic	Galar & Boiardi, 1995
2	*Arthrobacter*	Oxalic, malonic	Banik & Dey, 1982
3	*Bacillus firmus*	2-ketogluconic	Banik & Dey, 1982
4	*Bacillus megatherium*	Lactic, malic	Taha *et,al.*, 1969
5	*Bacillus subtilis*	Lactic, citric	Taha *et,al.*, 1969
6	*Micrococcus* Sp	Succinic, lactic	Taha *et,al.*, 1969
7	*Streptomyces* Sp	2-ketogluconic	Banik & Dey, 1982
8	*Pseudomonas* Sp	Citric, gluconic	Taha et,al., 1969

cepacia is the result of gluconic acid mediated dissolution of calcium phosphate. The gluconic acid is produced in the periplasmic space by direct oxidation pathway. The first step in this pathway is the oxidation of glucose to gluconic acid via the membrane bound glucose dehydrogenase (GDH). This enzyme contains 2,7,9-tricarboxyl-IH-pyrrolo(2,3,-f)-Quinoline-4,5-Quinone (PQQ) as a prosthetic group, so that the functional holoenzyme is quinoprotein (Ameyama, 1981; Goldstein *et al.,* 1993). Quinoproteins play a major role in the bioenergetic process in many gram negative bacteria including *Pseudomonas* species (Duine, 1991). The enzymes of the direct oxidation pathway are oriented in the cytoplasmic membrane such that glucose or other aldose sugars undergo upto three oxidations of two electrons and two protons in the periplasmic space. As a result, gluconic, 2-ketogluconic and / or 2,5-diketoglconic acid are formed in the periplasmic space (Goldstein, 1993). The first description of glucose dehydrogenase and its prosthetic group was given by Hauge in 1964. Direct oxidation of glucose to gluconic acid, generates a transmembrane proton motive force (PMF) that may be used for bioenergetic or membrane transport functions (Van Schie, 1985). The second oxidation, catalyzed by gluconate dehydrogenase results in the production of 2-keto gluconic acid, one of the strongest naturally occurring organic acids known and one which has historically been associated with bacteria selected for extremely high levels of calcium phosphate solubilization (Goldstein 1986).

2.6 BIOLOGY OF PQQ

A pyrroloquinoline quinone dependent glucose dehydrogenase from an isolate of *Erwinia* sp has been purified to homogeneity and the SDS-page showed a single band of 88.4 kDa. (Liucija, 1999). In 1964, Hauge demonstrated that the glucose dehydrogenase from *Acinetobacter*

calcoaceticus contained an unknown cofactor, which was proposed by Duine *et al.,* 1991 to be pyrroloquinoline quinone (PQQ). It was found that several species, such as *Escherichia coli* (Hommes *et al.,* 1984) could not synthesize PQQ but only possessed the capacity to synthesize the glucose dehydrogenase apo-enzyme. Addition of PQQ to the whole cells resulted in the production of gluconic acid from glucose. The *in vitro* reconstitution of apo-glucose dehydrogenase with PQQ was dependent on the presence of Ca^{2+} and Mg^{2+} (Van schie, 1987).

In *Klebsiella pneumoniae,* six genes constituting the *pqq* ABCDEF operon, required for the synthesis of the cofactor PQQ has been identified (Velterop, 1995). All the six genes were required for PQQ biosynthesis and excretion into the medium in sufficient amount to allow the growth of *E. coli* on glucose via the PQQ dependent glucose dehydrogenase. (Velterop, 1995).

It was concluded that *E. coli* cannot synthesize PQQ and that it lacks genes required for PQQ biosynthesis, but has the apo-GDH (Matsushita, 1997). However, the enzyme can be easily reconstituted to a functional dehydrogenase by the presence of PQQ in the environment because its active centre faces the periplasm (Hommes, 1984). The biosynthetic path / route of PQQ has not been elucidated yet, but it has been proposed that glutamate and tyrosine are precursors of PQQ (Houck *et al.,* 1988, 1991; Van Kleef and Duine, 1988). That *E. coli* is unable to produce PQQ is supported by the fact that the nucleotide sequence of the *E. coli* genome contained no ORFs that could encode proteins with similarity to other PQQ proteins. (Matsushita, 1997). In 1995, Babu-Khan *et al.,* reported the cloning of mineral phosphate solubilizing gene from *P. cepacia.* The plasmid construct containing a functional mineral phosphate-solubilizing gene from *P. cepacia* E-37 was

expressed in *E. coli* JM109 or HB101 which resulted in both dissolution of exogenous hydroxyapatite and production of gluconic acid (Babu-Khan, 1995). The MPS gene designated as *gab*Y showed the production of a 14.75 kDa translation product. The disruption of *gab*Y via site directed mutagenesis resulted in the loss of both the MPS phenotype as well as the ability to produce gluconic acid.

2.7 REGULATION OF PQQ SYNTHESIS

An investigation of PQQ synthesis and production of the apo or holoenzymes of dehydrogenase, in a variety of bacteria indicated that PQQ synthesis is not essential for apoenzyme production (Van Kleef and Duine, 1988). On the basis of DNA sequence analysis, it has been suggested that the *pqq* A and *pqq* B gene of *K. pneumoniae* and *M. extorquens* and the equivalent genes in *A. calcoaceticus* are co-transcribed. Moreover, *pqq* A encodes the precursor of PQQ and is needed in larger amounts than the products of other *pqq* genes, which presumably have catalytic role in processing the precursor. *Erwinia herbicola* genes necessary for gluconic acid production was cloned into *E. coli* HB101(Liu, 1992). As a result of gluconic acid production, *E. coli* HB101(pMCG898) showed enhanced mineral phosphate solubilizing phenotype due to acid dissolution of hydroxyapatite substrate. 2- keto-gluconic acid is another one of the strongest naturally occurring organic acid known, which has been associated with extremely high level of calcium phosphate solubilization (Goldstein, 1986).

2.8 BIOLOGY OF GLUCONATE DEHYDROGENASE

The gene cluster encoding three subunits of membrane-bound gluconate dehydrogenase (GADH) from *Erwinia cypripedii* was cloned in *E. coli* by performing a direct-expression assay. The positive clone converted D-gluconate to 2-Keto-D-gluconate(2 KDG) in the culture medium. Nucleotide sequence analysis of the GADH clone revealed that the cloned fragment contained the complete structural gene for 6.8 KDa dehydrogenase sub unit, a 47 KDa cytochrome C sub unit and a 24 kDa sub unit of unknown function and that the genes were clustered with the same transcriptional polarity. (Yum, 1997).

Direct oxidation is one of the four major metabolic pathways for glucose (aldose) utilization in bacteria. Furthermore, the activation of direct oxidation pathway or the presence of gluconic acid induces Entner- Douderoff pathway in *E. coli* (Egan, 1992). PQQ-GDH plays a bioenergetic role in energy metabolism of certain gram negative bacteria. In several bacterial species, it has further been shown that the efficiency of uptake of solutes such as alanine, lactose and proline is modified by PQQ-GDH mediated electron transfer (Van schie, 1987). Little is known however about the molecular, biochemical and genetic regulatory mechanisms by which the cells switches between the phosphorylative and periplasmic oxidative mode.

2.9 *Acetobacter diazotrophicus*

A diazotrophicus, an endophyte of sugarcane represents a model system for monocot-diazotroph association. In 1988, Cavalcante and Dobereiner reported an acid-tolerant N-fixing bacterium and called it *Saccharobacter nitrocaptans.* However, in the addendum of the paper they

proposed, based on the DNA/DNA TM values and DNA/DNA binding values, the name to be changed to *Acetobacter nitrocaptans*. Based on additional phenotypic and chemotaxonomic analysis, Gillis *et al.*, in 1989 proposed it to be renamed as *Acetobacter diazotrophicus. A. diazotrophicus* has been now referred to *Glucanobacter diazotrophicus* (Muthukumarasamy *et al.,* 2001). However, the name *Acetobacter diazotrophicus* continues to be synonymously used.

A. diazotrophicus is a gram negative, acid tolerant obligate aerobe, straight rods with rounded ends (0.7 - 0.9 m by 1-2 m) with one to three lateral flagella. It grows well at a temperature of 25-30^{0}C having a pH optima of 5.4 to 6.3 (John *et al.,*1994). The bacterium grows on high sucrose concentration (10% sucrose) and very low pH (3.0) and have the ability to fix N_2 under microaerophilic condition. (Cavalcante,1988; Hartman,1987; Stephan,1991). It colonizes the plant species *Saccharum* sp, *Pennisitum purpureum, Ipomea batatas* and *Coffea arabica* (Cavalcante, 1988; Egener, 1998, Fuentes-Ramirez, 1993). *A. diazotrophicus* produces different plant hormones in culture media, including cytokinins and indoleacetic acid. Some of the crops in which the organism colonizes are listed in Table 3. Most of the *A. diazotrophicus* isolates were found to be tolerant to streptomycin, tetracycline, rifampicin, ampicillin, erythromycin and roxithromycin (Muthukumarasamy, 2000; Mowade, 2000).

A. diazotrophicus possess a pyrroloquinoline quinone linked glucose dehydrogenase (PQQ-GDH) (Galar, 1995). In glucose containing media, oxidation of glucose to gluconate by *A. diazotrophicus* is essential for the start of exponential growth. The extra cellular of oxidation of glucose to gluconate plays a major role in the first step of glucose metabolism by *A.*

Table 3: **Plant sources from where *Acetobacter diazotrophicus* has been isolated**

Source	Part	Reference
Sugar cane	Root, root hair, stem, leaf.	Muthukumarasamy (2002)
Cameroon grass	Root stem	
Sweet potato	Root stem tuber	
Coffee	Root, rhizosphere, stem	
Ragi	Root, rhizosphere, stem	
Tea	Root	
Pine apple	Fruit	
Mango	Fruit	
Banana	Rhizosphere	
Others-mealy bugs, VAM Spores	Internal environment	
Sweet sorghum	Root	Bindu (2001)
Sesamum	Root	

diazotrophicus (Stephan *et al.*, 1991). The beneficial traits of *A. diazotrophicus* include fixation of dinitrogen inside sugarcane (Sevilla *et al.*, 1998; Sivella and Kennedy, 2000), production of significant amounts of plant growth harmones in culture (Fuentez - Ramirez *et al.*, 1993; Bastian *et al.*, 1998), solubilization of phosphate (Maheshkumar *et al.*, 1999) and enhancement of the growth of sugarcane in the presence of nitrogen fertilizer (Sevilla *et al.*, 1998).

2.10 REGULATION OF PHOSPHATE SOLUBILIZING ACTIVITY

The genetic characterization of regulation of the genes involved in MPS activity in *A. diazotrophicus* has not been attempted yet. Several approaches are available to understand the genetic basis of metabolic functions in bacteria. One approach is to develop mutants of the strain with knock out of the phenotype one is interested to study. Mutation can be induced using physical, chemical mutagens or by the use of transposable elements.

Mineral phosphate solubilization deficient and hyper expressive mutants were obtained by NTG mutagenesis in *Pseudomonas* sp. (Krishnaraj, 1996). Mutants deficient in MPS and mutants showing delayed solubilization have been developed through NTG mutagenesis (Santi, 1998 and Deepa, 2000).

Mutants of HB101, lacking gluconic acid production in presence of co-factor PQQ was obtained by Tn5 mutagenesis (Liu *et al.*, 1982). Transposon insertions at several loci have been used to define the smallest fragment of a larger clone that is necessary for PQQ activity in *E. coli* (Babu-khan *et al.*, 1995). Random Tn5 insertions into *Pseudomonas* genome was achieved using Tn5 present in suicidal vector pGS9 (Krishnaraj, 1996). The nature of pleiotropies shown by these mutants indicated that these mutational lesions might have occurred in some of the regulatory *mps* loci. A genomic library

was then used to isolate DNA fragments that transcomplemented the MPS defective mutants.

2.11 POLYMERASE CHAIN REACTION (PCR)

PCR has been called a "DNA photocopier". In a short time since its invention by Karry Mullis in 1983, PCR has revolutionized our approach to molecular biology. PCR copies the DNA in the test tube and uses the basic elements of the natural DNA replication processes. In a simple buffer system, a region of a template DNA molecule is copied by a DNA polymerase that uses deoxynucleotides as a building block of the new strands. PCR has now been adapted to serve a variety of applications, which has revolutionized our approach to research in the fields of medicine, forensic and environmental testing. Other applications include PCR based site directed mutagenesis, analysis of gene expression, gene cloning and genome analysis. PCR based RAPD markers help in diversity analysis as well as finger printing of individuals. Use of specific primers to identify a particular gene is of great value, both for studying the diversity and for further studies including cloning (Mc Pherson and Moller, 2000).

E. coli is capable of synthesizing the apo-GDH but not the cofactor PQQ, which is essential for the formation of the holoenzyme. Hence, this system could be utilized to identify and isolate *pqq* gene(s). An 800 bp PCR amplified *pqq* synthase gene was cloned and expressed in *Burkholderia cepacia* by Mahesh Kumar (2003). The PCR fragment was ligated into pET28 and mobilized into *E coli* BL21. The transformed *E. coli* BL21 (pMAK281) showed high solubilization of TCP. Since *E. coli* synthesises the apo-GDH, the glucose oxidation observed on the basis of MPS activity and the fact that the

cloned fragment was an amplification of pqq synthase specific primer, PQQ synthesis must have occurred and transcomplemented *E coli* BL21 to produce gluconic acid and solubilize TCP. This was the first report of cloning of pqq synthase gene from *Burkholderia cepacia.* In an earlier experiment, Babu-Khan *et al.,* (1995) presented an evidence to show that a totally different (392 bp) ORF designated as *gab*Y in *Pseudomonas cepacia* was sufficient to induce MPS phenotype and production of gluconic acid in *E coli* JM109.

Material and Methods

III. MATERIAL AND METHODS

Investigations were carried out at the Departments of Agricultural Microbiology and Biotechnology, University of Agricultural Sciences, Dharwad, on mineral phosphate solubilization by *A. diazotrophicus* Pal5, development of random MPS mutants of *A. diazotrophicus* Pal5, clone *gcd, gnd* and *pqq* genes and development of transgenic *Azospirillum.* The materials used and the strategies adopted for achieving the above objectives are detailed in this chapter.

3.1 PURIFICATION OF *A. diazotrophicus*

A. diazotrophicus Pal5 was gifted by Dr. Johanna Dobereiner Embrapa, Seropedica, R. J., Brazil. The culture was purified by four-way streak on Nutrient Agar Glucose (NAG) medium (Appendix I) and on LGI medium (Appendix I).

3.2 SCREENING OF *A. diazotrophicus* PAL5 FOR MINERAL PHOSPHATE SOLUBILIZATION (MPS) ACTIVITY

A. diazotrophicus was subjected to rapid screening for its MPS activity on MSM (Appendix I) and TCP agar (Appendix I). 10 µl of an overnight culture grown at 28^{0}C in Nutrient Broth with Glucose (NBG) developed in the earlier study (Bindu, 2001) was spotted on MSM and TCP plates and incubated at 28^{0}C. The solubilization of insoluble phosphate was observed through the development of clearing zone on the medium around the growth of the strain, after 36 hrs of incubation on MSM and 48 hrs of incubation on TCP agar.

The strain was additionally tested for its ability to release P_i in tricalcium phosphate (TCP) broth. For the analysis, 100 µl of overnight culture in Pikovskaya's broth was inoculated into 10 ml of fresh Pikovskaya's broth and incubated at 28°c on a shaker at 175 rpm. Three replications were maintained. Estimation of P_i was done at 3 and 5 days after incubation. A set of uninoculated broth was maintained as control. The broth culture was centrifuged at 10,000 rpm for 15 minutes in a microcentrifuge (Eppendorf, Centrifuge 5415D). The available P content in the supernatant was estimated by phosphomolybdic blue color method (Jackson, 1973). The method and reagents are detailed in Appendix II. The broth was also checked for the drop in the pH.

3.3 TESTING OF INTRINSIC ANTIBIOTIC RESISTANCE OF *A. diazotrophicus* PAL5

To mark the strain chosen for further detailed analysis, the intrinsic antibiotic resistance of strain PAl5 was carried out. Plates of NAG (Nutrient agar + 3% Glucose) with various added antibiotics were prepared. 10 µl of overnight grown culture was spotted on NAG plates and incubated at 28°C for 12 hours. The growth was qualitatively assessed in comparison with the growth on control NAG plate. The antibiotics used, their source, solubility and concentrations are mentioned in Table 4.

3.4 ORGANIC ACID PRODUCTION BY *A. diazotrophicus* Pal5

The organic acid released into TCP medium was analyzed to understand the spectrum of organic acid produced by Pal5 by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

Table 4: Drugs/Antibiotics used in the study and their source

Sl. No.	Antibiotics	Abbreviation	Solubility (Solvent)	Concentration used (μg/ml)	Source
1.	Ampicilin	Amp	H_2O	100	Himedia, Mumbai
2.	Chloramphenicol	Clm	Methanol	5	Sigma Chemicals, USA
3.	Gentamycin	Gen	H_2O	50	Sigma Chemicals, USA
4.	Kanamycin	Kan	H_2O	50	Himedia, Mumbai
5.	Nalidixic acid	Nal	0.1N NAOH	10	Himedia, Mumbai
6.	Rifampicin	Rif	Methanol	25	CDH, Mumbai
7.	Spectinomycin	Spc	H_2O	20	Sigma Chemicals, USA
8.	Streptomycin	Str	H_2O	100	Himedia, Mumbai
9.	Tetracyclin	Tet	70% Ethanol	10	Himedia, Mumbai

Overnight culture was grown in TCP broth and 100 µl of this was inoculated into fresh 10 ml TCP broth and incubated at 28°C for 200 rpm for five days. This was centrifuged at 10,000 rpm for 15 minutes. The supernatant was then taken and concentrated to nearly 1/10th of its original volume on a thermomixer (Eppendorf) at 60°C. The concentrate obtained was subjected to thin layer chromatography. The TLC Aluminium sheets (0.2mm) were obtained from S. D. Fine Chem Limited. Pure standard organic acids were prepared at a concentration of20mg/ml. 10 µl of the standard acid and culture supernatant was spotted on TLC plates separately and dried. An ascending chromatography was run using butanol:formic acid:water (10:2:15) solvent system in a chromatographic chamber presaturated with the solvent for six hours. The chromatogram was run for 16 hours, air dried in a hot air oven at 50°C for 3 – 4 hours, and developed by spraying 0.04 % bromocresol green dye in methanol. The chromatogram was then air-dried. The Rf values of standard organic acid spots and the sample were compared.

The presence of organic acids was also confirmed through high performance liquid chromatography (HPLC) using 0.1% orthophosphoric acid as mobile phase with a run rate of 1ml/minute at room temperature using Shodex RSpak KC-811 column along with guard column (Shodex KC – G (KC – 810P, WATERS). The samples were prepared by growing the wild type *A. diazotropicus* Pal5 and its derived mutants in 10 ml of TCP broth for 5 days at 28°C at 200 rpm. The culture supernatant was collected by centrifugation at 13,000 rpm for 5 minutes. This was passed through 0.45 µm syringe filter (Sartorius). 20 µl each of this filterate was injected into the system for organic acid analysis, with a run time of 15 minutes at a wavelength of 254 nm. The

peak profiles were compared with standard gluconic acid (Sigma Chemicals, USA).

3.5 MUTAGENESIS OF Pal5

To assess the mechanism of MPS activity, random mutagenesis of Pal5 was carried out to get MPS defective mutants. Mutagenesis was carried out using N-methyl-N'-nitro-N nitrosoguanidine (Fluka, Switzerland).

3.5.1 NTG mutagenesis

Single colony of an overnight streaked culture of Pal5 was inoculated into 5 ml of NBG in 30 ml culture tube and incubated at 28°C, 200 rpm for 12 hours. Equal amount of fresh NBG was added. An aliquot of this culture was taken for titre estimation. To the remaining, NTG was added @ 50 μg / ml and grown for 1 hour, 200 rpm, at 28°C. The treated cells were washed in 0.01 M $MgSO_4$ thrice and resuspended in 10 ml of NBG and an aliquot was taken for titre estimation. The resuspended cells were grown for 8 hours for fixation of the mutational lesions. The mutant library was stored at 4°C. An aliquot of the mutant library was plated on NAG and individual colonies were picked up and screened for MPS activity.

3.5.2 Isolation of mutants altered in MPS activity

Single colonies obtained by above mutagenesis were screened for MPS activity on MSM agar. The single colonies were picked with sterile toothpick, spotted and incubated at 28°C for 72 hours, with periodic observations for zone of solubilization. Colonies, which did not show any solubilization zone were picked up as MPS^- strains.

3.6 CHARACTERIZATION OF MUTANTS

The mutants obtained were characterized for the properties outlined below.

3.6.1 MPS activity on agar medium

All mutants were further screened for their MPS activity on TCP medium as described previously in section 3.2 of this chapter. Additionally the mutants were screened for their growth on an indicator medium *viz.*, MSM + BTB (0.04%) by spotting an overnight cultures of Pal5 and the mutants and incubated at 28^0 C. The mutants were observed for release of acid into the medium which would develop orange coloured colony.

3.6.2 Release of P_i from TCP

The amount of P_i released from TCP in the broth was determined by phosphomolybdic blue color method as described previously in section 3.2 of this chapter.

3.6.3 Determination of acidity in broth

One ml of over night culture of Pal5 and the mutants from TCP broth was inoculated into 50 ml TCP broth and incubated for 5 days at 28° C on a shaker. The culture was spun at 10,000 rpm for 15 minutes and the supernatant was collected in a fresh tube. The pH of the supernatant was checked using a digital pH meter (Mettler Toledo)

3.6.4 Intrinsic antibiotic resistance (IAR)

Intrinsic antibiotic resistance of the mutants was examined as described previously in section 3.3 of this chapter

3.6.5 Production of organic acids

The production of organic acids by mutants was analysed by thin layer chromatography and HPLC as described previously in the section 3.4.

3.6.6 Complementation with PQQ

To detect whether the mutants derived from Pal5 has defect in the loci controlling the synthesis of PQQ, complementation analysis was done by externally supplementing PQQ (Methoxanthin, Fluka, Switzerland) @ 30 μl of 0.1mM PQQ/l medium in MSM and TCP agar and the overnight cultures of mutants were spotted on it. *E. coli* DH5α (PQQ^-, GDH^+) and *E. coli* AG121 (PQQ^-, GDH^-) (Goldstein, A.H., Alfred University, USA) were used as positive and negative controls respectively, along with the wild type *A. diazotrophicus* Pal5.

3.6.7 Protein Profile of Pal5 and Mutants

To ascertain the effect of mutation in the loci controlling the MPS activity, Pal5 and the mutants were analysed for protein profile by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) following the protocol outlined by Sambrook and Russell (2001). The chemicals and reagents used are outlined in Appendix III.

Overnight culture of Pal5 and mutants were grown in 5 ml NBG medium at 200 rpm at 28°C. From this one hundredth volume was inoculated into

fresh 5 ml NBG and grown till log phase at 200 rpm, 28°C. One ml of each of the culture was separately transferred into sterile microcentrifuge tube and spun at 12,000 rpm for 1 minute for extraction of proteins. The pellet was resuspended in 100 µl of $T_{10}E_1$ (10 mM Tris, pH 8.0 and 1 mM EDTA, pH 8.0) and 100 µl of 2x -SDS gel loading buffer. It was mixed well and incubated at 95°C for 8 minutes. This was later spun at 5000 rpm for 5 minutes at room temperature. About 25 µl of the supernatant was loaded on 10% SDS-polyacrylamide gel along with broad range protein molecular weight marker (Range 3500-205000 Da, Bangalore Genei, Cat No. PMW-B).

3.6.8 PCR with specific primer

The total DNA of Pal5 and its mutants were isolated as described in section 3.6.9 of this chapter and subjected to PCR amplification using a 23S rDNA species-specific oligonucleotide universal primer (AD-1440) to identify *A. diazotrophicus* (Kirchoff *et al.,* 1998). Amplification reaction was performed in a total volume of 25 µl. The reaction mixture contained:

1. Sterile H_2O	18.0 µl
2. Taq DNA polymerase assay buffer (@1x)	2.5 µl
3. dNTP mix (dATP, dCTP, dGTP & dTTP 2.5 mM each)	1.0 µl
4. 23S r DNA specific oligonucleotide universal primer (50 pM)	1.0 µl
5. Species specific oligonucleotide primer (50 pM)	1.0 µl
6. Taq DNA polymerase enzyme (1.5 units)	0.5 µl
7. Template DNA (50 ng)	1.0 µl

The reaction was carried out in 0.2 ml microcentrifuge tubes in Eppendorf Mastercycler (R) gradient Thermal cycler. The thermalcycler was programmed for different steps as follows:

Steps	Process	Temperature (^{0}C)	Time minutes	No. of cycles
1.	Initial denaturation	95	5	1
2.	Denaturation	95	0.75	35
3.	Primer annealing	60	0.75	
4.	Primer extension	72	1.0	
5.	Final extension	72	10	1
6.	Hold	4	-	-

3.6.9 Isolation of total DNA of Pal5 and mutants

Total DNA was isolated from Pal5 and its derived mutants following the modified methodology of Stall and Flesher (1987). The chemicals and reagents required for DNA isolation are listed in Appendix IVa.

a) Growth and lysis

Pal5 and its mutants were grown for 36 hours in 25 ml of NBG at 28^{0}C, 200 rpm. The cells were centrifuged at 10,000 rpm in Sigma centrifuge (3K30) for 6 minutes at 4^{0}C in 50 ml Oakridge tubes and the pellet was resuspended in 2.5 ml of T_{10} $NaCl_{100}$ (10 mM Tris, pH 8.0 and 100 mM NaCl, pH 8.0) and centrifuged for 10,000 rpm, 6 minutes at 4^{0}C. The pellet was then resuspended in 2.5 ml of $T_{10}E_1$ containing lysozyme @ 1 mg/ml. It was incubated at 37^{0}C for 20 minutes. To this, RNAase was added @ 0.2 mg/ml and incubated at 37^{0}C for 10 minutes and treated with 2.5 ml 2%

Sarkosyl prepared in $T_{50}E_{20}$ and incubated at 50°C for 45 minutes. Later, Proteinase K was added to this @ 0.5 mg/ml and incubated at 55°C for 15 minutes. The DNA lysate was then extracted by phenol.

b) Phenol extraction

The lysate was treated with equal volume of phenol (PH > 7.8) and mixed well to form milky emulsion. It was spun at 15,000 rpm, 20 minutes at 4°C to separate organic and aqueous phase. The upper aqueous phase was collected in a fresh tube and to this equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added. The aqueous phase was collected in a fresh tube and equal volume of chloroform:isoamyl alcohol (24:1) was added to it. The components were mixed well and centrifuged at 15,000 rpm, 20 minutes at 4°C. The aqueous phase was finally subjected to ethanol precipitation.

c) Ethanol precipitation

To the aqueous phase, two volumes of ice-cold ethanol was added and mixed well. The thick precipitate of DNA was spooled using a sterile glass rod and dissolved in $T_{10}E_1$ (100 µl) in a micro centrifuge tube. It was stored at 4°C for further analysis.

3.6.10 Quantification of DNA

Total DNA was quantified by following the ethidium bromide spotting method as described in Sambrook and Russel (2001). A sheet of saran wrap was stretched over a transilluminator (Vilber lourmat). Equal volumes of a series of DNA concentration standards (DNA / *Hind*III digest of 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625 µg /ml) was spotted in an ordered array

on the saran wrap. Similarly, sample DNA was spotted in the lane below the standard DNA spots. Equal volume of $T_{10}E_1$ containing 2 mg /ml ethidium bromide (Appendix IVb) was added to each spot and mixed well using a micropipette. The concentration of the DNA was estimated by comparing the intensity of the fluorescence in the sample with that of standard DNA.

Electrophoresis

After completion of the above set program, the contents of the tube were electrophoresed on a 1% agarose gel (Appendix IVd) along with molecular weight standard marker DNA (λ DNA *Hind*III digest). The gel was observed under UV transilluminator (Uvi tech Cambridge, England) and documented.

3.7 CLONING OF *pqq, gcd* AND *gnd* GENE

Attempt was made to clone *pqq, gcd* and *gdh* from *A diazotrophicus* Pal5. PCR based cloning was attempted by designing allele specific primer. The primers were designed by following the set protocols (Kamel, 2003) using the sequences downloaded from database (http://www.ncbi.nlm.nih.gov/). The primers were custom synthesized at Bangalore Genei Pvt. Ltd Bangalore. The sequences used to amplify *pqq, gcd* and *gnd* are mentioned below.

pqq	Forward: 5'CGGAATTCATGGTGGCATTTCTGCCGTGGCCCA3' Reverse : 5' CCGCTCGAGTCATGCGTGACTTACCAATGGA3'
gcd	Forward: 5' CCGGGATTCATGGCAATTAACAATACAGGC 3' Reverse: 5'CCCAAGCTTACTTCACATCATCCTGCA 3'
gnd	Forward: 5' CCGGAATTCATGTCCAAGCAACAGATCGGCGT 3' Reverse: 5' CCCAAGCTTAATCCAGCCATTCGGT 3'

The methods followed for DNA amplification, ligation, transformation, selection are outlined here:

3.7.1 Optimization of PCR conditions

PCR amplification of *pqq gcd* and *gdh* was optimized for annealing temperature using gradient PCR. The gradients and the annealing temperature for *pqq, gcd, gnd* are as follows.

Gene	Gradient	Annealing temperature range(^{0}C)				
pqq	2	42.0	43.1	44.1	45.2	46.0
gcd	10	43.3	45.1	47.4	50.0	52.8
gnd	10	43.3	45.1	47.4	50.0	52.8

The reaction mixture and PCR conditions are as follows:

Reaction mixture

Components	Volume
1. Sterile H_2O	18.0 µl
2. Taq DNA polymerase assay buffer (@1x)	2.5 µl
3. dNTP mix (2.5 mM each)	1.0 µl
4 Forward primer (5 pM)	1.0 µl
5. Reverse primer (5 pM)	1.0 µl
6. Taq DNA polymerase enzyme (1.5 units)	0.5 µl
7. Template DNA (50 ng)	1.0 µl

PCR Conditions

Steps	Process	Temperature (°C)			Time (minutes)	No. of cycles
		pqq	*gcd*	*gnd*		
1.	Initial denaturation	94°C	95°C	95°C	5	1
2.	Denaturation	94°C	95°C	95°C	2	35
3.	Primer annealing	44.1°C	45.1°C	45.1°C	2	35
4.	Primer extension	72°C	72°C	72°C	2	35
5.	Final extension	72°C	72°C	72°C	30	1
6.	Hold	4°C	4°C	4°C	-	-

The primers of *pqq, gcd* and *gnd* gene are supposed to amplify DNA fragments corresponding to approximately 800 bp, 750 bp and 1200 bp respectively.

The PCR products were checked on 1% agarose gel along with standard marker DNA (λ DNA Hind III digest).

3.7.2 Elution of PCR product and purification

The PCR products of the above three genes were run separately on 0.7% low melting agarose gel, along with λ DNA *Hind* III digest. After the samples were run sufficiently, the expected products of the three primers were cut separately using a sterile scalpel from the agarose gel on a low intensity UV transilluminator (Vilber lourmat). The agarose gel slices containing the band were collected separately in a preweighed sterile 1.5 ml

microcentrifuge tube. The products were purified using Quiagen gel extraction kit. The DNA was finally eluted in 30 µl of elution buffer (pH 8.5).

3.7.3 Quantification of the PCR product

The eluted DNA from agarose gel was quantified by ethidium bromide spotting method (Sambrook and Russel, 2001) as described earlier in section 3.6.10 of this chapter.

3.7.4 Ligation

Ligation was done using Inst T/ A clone ™ PCR product cloning kit (MBI Fermentas). Using the conversion table, the amount of PCR product required per ligation reaction was determined (Appendix Va). The ligation reaction mix for *pqq, gcd* and *gnd* are tabulated in appendix Vb. Ligation was carried out at 22^{0}C over night. pTZ57R provided in the kit was used as a cloning vector. This vector has a multiple cloning site with the *lacZ* genetic marker. The vector was pre-cleaved with *Eco*321 (an isoschizomer of EcoRV) and treated with terminal deoxynucleotidyl transferase to create 3'- dT over hangs at both ends.

3.7.5 Transformation

Transformation was carried out using transform Aid™ bacterial transformation system (Inst/A clone ™ PCR product cloning kit, MBI fermentas).

3.7.6 Preparation of competent cells

The competent cells were prepared using XL1 Blue strain of *E. coli* by picking single colony from overnight grown culture on LA plate with subsequent, inoculation into 1.5 ml of C-medium provided in the kit. The culture was inoculated into pre-warmed C-medium in 30 ml culture tube (1.5 ml / two transformations) and incubated at 37°C, 200 rpm for 2 hours. The culture was spun at 12,000 rpm for 1 minute at 4°C. The pellet was resuspended in 300 µl of transform Aid™ solution [(Prepared by mixing equal volume of T-solution (A) and T-solution (B)] and incubated on ice for 5 minutes. It was then spun at 12,000 rpm for 1 minute at 4°C. The pellet was later resuspended in 120 µl of transform AID™ T-solution. To 50 µl of competent cells about 2.5µl of ligation mixture (~14 ng of DNA) was added, mixed and incubated on ice for 5 minutes. Cells were plated on prewarmed LA Ampicillin plates (100 µg/ml Ampicillin) with X-Gal IPTG (Appendix V).

Appropriate controls were maintained by transforming control ligation of pTZ57R with the PCR fragment provided with the kit and pTZ57R vector (Appendix Vc).

3.7.7 Selection of Recombinant clones

The white colonies on LA A_{100} X-Gal IPTG plates were selected as recombinants.

3.7.8 Confirmation of clones

Plasmids were isolated from the white colonies. The difference in the profile of plasmid preparations from white colonies and control vector were compared by running the plasmid DNA on 0.7% agarose gel.

Further confirmation was done by PCR amplification of the recombinant plasmid DNA of the clones, Pal5 DNA and the cloning vector using the allele specific primers already mentioned in section 3.7.3 of this chapter. Pal5 DNA and the vector DNA pTZ57R were taken as positive and negative control respectively.

Restriction analysis of the plasmids of the selected clones and control vector was done for further confirmation using *EcoR*I, *Bam*H1 and *Hind*III restriction endonucleases (Appendix VI). The restricted products were analyzed on 0.7% agarose.

3.7.9 Transformation of *pqq* clone into BL21

One of the recombinants pJKQ1 was transformed into *E coli* BL21 pLysS to study the expression of *pqq* synthase gene. $CaCl_2$ competant cells of BL21 was prepared as per the protocol outlined in section 3.8.7 of this chapter.

The recombinant construct pJKQ1 was transformed into BL21 pLysS as per the protocol outlined in section 3.8.8 of this chapter. The recombinants were selected on LA Amp_{100} and checked for its expression by spotting 10 μl of overnight grown culture of the BL21pLysS (pJKQ1) along with XL1Blue (pJKQ1) and AG121 as controls.

3.8 DEVELOPMENT OF TRANSGENIC *Azospirillum*

Attempt was made to develop transgenic *Azospirillum* using the construct pMCG 898 carrying *pqq* gene(s) (A gift from Dr. Goldstein,Alfred University, USA). The methodology followed is outlined below.

3.8.1 Complementation of MPS activity of *Azospirillum by* PQQ

The wild type *Azospirillum* IABT-1 obtained from the culture collection was checked for MPS activity by spotting 10 µl overnight grown *Azospirillum* culture on MSM Agar and MSM Agar+PQQ @ 30µl of 0.1mM PQQ/ liter medium.

3.8.2 Intrinsic Antibiotic Resistance of *Azospirillum*

Azospirillum IABT-1 was checked for its IAR for various antibiotics, viz., Ampicllin (Amp_{100}), Kanamycin (Kan_{50}), Spectinomycin ($Spec_{20}$).

3.8.3 Development of *pqq* construct with Kanamycin marker

Since the *Azospirillum* strain used in the study was resistant to ampicillin, the *pqq* synthase gene in pMCG898 was cloned into pET28 vector which possesses kanamycin resistant marker.

3.8.4 Plasmid isolation

Plasmids from pMCG898 and pET28 were isolated essentially following the alkaline lysis method as outlined by Sambrook and Russell (2001).The reagents required for plasmid isolation are given in Appendix VIIa.

Both pET28 and pMCG898 were restricted with *Bam*H1 at 37°C for 5 hours. (Appendix VIIb). An aliquot was checked on 0.7% agarose gel for complete linearization using uncut pET28 and pMCG898 as control. pET28 was later dephosphorylated using calf intestine alkaline phosphatase (CIAP) by incubating for 1 hour at 37°C (AppendixVIIc). The CIAP after completion of the reaction was inactivated by incubating at 85°C for 15 minutes. The

restricted sample of pMCG898 and CIAP treated pET28 was cleaned using gel purification kit [QIAquick^{R} Gel Extraction Kit]. The samples were finally eluted in 10µl of elution buffer out of which one µl was checked on 0.7% agarose gel.

3.8.5 Ligation

For 1:1 molar ratio of pET28 and pMCG898 50 ng of pET28 and 50 ng pMCG898 were mixed in a ligation mixture of 15µl. (Appendix VIId) and incubated at 16^{0}C over night.

3.8.6 Transformation

The ligated product was transformed into calcium chloride competent DH5α cells.

3.8.7 Preparation of $CaCl_2$ competent cells

A loop full of culture from over night grown DH5α on LA + Nalidixic acid (Nal_{10}) was inoculated into 25 ml of Luria broth and grown at 37^{0}C, 200 rpm till the OD of the culture reached 0.35 to 0.4 (A_{600}). The culture was transferred to 50 ml polypropylene tube, and incubated on ice for 30 minutes and then centrifuged at 6000 rpm, 7 minutes at 4^{0}C. The pellet was resuspended in 12.5 ml of ice cold 0.1 M $CaCl_2$ and incubated in ice for 20-30 minutes. The cells were pelleted at 4000 rpm, for 5 minutes at 4^{0}C. The media was completely drained and the pellet was resuspended in 1 ml of 0.1M ice cold $CaCl_2$ and stored at 4^{0}C.

3.8.8 Transformation

100 µl of competent DH5α cells were mixed with 4 µl of ligated mixture (containing 25 µg of DNA) and incubated in ice for 30 minutes. It was then incubated at 42°C for 2 minutes and immediately transferred to ice and incubated further for 2 minutes. To this 900 µl of fresh LB was added from which 100 µl was plated on Luria Agar containing Amp_{100} + Kan_{50} and incubated at 37°C over night.

3.8.9 Confirmation of transformants

The transformants obtained were subjected to plasmid isolation by Alkaline lysis method as outlined by Sambrook and Russell (2001). The plasmid preparations were loaded on 0.7% agarose gel along with λDNA *Hind* III digest.

Further confirmation was done by restriction of the recombinant plasmid (pJSK15) with *Bam*HI to release the two fragments (Appendix VIIe).

3.8.10 Tri parental mating

To mobilize the *pqq* construct pJSK15 with kanamycin marker, triparental mating was done using *Azospirillum* IABT-1, pJSK15 (the *pqq* construct) and pRK2073 as recipient, donor, and helper respectively. *Azospirillum* IABT-1, pJSK15 and pRK2073 were grown over night in 10 ml LB at 37°C, 200 rpm with their respective antibiotics viz.,

Azospirillum IABT-1 : LB + $Ampicillin_{100}$

E. coli DH5 (pRK2073) : LB + $Spectinomycin_{20}$

E. coli DH5 (pJSK15) : LB + $Kanamycin_{50}$ + $Ampicillin_{100}$

One ml of the cultures were taken separately in microcentrifuge tubes and spun at 10,000 rpm, 5 minutes. The pellet was washed with 0.01 m $MgSO_4$ and spun at 10,000 rpm, 5 minutes. The pellet was then resuspended in 500 µl of fresh LB. Donor *E. coli* DH5α(pJSK15), Recipient (*Azospirillum* IABT-1) and Helper *E. coli* DH5α (pRK2073) were mixed in two different ratios *viz.*, 1:1:1 and 2:1:2 and patch mated on LA plate. Plates were incubated at 28°C over night. The growth was scraped off into one ml LB medium. 100µl of this was plated on M9 glucose minimal medium containing Kanamycin (Kan_{50}) and Ampicillin (Amp_{100}) and incubated at 28°C for 15 hours.

3.8.11 Confirmation of transconjugants

The transconjugants were confirmed by isolation of plasmids. Further confirmation was done by spotting over night culture on MSM agar medium + Amp_{100} + Kan_{50} along with appropriate controls, *viz.*, *E. coli* AG121 as negative control and *E. coli* DH5α (pJSK15) as positive control.

Experimental Results

IV. EXPERIMENTAL RESULTS

Acetobacter diazotrophicus Pal5 is reported to possess the property of solubilizing mineral phosphates. The present study attempted to dissect the mechanism of mineral phosphate solubilization of tricalcium phosphate and to isolate certain genes involved in this phenomenon. An attempt was also made to clone *pqq* synthase gene(s) into *Azospirillum*. The results obtained during the study are presented in this chapter.

4.1 CHARACTERIZATION OF MPS FUNCTION

4.1.1 MPS activity

The solubilization of *A diazotropicus* Pal5 was quickly tested on TCP Agar and MSM Agar for zone of clearence of insoluble phosphate. Pal5 showed clear zone of solubilization on both MSM and TCP (Plate 1). The release of P_i from TCP by *A diazotrophicus* Pal5 was tested by growing the strain in TCP broth for 5 days at 28°C on a shaker at 200 rpm. The results are presented in Table 5. The P_i release by Pal5 at 3rd and 5th DAI (Days after incubation) was 47.8 % and 52.88 % respectively.

4.1.2 Intrinsic antibiotic resistance (IAR)

To define the genetic markers of the strain Pal5 for further genetic studies it was tested for its IAR. The inhibition of growth of Pal5 in presence of nine antibiotics is indicated in Table 6. Pal5 showed resistance to ampicillin, spectinomycin, chloromphenicol and nalidixic acid at a concentration of 100 ppm, 20 ppm, 5 ppm and 10 ppm, respectively.

4.1.3 Organic acid profile of Pal5

The culture supernatant of Pal5 grown in TCP broth for 5 days was subjected to organic acid analysis by thin layer chromatography using butanol: formic acid:water (10:2:15) solvent system. The organic acid spots were developed using bromocresol green indicator. The results indicate the production of gluconic acid by Pal5 (Plate 2).

4.1.4 Isolation and characterization of mutants of Pal5 with altered MPS activity

The conditions for Pal5 mutagenesis were standardized for optimum killing percent. Treatment with 50 μg/ml of NTG for 1 hour resulted in a kill of 99.99%. The mutagenised stock after fixation was preserved in DMSO. Plating of this stock was done to get single colonies on NAG. A total of 2000 colonies were screened on MSM agar for MPS activity. The screening yielded six mutants that failed to show any solubilization zone even after 96 hours of incubation. These were designated as MPS^- phenotype. One mutant (ADM-7) which showed solubilization zone lesser than that of Pal5, was designated as leaky mutant. All the selected mutant strains were subjected to single colony purification several times and were retested for extent of solubilization on MSM Agar for consistency of the phenotype as shown in Plate 3.

4.1.5 MPS activity on Agar medium

The mutants along with Pal5 were tested for their growth on an indicator medium viz., MSM + BTB and compared with the phenotype on MSM agar. On MSM + BTB, the wild type appeared dark orange in colour while the mutants appeared light orange (Plate 4).

Plate 1: Zone of solubilization of *Acetobacter diazotrophicus* Pal5 on MSM

Plate 2: Gluconic acid production by Pal5

1. Standard gluconic acid
2. Gluconic acid of *Acetobacter diazotrophicus* Pal5

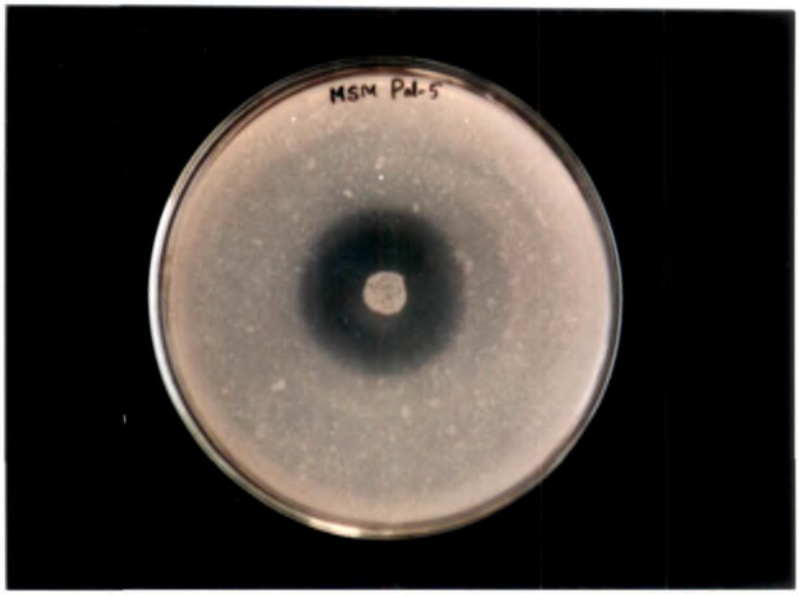


Plate 1. Zone of solubilization of *Acetobacter diazotrophicus* Pal5 on MSM

1 2

Plate 2. Gluconic acid production by Pal5

Plate 3. ***A. diazotrophicus*** **Pal5 and its derived mutants on MSM**

1. *A. diazotrophicus* Pal5
2. Mutant ADM-1
3. Mutant ADM-2
4. Mutant ADM-3
5. Mutant ADM-4
6. Mutant ADM-5
7. Mutant ADM-6
8. Mutant ADM-7

Plate 4. ***A. diazotrophicus*** **Pal5 and its derived mutants on MSM + BTB**

1. *A. diazotrophicus* Pal5
2. Mutant ADM-1
3. Mutant ADM-2
4. Mutant ADM-3
5. Mutant ADM-4
6. Mutant ADM-5
7. Mutant ADM-6
8. Mutant ADM-7

Plate 5. ***A. diazotrophicus*** **Pal5 and its derived mutants on MSM + PQQ**

1. *A. diazotrophicus* Pal5
2. Mutant ADM-1
3. Mutant ADM-2
4. Mutant ADM-3
5. Mutant ADM-4
6. Mutant ADM-5
7. Mutant ADM-6
8. Mutant ADM-7
9. DH5 α
10. AG121

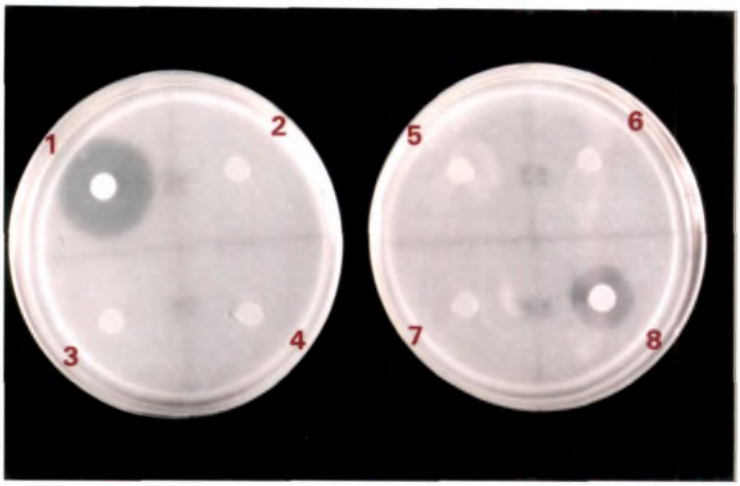


Plate 3. *A. diazotrophicus* Pal5 and its derived mutants on MSM

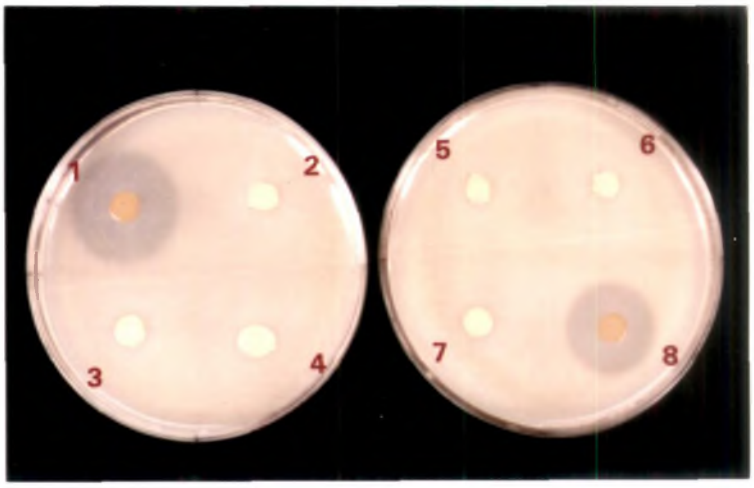


Plate 4. *A. diazotrophicus* Pal5 and its derived mutants on MSM + BTB

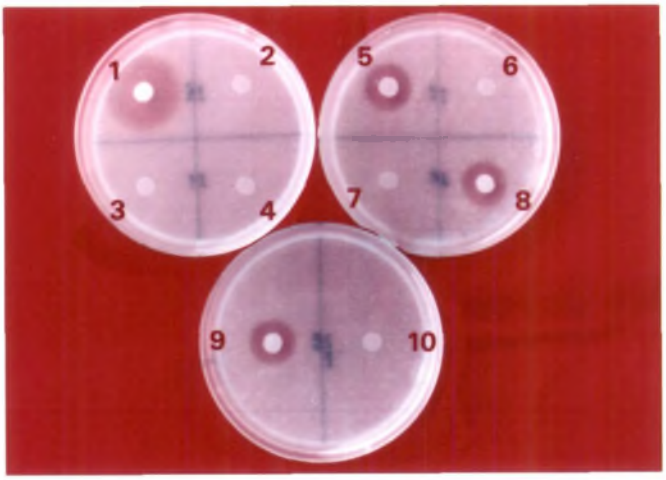


Plate 5. *A. diazotrophicus* Pal5 and its derived mutants on MSM + PQQ

4.1.6 Release of P_i and pH drop in TCP broth

The P_i release in Pikovskaya's broth medium by wild type and mutants was studied at 3rd and 5th day after incubation. The results are given in Table 5. The percent P_i released by derived mutants ranged from 9.6% (ADM-7) to 42.9 % (ADM- 5). The leaky mutants (ADM-7) released 42.9 % of P_i at 5 th DAI. The wild type strain Pal 5 released 52.88 % P_i. Pal5 showed a pH drop from 6.8 to 4.02 in the external medium. The growth of the mutant mutant ADM-6 dropped the pH to 6.3. However, the leaky mutant ADM-7 registered a drop to 5.0 (Table 7).

4.1.7 Intrinsic Antibiotic Resistance (IAR)

The IAR of wild type and the derived mutants were studied on NAG medium with 9 different antibiotics. The results are presented in Table 6. All the mutants were found to grow luxuriantly on NAG without antibiotics. Both the mutants and wild type Pal5 were resistant to nalidixic acid at 10 ppm, ampicillin at 100 ppm, spectinomycin at 20 ppm and chloramphenicol at 5ppm.

4.1.8 Production of organic acid

The culture supernatant of Pal5 and its MPS defective mutants were subjected to organic acid analysis by thin layer chromatography (TLC) and High performance liquid chromatography (HPLC).

The concentrated culture supernatants of Pal5 and its mutants were subjected to TLC using butanol:formic acid:water (10:2:15) as solvent system. The results indicated that Pal5 produced gluconic acid. ADM-7, the leaky mutant showed appreciable amount of gluconic acid. All other mutants

Table 5 : Release of Pi (percent) by *A. diazotrophicus* Pal5 and its mutants

Strains		Incubation period	
		3 days	5 days
		Pi release (%)	
Wild type	Pal-5	47.8	52.88
Mutant	ADM-1	10.6	10.9
Mutant	ADM -2	8.7	10.5
Mutant	ADM -3	11.1	12.2
Mutant	ADM -4	9.7	11.3
Mutant	ADM -5	8.3	9.6
Mutant	ADM -6	11.3	12.6
Mutant	ADM -7	38.2	42.9

Table 6: Intrinsic antibiotic resistance of *A. diazotrophicus* Pal5 and its mutants

Strain	Antibiotic								
	Amp_{100}	Clm_{5}	Kan_{50}	Nal_{10}	Spc_{20}	Str_{100}	Tet_{10}	$Gent_{50}$	Rif_{25}
Pal-5	+	+	-	+	+	-	-	-	-
M-1	+	+	-	+	+	-	-	-	-
M-2	+	+	-	+	+	-	-	-	-
M-3	+	+	-	+	+	-	-	-	-
M-4	+	+	-	+	+	-	-	-	-
M-5	+	+	-	+	+	-	-	-	-
M-6	+	+	-	+	+	-	-	-	-
M-7	+	+	-	+	+	-	-	-	-

+ :Resistance

- : Susceptible

Table 7: Change in pH of TCP broth by *A. diazotrophicus* Pal5 and its mutants

A. diazotrophicus	pH
Pal-5	4.02
ADM-1	5.88
ADM -2	6.18
ADM -3	5.84
ADM -4	5.90
ADM -5	6.28
ADM -6	6.36
ADM -7	5.02

Initial pH of the medium: 6.8

showed comparatively very less amount of gluconic acid. This was further confirmed by HPLC analysis.

The filter sterilized culture supernatant of Pal5 and its derived mutants were subjected to HPLC using 0.1% orthophosphoric acid as a mobile phase in a Shodex Rspak KC-811 column. 20 µl of the sample was injected and checked for organic acid peak. The peak profile of Pal5 and its derived mutants are shown in Fig 1. The culture filterate Pal5 and its mutants showed the presence of gluconic acid. The peak profile of standard gluconic acid is shown in Fig 5.

4.1.9 Complementation with PQQ

On the MSM agar supplemented with PQQ, only ADM-4 showed solubilization zone. The negative control, *E. coli* AG121 (GDH$^-$), did not show any MPS activity. The positive control *E. coli DH5*α showed solubilization (Plate 5).

4.1.10 Protein profile of Pal5 and mutants

The crude protein extracted from Pal5 and its mutants were separated on 10% SDS PAGE along with broad range protein molecular weight marker. Pal5 and the mutants showed identical protein profile (Plate 6).

4.1.11 PCR with specific primer

The genomic DNA of Pal5 and its mutants were subjected to PCR using *A. diazotrophicus* species specific universal oligonucleotide primer (AD-1440). The gel analysis showed the presence of a 411 bp amplified product in both wild type and mutants (Plate 8).

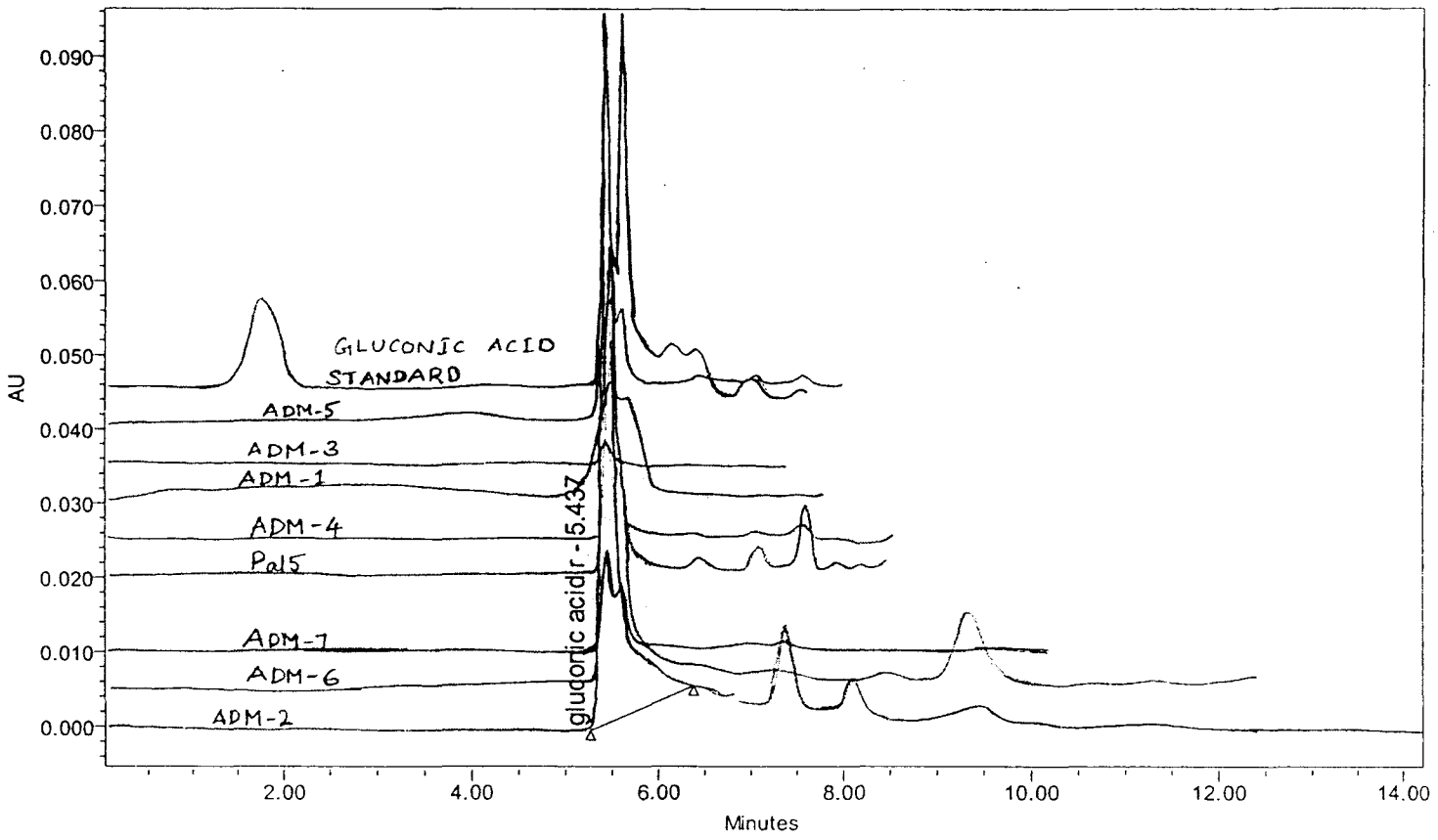


Fig. 1: HPLC analysis of gluconic acid

Plate 6: Protein profile of *A. diazotrophicus* Pal5 and its derived mutants

1. Broad range protein molecular weight market
2. Crude protein of *A. diazotrophicus* Pal5
3. Crude protein of mutant ADM-1
4. Crude protein of mutant ADM-2
5. Crude protein of mutant ADM-3
6. Crude protein of mutant ADM-4
7. Crude protein of mutant ADM-5
8. Crude protein of mutant ADM-6
9. Crude protein of mutant ADM-7

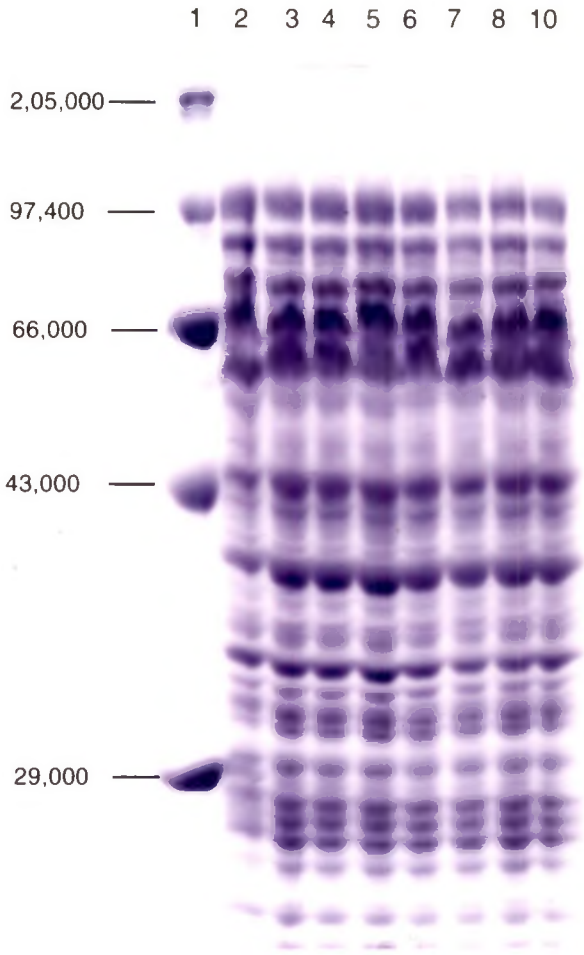


Plate 6. Protein profile of *A. diazotrophicus* Pal5 and its derived mutants

4.2 ISOLATION OF TOTAL DNA

Total DNA from Pal5 and its mutants were obtained from 10 ml of culture grown for 24 hours at 28°C, 200 rpm. The size of the DNA fragment obtained and purity is presented in Plate 7. It was stored at 4°C for further analysis.

4.3 CLONING OF *pqq, gcd* AND *gnd*

4.3.1 PCR amplification of *pqq, gcd* and *gdh* gene

PCR amplification of the *pqq, gcd* and *gnd* genes were optimized using Eppendorf gradient PCR that was programmed with a gradient of 2, 10 and 10 for *pqq, gcd* and *gnd* respectively. Good yield was obtained at an annealing temperature of 44.1°C, 45.1°C and 45.1°C for *pqq, gcd* and *gnd* respectively (Plate 9,10,11).

At their respective optimized annealing temperature, amplification was done on a large scale. The PCR amplification of *pqq, gcd* and *gnd* gave amplicons of approximately 800 bp, 750bp, and 1200 bp. The yield of the PCR product was 90 ng/µl, 30ng/µl and 30ng/µl for *pqq, gcd* and *gnd* respectively.The amplified fragments of *pqq, gcd* and *gnd* gene was cloned into pTZ57R/T cloning vector (Fig 2) by A, T tailing and transformed into competent XL1 blue strain of *E coli.* The recombinant clones which were white, were selected and restreaked on LA + amp_{100} + X-Gal IPTG. Of the several clones obtained, five from each ligation reaction were maintained. They included pJKQ-1, pJKQ-2, pJKQ-3, pJKQ-4and pJKQ-5 (*pqq* clones), pJKS-1 pJKS-2, pJKS-3 pJKS-4 and pJKS-5 (*gcd* clones), pJKN-1, pJKN-2, pJKN-3, pJKN-4 and pJKN-5 (*gnd* clones).

Plate 7: **Total DNA preparation of *Acetobacter diazotrophicus* Pal5 and its derived mutants**

1. DNA *Hind* III digest
2. *A. diazotrophicus* Pal5
3. Mutant ADM-1
4. Mutant ADM-2
5. Mutant ADM-3
6. Mutant ADM-4
7. Mutant ADM-5
8. Mutant ADM-6
9. Mutant ADM-7

Plate 8. **PCR amplification of Pal5 and its derived mutants with *A. diazotrophicus* specific primer**

1. 100 bp ladder
2. *A. diazotrophicus* Pal5
3. Mutant ADM-1
4. Mutant ADM-2
5. Mutant ADM-3
6. Mutant ADM-4
7. Mutant ADM-5
8. Mutant ADM-6
9. Mutant ADM-7

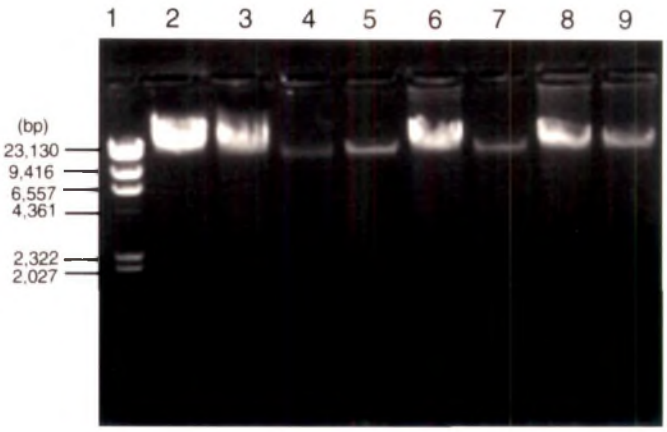


Plate 7. Total DNA preparation of *Acetobacter diazotrophicus* Pal5 and its derived mutants

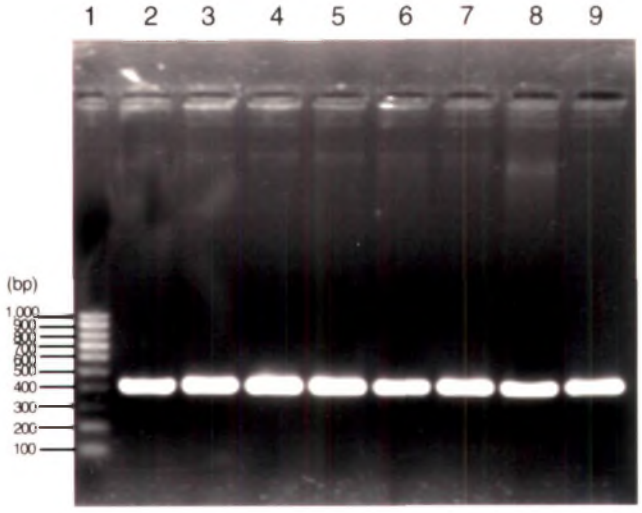


Plate 8: PCR amplification of Pal5 and its derived mutants with *A. diazotrophicus* specific primer

Plate 9: Optimization of annealing temperature for *pqq* specific primer

1. 42.0°C
2. 43.1°C
3. 44.1°C
4. 45.2°C
5. 46.0°C
6. λ DNA *Hind* III digest

Plate 10. Optimization of annealing temperature for *gcd* specific primer

1. 43.3°C
2. 45.1°C
3. 47.4°C
4. 50.0°C
5. 52.8°C
6. λ DNA *Hind* III digest

Plate 11. Optimization of annealing temperature for *gnd* specific primer

1. 43.3°C
2. 45.1°C
3. 47.4°C
4. 50.0°C
5. 52.8°C
6. λ DNA *Hind* III digest

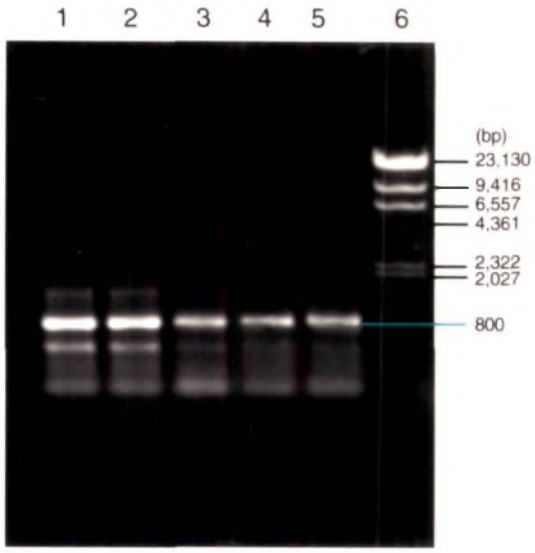


Plate 9. Optimization of annealing temperature for *pqq* specific primer

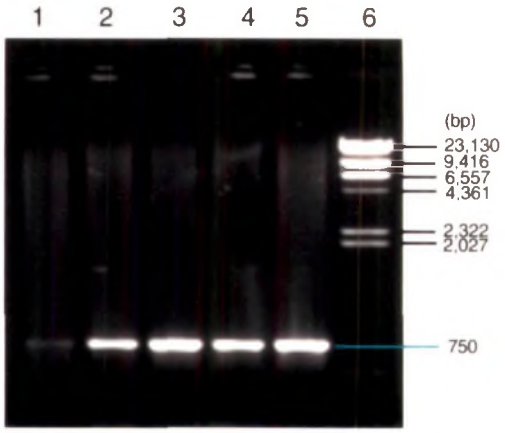


Plate 10. Optimization of annealing temperature for *gcd* specific primer

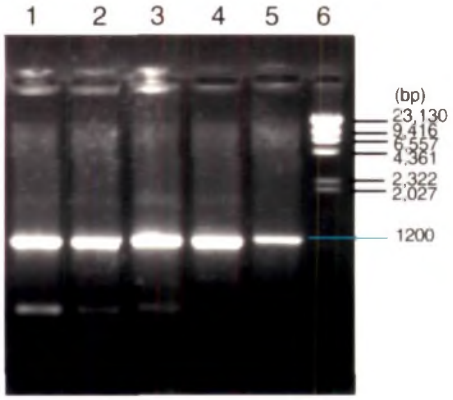


Plate 11. Optimization of annealing temperature for *gnd* specific primer

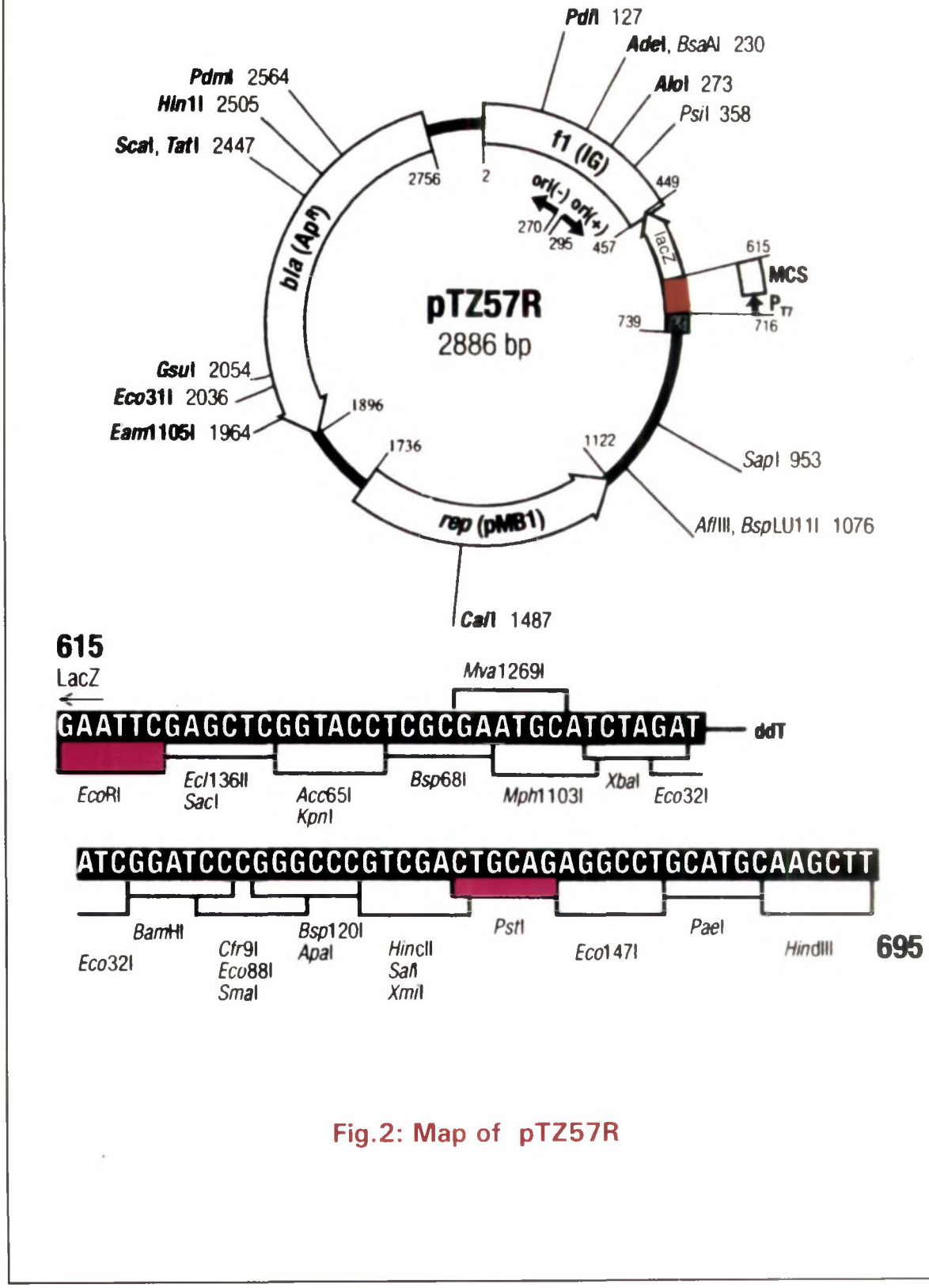


Fig.2: Map of pTZ57R

Restriction analysis of the clones indicated the presence of the 800 bp *pqq*, 750 bp *gcd* and 1200 bp *gnd* fragments (Plate 12, 13, 14).

Finally, the selected clones were subjected to PCR confirmation. The plasmid DNA of the selected clones, vector DNA (-ve control) and Pal5 total DNA (+ve control) were used as template and PCR checked with *pqq, gcd* and *gnd* specific primer. Agarose gel electrophoresis of amplified products with appropriate control showed the presence of insert in the recombinant vector (Plate 15, 16, 17).

4.3.2 Transformation of pqq clone into BL21

The recombinant pJKQ1 was transformed into *E coli* BL21 to know its expression. The 800 bp cloned fragment in pTZ57R would be transcribed by T_7 RNA Polymerase. *E coli* BL21 pLysS provides this function to express the protein by the cloned fragment. The transformants obtained were then screened for their expression by spotting overnight grown culture of BL21 (pJKQ1) on MSM. The transformants showed zone of solubilization around the colony (Plate 18).

4.3.3 Sequence analysis of pJKN1

The 1200 bp PCR product of *gnd* primer that was cloned into pTZ57R by T/A cloning strategy was custom sequenced at Bangalore Genei Pvt. Ltd., Bangalore. With the M13 forward primer, a sequence of 696 bp and with M13 reverse primer a sequence of 697 bp was available (Table 8a, 8b). After curing, M13 primer sequences, 428 bp sequence from 3' end of the gene and 661 bp sequence from 5'end of the gene was available. (Table 9) The available sequence information from cloned fragment was used for BLAST search. The homology results are presented in Table 10a and 10b. Both the

Plate 12: Restriction analysis of *pqq* clones

1. λ DNA *Hind* III digest
2. Uncut pJKQ1
3. PJKQ1 restricted with *Bam* H1
4. PJKQ1 restricted with *Eco*R 1
5. PJKQ1 restricted with *Hind* III
6. PTZ57R restricted with *Eco*R 1
7. Uncut PTZ57R
8. 1 kb ladder

Plate 13. Restriction analysis of *gcd* clones

1. λ DNA *Hind* III digest
2. Uncut pJKS1
3. PJKS1 restricted with *Bam* H1
4. PJKS1 restricted with *Eco*R 1
5. PJKS1 restricted with *Hind* III
6. PTZ57R restricted with *Eco*R 1
7. Uncut PTZ57R
8. 1 kb ladder

Plate 14. Restriction analysis of *gnd* clones

1. λ DNA *Hind* III digest
2. Uncut pJKN1
3. PJKN1 restricted with *Bam* H1
4. PJKN1 restricted with *Eco*R 1
5. PJKN1 restricted with *Hind* III
6. PTZ57R restricted with *Eco*R 1
7. Uncut PTZ57R
8. 1 kb ladder

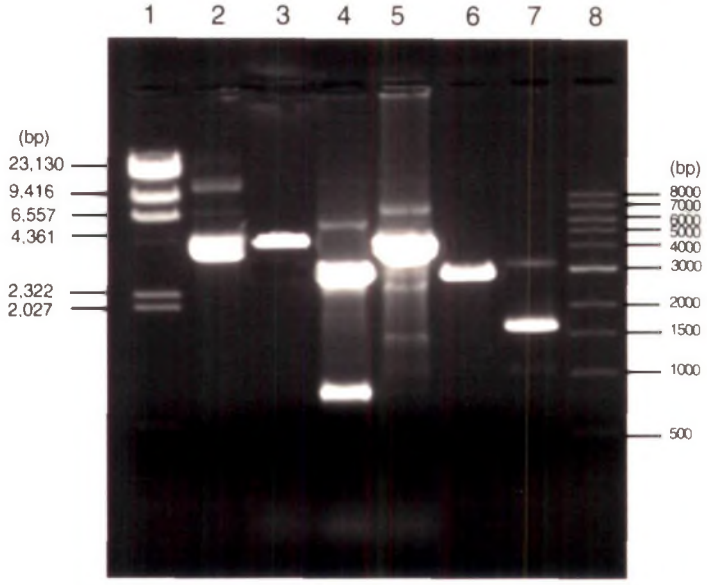


Plate 12. Restriction analysis of *pqq* clones

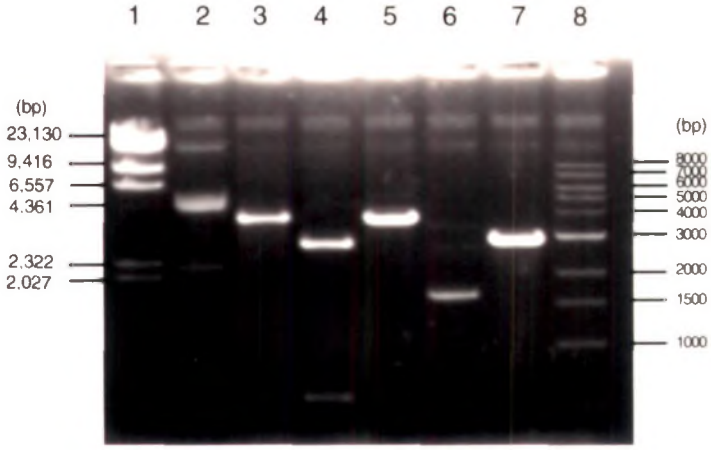


Plate 13. Restriction analysis of *gcd* clones

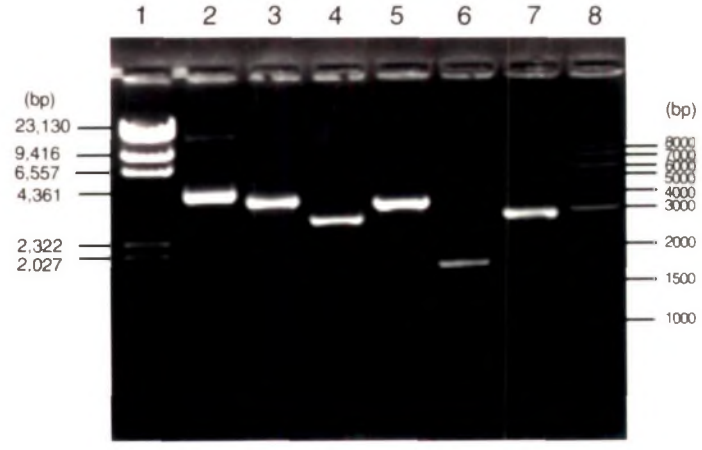


Plate 14. Restriction analysis of *gnd* clones

Plate 15: **PCR confirmation of clones with *pqq* specific primers**

1. λ DNA *Hind* III digest
2. pJKQ1
3. pJKQ2
4. pJKQ3
5. pJKQ4
6. pJKQ5
7. PTZ57R
8. Pal5

Plate 16: **PCR confirmation of clones with *gcd* specific primers**

1. λ DNA *Hind* III digest
2. pJKS1
3. pJKS2
4. pJKS3
5. pJKS4
6. pJKS5
7. PTZ57R
8. Pal5

Plate 17: **PCR confirmation of clones with *gnd* specific primers**

1. λ DNA *Hind* III digest
2. pJKN1
3. pJKN2
4. pJKN3
5. pJKN4
6. pJKN5
7. PTZ57R
8. Pal5

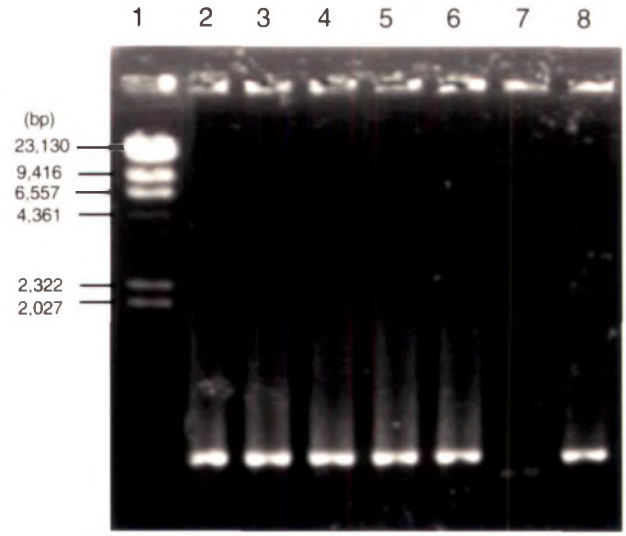


Plate 15. PCR confirmation of clones with *pqq* specific primers

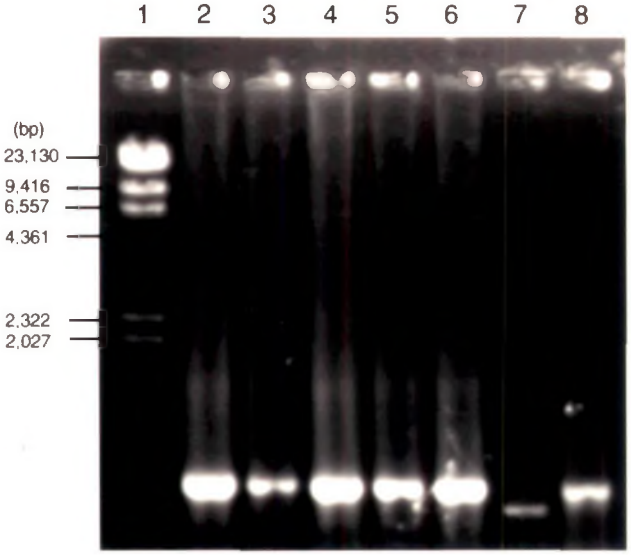


Plate 16. PCR confirmation of clones with *gcd* specific primers

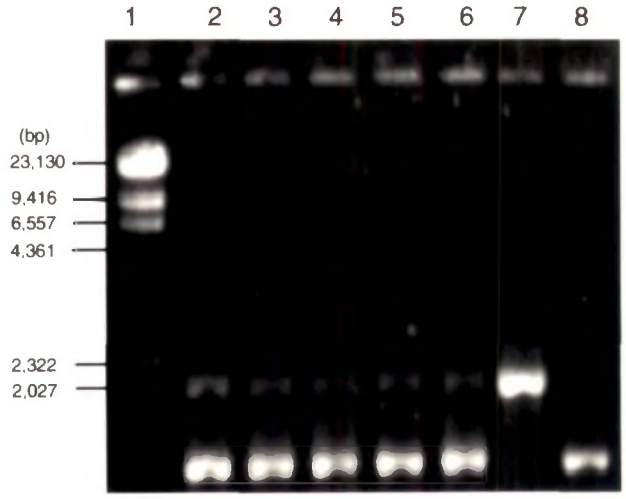


Plate 17. PCR confirmation of clones with *gnd* specific primers

Plate 18: ***E coli*** **BL21 (pJKQ1) on MSM**

1. BL 21 (pJKQ1)
2. XL1 Blue (pJKQ1)

Plate 19: **DH5α pJSK15 on MSM**

1. pJSK 15
2. pMCG898
3. pET28

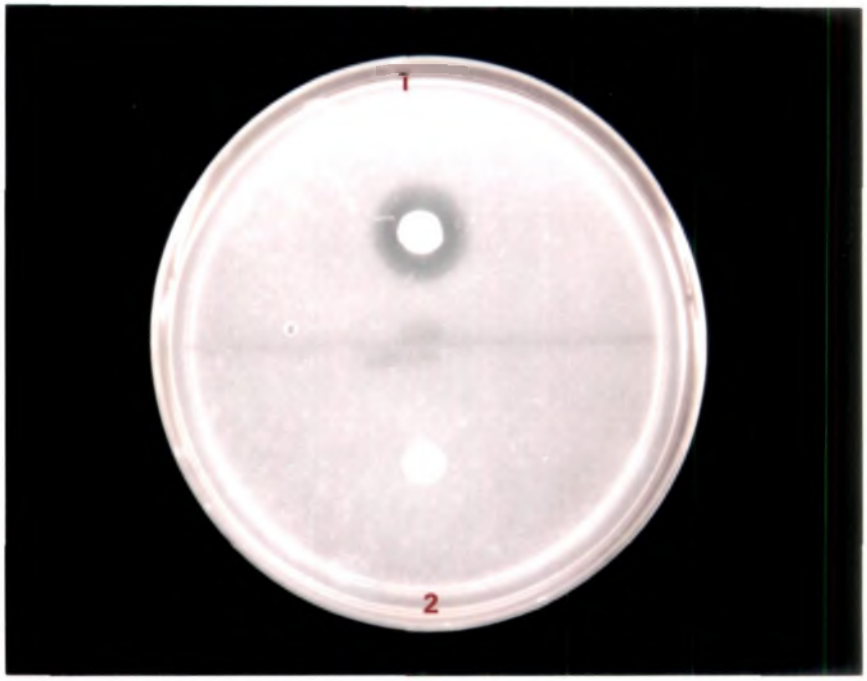


Plate 18. BL21 (pJKQ1) on MSM

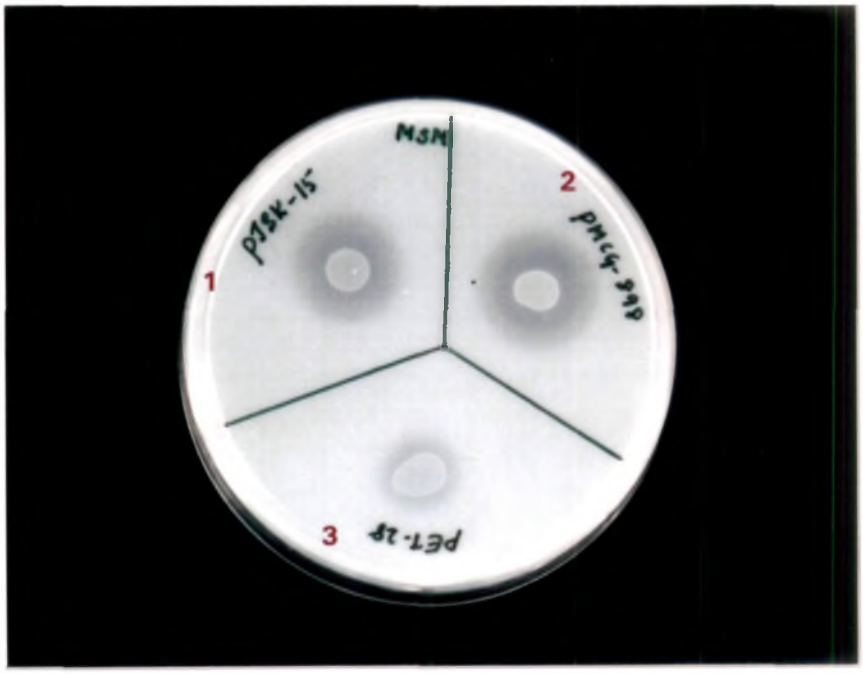


Plate 19. pJSK15 on MSM

Table 8a: Sequence of pJKN1 analysed by using M13FP

	10	20	30	40	
1	AAAGCTACGA	CCATGATTAC	GCCAAGCTCT	AATACGACTC	40
41	ACTATAGGGA	AAGCTTGCAT	GCAGGCCTCT	GCAGTCGACG	80
81	GGCCCGGGAT	CCGATTCCGG	AATTCATGTC	CAAGCAACAG	120
121	ATCGGCGTTT	CCTATGACCG	GAGCGCGCCC	CGCCGCCCGG	160
161	TCAATCTGTC	GCTCAACACC	GATCTGCTGG	CGCAGGTCCG	200
201	GGAAGTGACG	CCCAATCTTT	CGGCGACGGT	CGAGACCCTG	240
241	CTGGGCGACT	ACCTGCAATC	CGCACGCAAG	CAACGCGAGG	280
281	ACGAACAGCG	CAAGCTCGAC	GGCGTGATCG	ACGCAGTGAA	320
321	CGATCTGCAC	GCGCGGCACG	GTTTCCTGAG	CGACGAATTC	360
361	TCGACGCTCT	AATCCGATGC	CGCAATTCGC	GATCTACCGG	400
401	AACCCCGGCC	GAAACCAGGA	CATCCCGTTC	GTGGTCCAGA	440
441	TCCAGAGCAG	CCGCCTGGAG	CGCAGCATGG	GCCGCGTTGT	480
481	CATGCCGCTC	GTCAGACGAT	CGGGCAGCGC	GCCGCCGGAT	520
521	CACCCGCTGA	CGCCGTATCT	GCATGTCGAG	GGAGAGGACG	560
591	TCTTTGCCAA	CCCGTTCGAC	CTGGCGACCA	TCCCTGCCGC	600
601	GCGGCTTGGA	ACCGCCGTCG	GTGTCCTGGC	CGAACGCGAT	640
641	CAGGATAAGA	TCATAAGAGC	ACTGGACGAC	TGGTTAGCAG	680
681	GGGTGGAGTG	GCGCGA			696

Table 8b: Sequence of pJKN1 analysed by using M13RP

	10	20	30	40	
1	AATTCGAGCT	CGGTACCTCG	CGAATGCATC	TAGATTCCGG	40
41	AATTCATGTC	CAAGCAACAG	ATCGGCGTCT	CCTTGATCGG	80
81	ATCAAGGCTG	TTCATGGCGA	GAATGCGGTT	ATCAACGTGT	120
121	TTCCCGCTTT	GCCGAATTCA	GCGGCGGTTG	AGGTCGGGCG	160
161	CGTGTGGATG	CCTAAGGCCG	ATTTGCCCAT	GCAGATCTAT	200
201	GATCAGAATC	GGGCCGTCGG	CGGGTTCATT	CCTACCTTAT	240
241	GTATCGCGAA	TTAGAAGAGG	GCCGCCTCCC	CCTTCGTCGA	280
281	TCGGCTAAAG	CTACCGATAT	GCAACCCACT	CTAAGTGACA	320
321	ATAGAAGTTT	GCACTGGTCA	TGTCCGTTAT	GCGGAAAGTG	360
361	CACTGTGCGT	TACATGTTCG	GAAGGGAGGG	CGTAAACGGA	400
401	AGTTCCGCGC	CACTCACGCC	CTGCTAACCA	GTTCGTCCAG	440
441	TGCTCTTATG	ATCTTATCCT	GATCGCGTTC	GGCCAGGACA	480
481	CCGACGGCGG	TTCCAAGCCG	CGCGGCAGGG	ATGGTCGCCA	520
521	GGTCGAACGG	GTTGGCAAAG	ACGTCCTCTC	CCTCGACATG	560
561	CAGATACGGC	GTCAGCGGGT	GATCCGGCGG	CGCGCTGCCC	600
601	GATCGTCTGA	CGAGCGGCAT	GACAACGCGG	CCCATGCTGC	640
641	GCTCCAGCGG	CTGCTCTGGA	TCTGGACCAC	GAACGGATGT	680
681	CTGTTTCGCC	GGGGTCG			697

Table 9 : Sequence data of pJKN1

Sl. No.	Primer used	Recombinant sequenced	No. of bp sequenced	Sequence after curing
1.	M13FP	PJKN1	696	428
2.	M13RP	PJKN1	697	661

Table 10a: BLASTn search result for 428 bp sequence obtained from pJKN1 using M13 forward primer

Sl. No.	Accession No.	*Gluconate dehydrogenase gene*	% Homology
1.	X15651	*Salmonella enterica gnd* gene 6, Phospho Gluconate dehydrogenase	100
2.	AF176373	*E. coli* DEC2B 6, Phospho Gluconate dehydrogenase	100
3.	AF176372	*E. coli* DEC2A 6, Phospho Gluconate dehydrogenase	100
4.	AF176371	*E. coli* DEC1B 6, Phospho Gluconate dehydrogenase	100
5.	AF176370	*E. coli* DEC1A 6, Phospho Gluconate dehydrogenase	100
6.	AF176369	*E. coli TB182A* 6, Phospho Gluconate dehydrogenase	100
7.	M64332	*Salmonella typhimurium* 6, Phospho Gluconate dehydrogenase	100
8.	M18959	*Salmonella typhimurium* 6, Phospho Gluconate dehydrogenase	100
9.	M63828	*E. coli* 6, Phospho Gluconate dehydrogenase	100
10.	M23181	*E. coli* 6, Phospho Gluconate dehydrogenase	100
11.	M18958	*E. coli gnd* gene 6, Phospho Gluconate dehydrogenase	100

Table 10b: BLASTn search result for 661 bp sequence obtained from pJKN1 using M13 reverse primer

Sl. No.	Accession No.	*Gluconate dehydrogenase gene*	% Homology
1.	X15651	*Salmonella enterica gnd* gene 6, Phospho Gluconate dehydrogenase	100
2.	X60666	*Salmonella enterica* rfbP, orf17 and *gnd* gene	100
3.	AF176373	*E. coli* strain DEC2B 6, Phospho Gluconate dehydrogenase	100
4.	AF176372	*E. coli* strain DEC2A 6, Phospho Gluconate dehydrogenase	100
5.	AF176371	*E. coli* strain DEC1B 6, Phospho Gluconate dehydrogenase	100
6.	AF176370	*E. coli* strain DEC1A 6, Phospho Gluconate dehydrogenase	100
7.	AF176369	*E. coli* strain TB182A 6, Phospho Gluconate dehydrogenase	100
8.	STYGNDA	*Salmonella typhimurium* (strain LT2) 6, Phospho Gluconate dehydrogenase	100
9.	STYGND	*Salmonella typhimurium* (3436) 6, Phospho Gluconate dehydrogenase	100
10.	EcoR47	*E. coli* 6, Phospho Gluconate dehydrogenase	100
11.	EcoGNDF	*E. coli* 6, Phospho Gluconate dehydrogenase	100
12.	EcoGNDC	*E. coli* 6, Phospho Gluconate dehydrogenase	100
13.	EcoGND	*E. coli* 6, Phospho Gluconate dehydrogenase	100

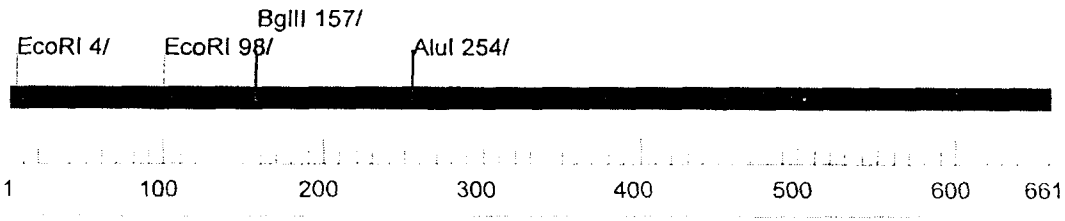
forward and reverse primer sequences showed 100% homology with *Salmonella typhimurium gnd gene, Salmonella enterica gnd gene,* and *E. coli gnd gene.*

The available sequence information was used to know the correct orientation of the gene. The sequences of pJKN1M13FP and pJKN1M13RP were matched with the sequences of *gnd* forward and reverse primer. PJKN1M13FP showed the presence of forward primer and pJKN1M13RP showed the presence of reverse primer indicating the correct orientation of *gnd* gene in the recombinant clone.

The available sequence information has been used to find the restriction sites and to obtain the sequence data by using BTI Gene Tool Software. The restriction map of 661 bp sequence of pJKN1M13FP reveals the presence of *Eco*RI site at 4 bp and 98 bp sequence, *Bgl*IIIsite at 157 bp sequence and *Alu*I site at 254 bp sequence from 3' end of 1.2 kb gene (Fig. 3a). The restriction map of 428 bp sequence of pJKN1M13RP indicates the presence of *Alu*I site at 4bp, 26 bp, 53 bp and 294 bp sequence, *Hind*III site at 51 bpsequence,*Pst*I site at 73 bp sequence, *Sal*I site at 74bp sequence, *Sma*I site at 85 bp sequence, *Bam*HI site at 87 bp sequence and *Eco*RI site at 100 bp and 355 bp sequences from 5' end (Fig. 3b). Other sequence data for these sequences are given in Appendix IX.

4.4 DEVELOPMENT OF TRANSGENIC *Azospirillum*

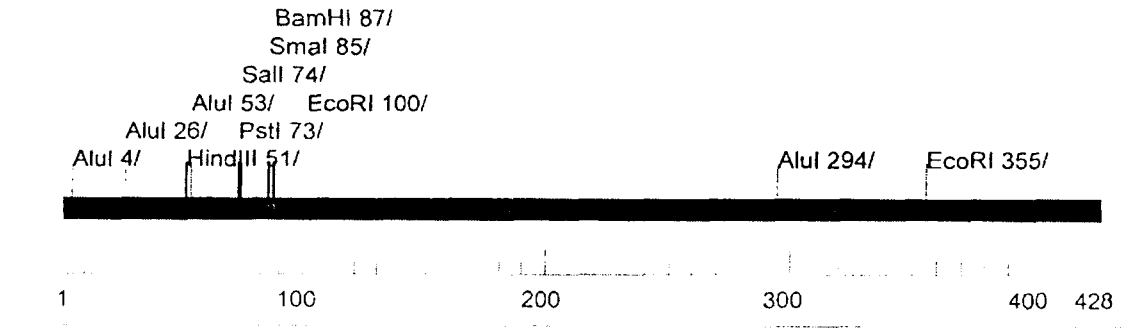
An *Azospirillum* strain IABT-1 that showed complementation with external supply of PQQ (Plate 22, 23) was used for development of transgenic *Azospirillum* by mobilizing a *pqq* synthase construct into it. IAR analysis showed that the strain was ampicillin resistant and susceptible to kanamycin. As the recombinant pMCG898 containing *pqq* synthase gene(s) had amp^R as



Sequence Name: 661 bp pJKN1M13FP

Organism: Acetobacter diazotrophicus

Fig. 3a: Restriction map of 661 bp sequence of pJKN1 upstream



Sequence Name: 428 bp pJKN1M13RP

Organism: Acetobacter diazotrophicus

Fig. 3b: Restriction map of 428 bp sequence of pJKN1 downstream

Plate 20: Restriction analysis of pJSK15

1. λ DNA *Hind* III digest
2. pET 28
3. pMCG898
4. pJSK15
5. pJSK15 restricted with *Bam* H1

Plate 21: Plasmid preparation of transgenic *Azospirillum* IABT1

1. λ DNA *Hind* III digest
2. *Azospirillum* IABT-1
3. Transgenic *Azospirillum* IABT-1

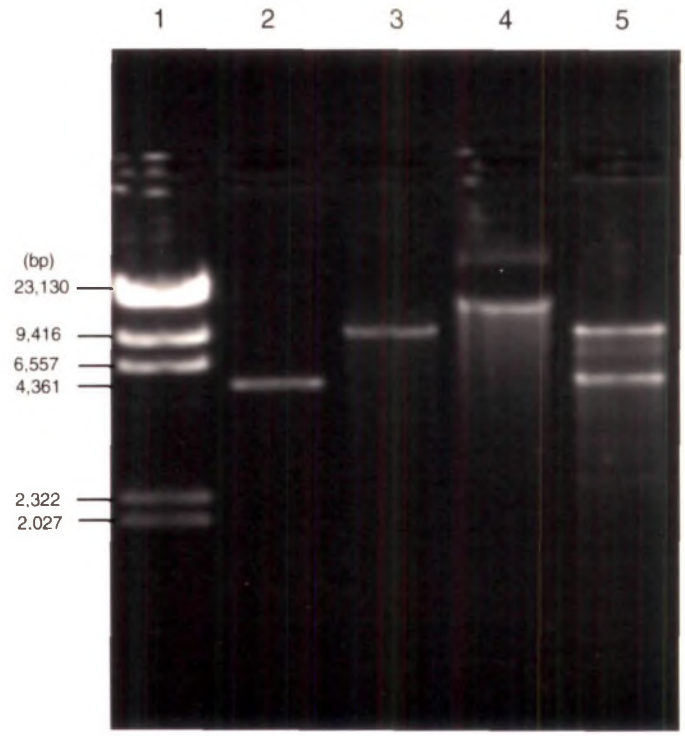


Plate 20. Restriction analysis of pJSK15

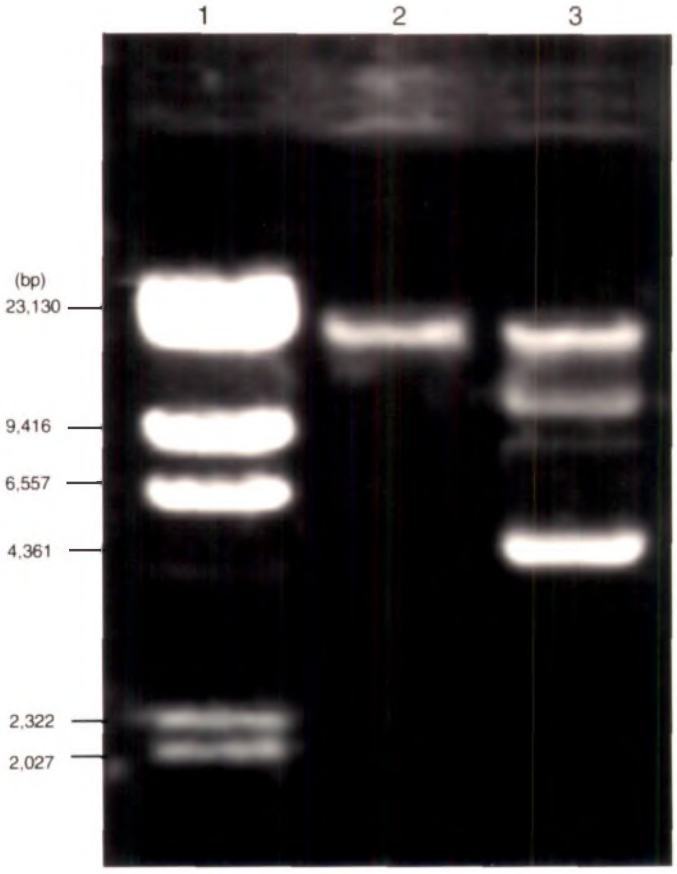


Plate 21. Plasmid preparation of transgenic *Azospirillum* IABT1

its marker, a construct containing kanamycin resistance gene as marker was developed (Fig 4). This was obtained by ligation of 1:1 molar ratios of *Bam*HI linearized pMCG898 into *Bam*HI site of pET28. The recombinant pJSK15 was maintained in *E. coli* DH5α. The expression of pJSK15 in *E. coli* was studied by noting the extent of solubilization on MSM agar and TCP broth. The results indicate solubilization on MSM (Plate19) and a P_i release of 38.54%. The Pi release of the controls DH5α, DH5α(pET28), DH5α(pMCG898) were14.18%, 15.88% and 40.54% and respectively.

The presence of *pqq* synthase insert was confirmed by restriction analysis. The plasmid preparation of pMCG 898, pET28 and the recombinant pJSK-15 was restricted with *Bam*H1. The recombinants after restriction separated into pMCG 898 and pET28 (Plate 20). The construct pJSK15 was now mobilized into *Azospirillum* IABT-1 by triparental mating. *E. coli* DH5α(pRK 2073) (Spc^R) was used as a helper. The colonies obtained on M9 minimal glucose media with kan50 were picked up as transconjugants. The transgenic *Azospirillum* was then tested for MPS activity on MSM medium and TCP broth. The transconjugants were spotted on MSM media along with *E. coli* AG121 and *E. coli* DH5α(pMCG 898) as negative and positive controls. But the transgenic *Azospirillum* did not show any sign of solubilization on MSM agar (Plate 24) and showed a Pi release of 13.7% as against 12.1% by wild type *Azospirillum* IABT-1.

The presence of the recombinant plasmid in the transgenic *Azospirillum* was analysed by isolation of plasmids from wild *Azospirillum* IABT-1 and one of the transconjugants. The plasmids were loaded onto 0.7% agarose gel. The difference in the plasmid profile between the wild and transgenic *Azospirillum* confirmed the presence of 14.8 kb pJSK-15 plasmid in the *Azospirillum* transconjugant (Plate 21).

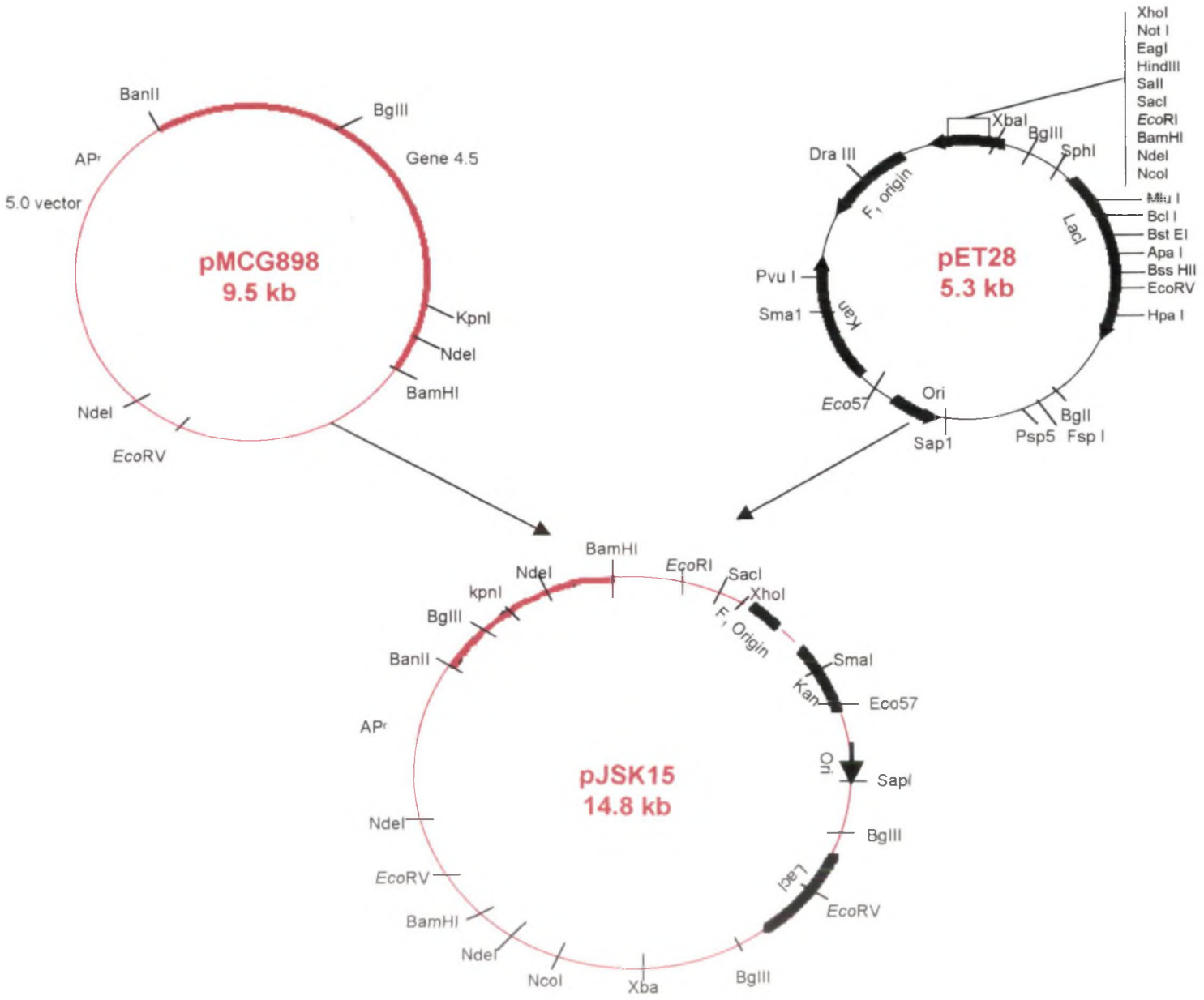


Fig. 4: pJSK15 construct

Plate 22: ***Azospirillum* IABT1 on MSM**

1. *Azospirillum* IABT1
2. pJSK15
3. AG 121

Plate 23: ***Azospirillum* IABT1 on MSM + PQQ**

1. AG 121
2. *Azospirillum* IABT1
3. DH5 α

Plate 24: **Transgenic *Azospirillum* IABT1 on MSM**

1. pJSK15
2. Transgenic *Azospirillum* IABT1
3. AG 121

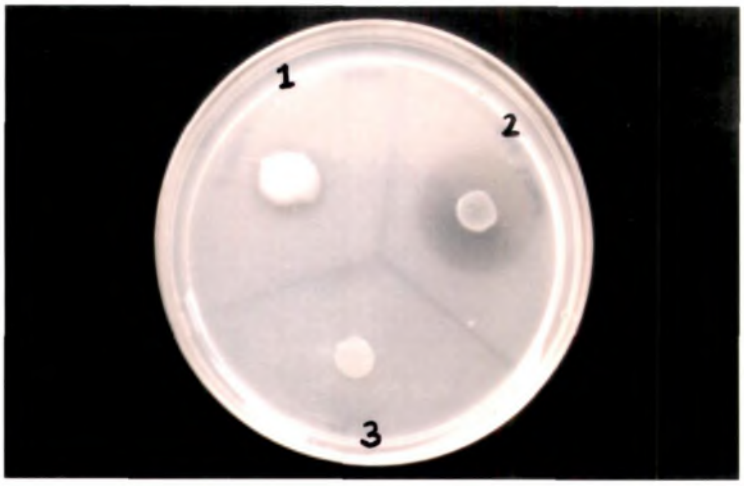


Plate 22. *Azospirillum* IABT1 on MSM

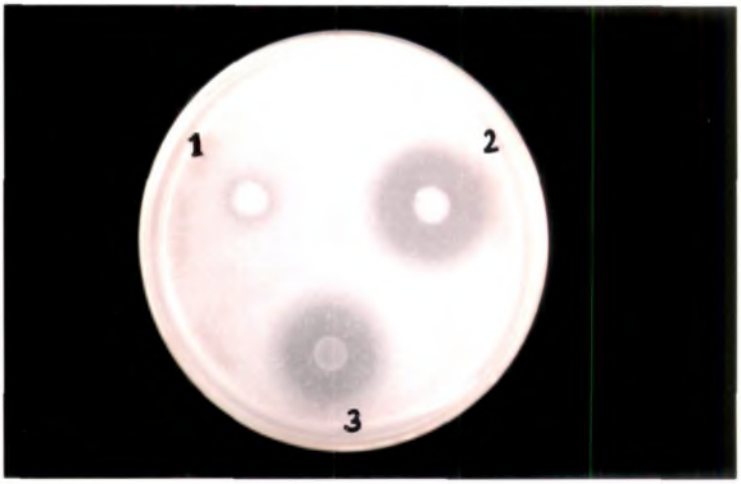


Plate 23. *Azospirillum* IABT1 on MSM + PQQ

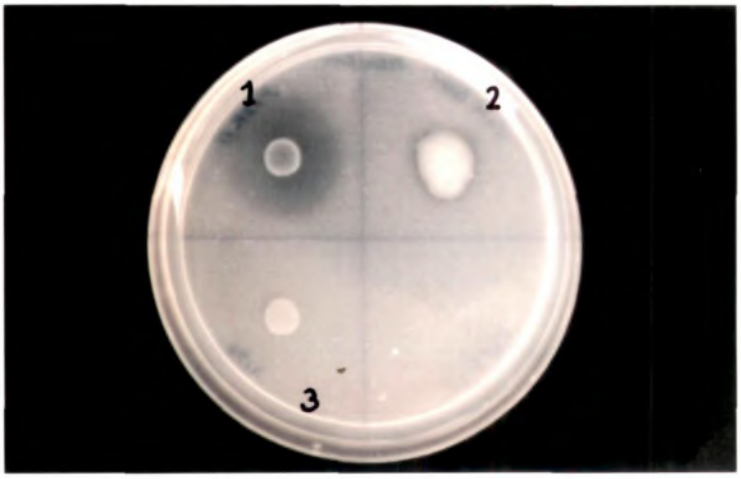


Plate 24. Transgenic *Azospirillum* IABT1 on MSM

Discussion

V. DISCUSSION

Phosphorus is an essential macronutrient required by cells and is involved from energy metabolism to the structure of the genetic material. Phosphorus represents the world's second largest bulk agricultural chemical product (Goldstein, 2000; Larsen, 1967). The eco-physiological paradox is that while most organisms can only assimilate Pi or low molecular weight soluble organic phosphates, most of the P pool in the soil is made up of poorly soluble mineral phosphate precipitates and high molecular weight organic 'P' (Eg. primary cell degradation products such as RNA and phospholipids). These P must be recycled from these unavailable forms back into available forms. Under these circumstances, a group of microorganisms allow the plants to absorb phosphorus from sources that are otherwise less available (Kucey *et al.*, 1989). Ever since Gerretsen (1942) demonstrated the ability of microorganisms to convert fixed phosphates in the soil into available forms, extensive research has gone into isolation of efficient organisms, understanding the biochemical basis and more recently the molecular basis of this phenomenon in gram-negative bacteria. The phenomenon of transforming the insoluble inorganic phosphates into available form is termed as mineral phosphate solubilization (MPS) (Goldstein, 1986).

Mineral phosphate solubilizing microorganisms thus, have provided a natural way for increasing the availability of phosphorus in soil rich in fixed phosphates. Further, enhancement of the efficiency is possible through selection or directed improvement towards better strains. The directed genetic improvement of the strain requires detailed information at genetic level for the process of solubilization. Information especially on the molecular aspects and

processes occurring at cell surfaces is scanty and need attention (Illmer and Schinner, 1992).

In the present study, genetic analysis on MPS function has been attempted through isolation of MPS mutants, complementation through PQQ, PCR cloning of *pqq, gcd* and *gnd* from *A. diazotrophicus* Pal5. The results obtained in the course of the investigation are discussed herein.

A diazotrophicus Pal5 was earlier reported to solubilize tricalcium phosphate (Mahesh kumar *et al.*, 1999). The strain was tested for stability of phenotype as earlier reports indicated that the MPS activity is lost during repeated subculture (Sperber, 1958;Kucey, 1983). The medium used for the study was modified Sperber's medium (MSM) and TCP medium. Even on repeated subculturing Pal5 showed large zone of solubilization both on MSM and TCP indicating that the MPS activity in *A. diazotropicus* Pal5 was a stable phenotype. Pal5 was further tested for the ability to release Pi in broth medium having tricalcium phosphate. The amount of Pi released was 58.88%. Pal5 showed a pH drop from 6.8 to 4.02 in the external medium.

An indicator medium was used to characterize Pal5 and the derived mutants at the colony surface level. The commonly used indicator, bromothymol blue was used, since it is blue at neutral pH and yellow in acidic solutions. Pal5 showed yellow colony and mutants showed light yellow coloured colony. The decrease in the coloration indicated the loss of ability of the mutants to acidify the external milieu.

The mechanism of MPS has been for long a subject of great curiosity. Extensive release of organic acids (Gaur, 1990; Illmer and Schinner, 1992; Kim *et al.*, 1997; Di Simine, 1998) or pH reduction of external medium (Illmer

et al., 1995; Krishnaraj, 1996) has been hypothesized to be the major cause of MPS activity in most of the organisms. The production of organic acids like lactic acid, gluconic acid, succinic acid, acetic acid, citric acid, oxalic and malic acid by phosphate solubilizing microorganisms has been reported earlier (Venkateshwaralu *et al.*, 1984; Illmer *et al.*, 1995). Hence, an attempt was made to study the profile of organic acids produced by the organism through TLC and HPLC. The result reveals that gluconic acid was the organic acid produced by Pal5.

The release of gluconic acid indicates the existence of a direct oxidation pathway involving the membrane bound dehydrogenase for glucose utilization. The involvement of such oxidation pathway is known for *Pseudomonas* (Babu-Khan *et al.*, 1995), *E. herbicola* (Liu, 1992), Enterobacter (Kim *et al.*, 1997) and *B. cepacia* (Maheshkumar 2003). The presence of a membrane bound glucose dehydrogenase in *A. diazotrophicus*, in fact, has been observed by Galar and Boiardi, 1995.

To genetically mark the strain for further characterization, its IAR was done.The IAR of Pal5 showed that it was resistant to ampicillin (100ppm), chloramphenicol (5 ppm), spectinomycin (20 ppm) and nalidixic acid(10 ppm) and sensitive to kanamycin (50), gentamycin (50 ppm), tetracycline (10ppm), streptomycin (100ppm) and rifampicin (25 ppm) among the nine antibiotics / drugs tested.

In a forward genetic analysis approach to understand the molecular basis of the MPS phenotype of *A. diazotrophicus*, the strain was subjected to mutagenesis. A collection of desired independently isolated random mutants

was obtained through chemical mutagenesis employing NTG. Characterization of such mutants provided the following information.

Seven independent mutants defective in MPS activity, isolated through screening 2000 NTG survivours from mutagenised culture showed distinct MPS phenotype(s) compared to the wild type strain Pal5. The mutants were confirmed as derivatives of *A. diazotrophicus* Pal5 as they showed 411 bp PCR product when genomic DNA from NTG mutants were amplified using *A. diazotrophicus* species specific primers (Kirchoff *et al.*, 1998).

Broadly, these mutants could be classified into two categories.

i) MPS negative mutants: Six of the seven mutants showed no zone of solubilization on MSM and released negligible Pi from TCP in broth medium. They were designated as MPS$^-$ phenotype.

ii) Leaky mutant: One mutant showed weak solubilization (42.9%) as compared to that of wild type. This was designated as a leaky mutant.

The nature of the above two classes of mutants defined clearly by their phenotype and biochemical analysis indicates the involvement of more than one genetic loci directly or indirectly in phosphate solubilization. Phosphate regulated genes belong to a distinct regulon (Makino *et al.*, 1989) which may be involved in cellular metabolism in a major way controlling several other activities (Wanner, 1987). Such genes showing complex roles can lead to pleiotropic effect in mutated state. Thus, the mutants selected for altered MPS phenotype were also tested for other growth and metabolic characteristics like cell surface properties, ability to lower external pH etc.

Six of the seven mutants showed faster growth on MSM, TCP and NAG medium taking 12-16 hours to form a streak of growth while the wild type and one mutant showed slow growth, taking 24-36 hours. In addition, all the mutants did not take up dye and appeared light yellow on MSM (BTB). These pleiotropic effects implicate related phenotype to be having common genetic control where phosphorus plays an important role.

The altered phenotypic effect indicates, that MPS^- mutants do not excrete acids. Hence no yellow colour developed on MSM (BTB). Such pleiotrophy had been reported earlier for *Pseudomonas* sp. Psd201 (Krishnaraj, 1996).

The mechanism of MPS activity has been attributed to organic acid release (Mehta *et al.*, 1979; Banik and Dey, 1983) or direct acidification (Illmer and Schinner, 1992). The arguments on the actual mode remains so far unresolved (Illmer *et al.*, 1995). Hence, the mechanism responsible for MPS was analysed using MPS^+ and MPS^- mutants in relation to the organic acid production and related traits.

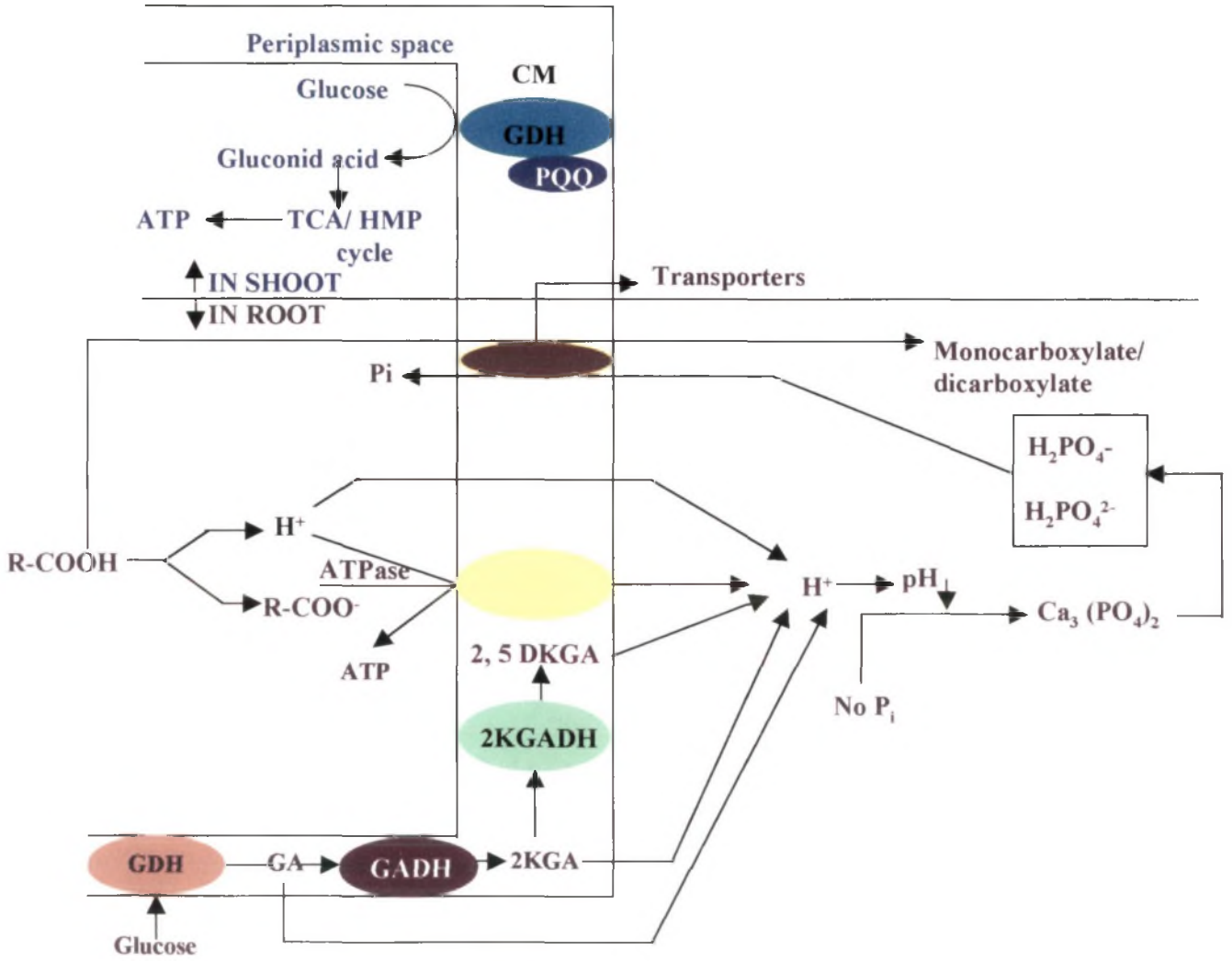
All the MPS defective mutants had lower Pi release in TCP broth than wild type. The Pi released by MPS mutants ranged from 9.6% (ADM-5) to 42.9% (ADM-7) where as the wild type showed a Pi release of 52.88%. Such MPS defective mutants have been isolated earlier by Krishnaraj (1996) in *Pseudomonas, S. marcecens* by Santi (1998) and *P. fluorescens* by Deepa (2000). The wild type showed the maximum reduction of pH and brought down the pH from 6.8 to 4.02. All the mutants except ADM-7 caused lower pH drop, which showed pH drop higher than other mutants but lesser than the wild type. The TLC analysis showed that all the mutants except ADM-7

showed reduced production of gluconic acid. The wild type Pal5 under identical conditions showed extensive production of gluconic acid.

Transcomplementation analysis of the mutants was attempted by providing PQQ supplemented externally in the MSM medium. Of the six MPS$^-$ mutants, only ADM-4 showed MPS activity in the presence of PQQ. PQQ is a cofactor for glucose dehydrogenase activity and is required for production of gluconic acid production in many gram-negative bacteria (Liu *et al.*, 1992). The enzyme is membrane bound and faces the periplasm. Thus mutant ADM-4 now serves as a strain for isolating the gene(s) involved in the synthesis of PQQ in *A. diazotrophicus.* The other MPS$^-$ mutants can further be used to isolate genes involved in MPS activity by transcomplementation analysis of the mutants through mobilization of clones of a genomic library of *A. diazotrophicus* Pal5.

The analysis of various parameters involved in gluconic acid production and the mutants defective in MPS activity reveal the mechanism of phosphate solubilization. The cloning of of *pqq, gcd* and *gnd* via PCR cloning also indicate the presence and utility of the biochemical pathway involving gluconate synthesis and metabolism in *A. diazotrophicus.* The overall role of the gluconic acid synthesis in *A. diazotropicus* and its use subsequently both for the bacteria and in the soil has been brought out in Fig 6.

The extrusion of gluconic acid into the soil is involved in the solubilization of fixed P. In addition, the gluconic acid is also involved in energy generation by entering into TCA and HMP pathway that the organism utilizes for nitrogen fixation (Maria *et al.*, 2002). The organic acid also provide a source of H^+ ions in the cells which can be released in the external



CM - Cytoplasmic membrane
GADH - Gluconate dehydrogenase
2,5DKGA - 2,5 di keto gluconic acid

GDH - Glucose dehydrogenase
2KGA - 2 keto-gluconic acid
TCA - Tricarboxylic acid

PQQ - Pyrrolo quinoline quinone
2KGADH - 2keto gluonate dehydrogenase
HMP - Hexose mono phosphate

Fig. 5: Importance of gluconic acid synthesis in *A. diazotrophicus*

environment through the involvement of membrane bound ATPase. The ATPase, causes higher trans membrane potential and greater capacity to acidify the medium. Such functions of ATPase consequent to increase of organic acid production in cells and phosphate stress to release H+ ions, maintain internal pH of cells at set values is known to be of prime importance in plants (Le Bot *et al.*, 1994). Based on the results obtained and the change in pH, Pi release, presence of organic acid in the culture filtrate and related literature available, a hypothetical model for the mechanism of MPS and the role of gluconic acid in the bacteria is presented.

The figure indicates that the bacterial cell in response to PO_4 starvation accumulate organic acids intercellulary. On further accumulation, they serve as a source of protons, which are released into the medium. The released proton may account for solubilization of TCP with subsequent release of Pi. The Pi so released is probably imported into PO_4 starved cells by monocarboxylate or dicarboxylate transporters that are membrane bound proteins. These proteins transport Pi with concomitant exchange of tricarboxylate such as succinate (Vivekanand *et al.*, 1988) and monocarboxylate (Vivekanand and Oliver, 1990; Oliver and McIntish, 1994). The organic acid so released might explain the presence of organic acid in culture filtrate by wild type and MPS used in the study.

In addition to assisting the solubilization of 'P' in the soil, the gluconic acid produced by *A. diazotrophicus* involves in the energy generation, via the direct oxidation pathway. Yet another pathway into which gluconic acid is implicated is through the hexose monophosphate pathway and TCA cycle

(Maria *et al.,* 2002). The gluconic acid ($C_2H_{12}O_7$) metabolized through this pathway ultimately leads to the production of ATP via the following equation.

$C_2H_{12}O_7 \longrightarrow 6CO_2 + 11\ NADH + 2\ ATP$ (Maria et al., 2002)

A. diazotrophicus is also known for its nitrogen fixing ability and needs high energy in presence of sugars and under N_2 fixing condition found inside the plants. Thus, the PO_4 starvation induced periplasmic oxidation and the HMP-TCA cycle may just provide that. Metabolic engineering of the gluconate metabolism would lead to increased availability of energy for the cellular processes.

An attempt was made to clone *pqq,gcd* and *gnd* gene from *A. diazotropicus* by PCR based cloning. This was done by designing allele specific primer using informations on the sequences of the gene available in the database. The amplicon generated on PCR amplification of the total DNA of *A. diazotropicus* specific to *pqq,gcd* and *gnd* primers(800bp, 750bp, 1200bp) were gel eluted and cloned into pTZR57/T cloning vector. The white colonies obtained on LA + Amp_{100} +X-Gal IPTG were confirmed for the presence of inserts by subjecting their plasmid preparations to PCR amplification using allele specific primers.The amplification in the selected clones and not in the control vector pTZ57R indicated the presence of inserts. Cloning of pqq synthase gene was earlier reported in *B. cepacia* (Maheshkumar, 2002) using a 800 basepairs PCR amplicon of *pqq* synthase gene. Genes involved in *pqq* synthase has also been reported to be cloned from *Erwinia herbicola* (Liu *et al.,* 1992) and *P. cepacia* (Babu-Khan, 1995).

The recombinant construct pJKQ1 containing *pqq* gene(s) was transformed into *E. coli* BL21 to know the expression of recombinant plasmid.

The cloned fragment in pTZ57R would be transcribed by T_7 RNA polymerase. *E. coli* BL21 PLysS provides this function to express the protein by the cloned fragment. The transformants obtained were assessed for desired function on MSM. The transformants showed zone of solubilization which was not shown by the same transformants in XL1Blue. This indicates the expression of MPS activity in *E.coli* BL21. Thus, it can be inferred that the fragment required for gluconic acid production through transcomplementation of apo GDH in *E.coli* has been cloned. The gram-negative bacteria have evolved a unique periplasmic oxidation system coupled with respiratory chains, which is mainly initiated by Quinoprotein dehydrogenases bearing a PQQ as a non covalently bound prosthetic group (Mamoru, 2003).

The clone pJKN1 was custom sequenced at Bangalore Genei Pvt. Ltd., Bangalore using an automated DNA sequences (Applied Biosystems AB1100 Version 3.2). The sequence was analysed using the nucleotide-nucleotide (BLASTn) search using program available at NCBI website (http://www.ncbi.nlm.nih.gov/). Homology search of sequences obtained from pJKQ1 using M13 forward and reverse primer revealed that the fragment (661 bp and 428 bp respectively) had 100 per cent homology with the other reported *gnd* gene from *E. coli, S. enterica* and *S. typhimurium*. The map of construct pJKN1 containing *gnd* gene is shown in Fig. 7. The position of the primer sequence in the overall sequence indicates that the fragment has been cloned in correct orientation.

In this study, one of the *Azospirillum* strains, IABT-1 was observed to solubilize phosphate in MSM medium in presence of externally added PQQ. This indicates that, *Azospirillum* possesses an apo GDH but doesn't have the genes for synthesis of PQQ. Hence, an attempt was made to clone *pqq*

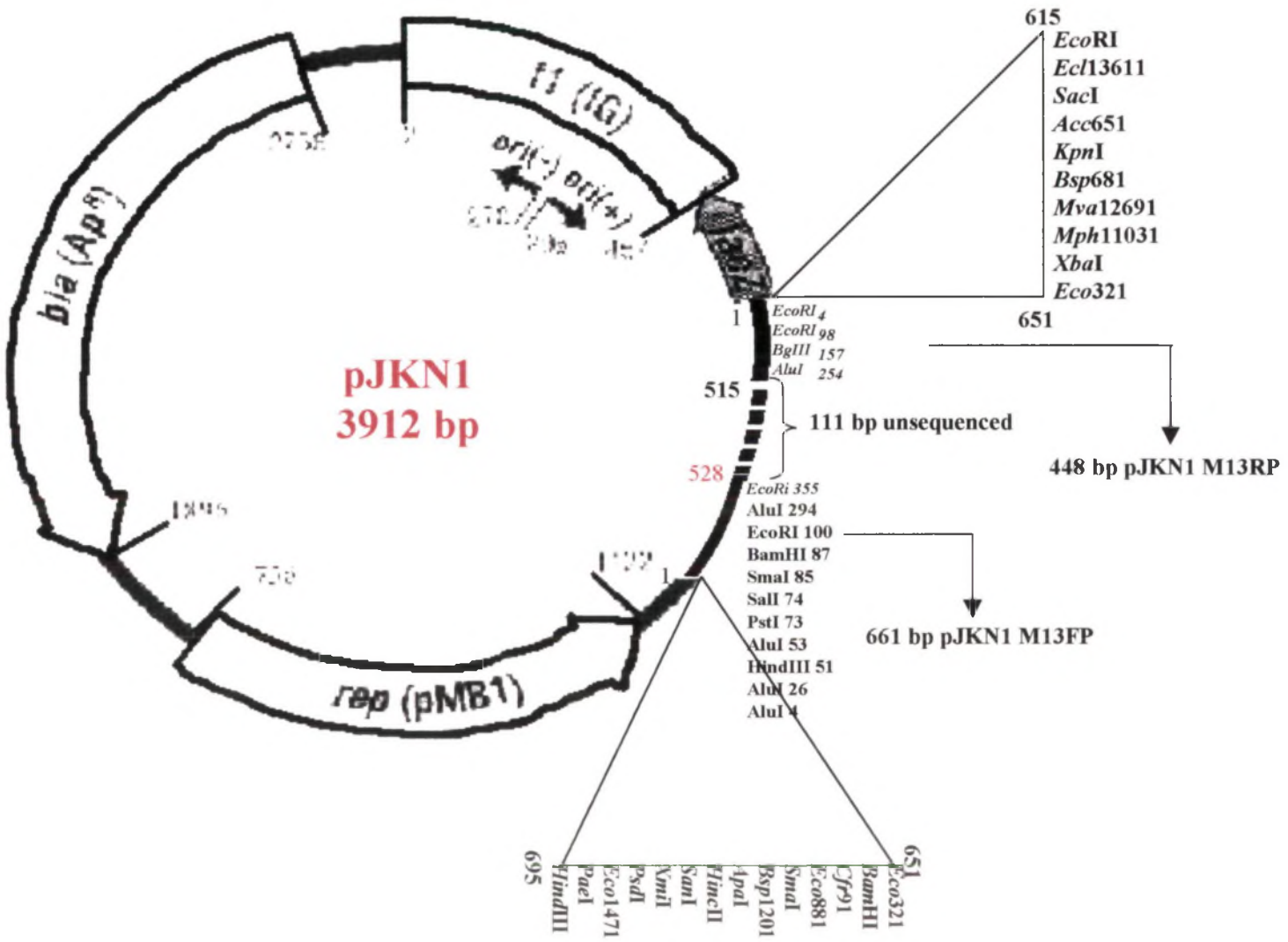


Fig. 6: pJKN construct

gene(s) present in pMCG898 (Babu-Khan, 1995) that transcomplemented *E. coli* HB101 into showing MPS activity. The *Azospirillum* IABT-1 being Amp^R (Marker for pMCG898), a construct was developed by cloning linearised pMCG898 into *Bam*HI site of pET28. The resulting construct pJSK-15 (Amp^R , Kan^R) was then mobilized into IABT-1 by triparental mating. The transgenic IABT-1 was confirmed for the presence of pJSK15 by miniprep analysis. However, the transgenic IABT-1 (pJSK-15) did not show MPS activity. Thus, it indicates that the pqq gene(s) present in pMCG898 wasn't enough to transcomplement the apo GDH present in *Azospirillum* IABT-1. *Azospirillum* IABT, therefore doesn't either posses the entire complement of pqq genes(s) required for PQQ synthesis or doesn't contain a fragment that is not present in pMCG898, to complement it. There are several reports that *E. coli* strain carrying heterologous *pqq* genes can synthesise PQQ and can therefore make active glucose dehydrogenose. However, the number of genes needed to obtain PQQ synthesis varies, depending on the source of heterologous DNA. When *K. pneumoniae* DNA was used, all six *pqq* genes seem needed for PQQ production by *E. coli* (Meulenberg *et al.,* 1992). This is consistent with the report that four *A. calcoaceticus* pqq genes IV, I, II and III equivalent to *pqq* A, C, D and E of *K. pneumoniae)* were required for PQQ synthesis by *E. coli* (Goosen *et al.,* 1989). This variability and the results obtained in this study on the lack of expression of MPS activity by *Azospirillum* IABT-1 (pJSK15) indicates the necessity to clone the entire complement of pqq genes available in *K. pneumoniae* and transform into *Azospirillum* to make it an MPS transgenic bacteria.

Summary

VI. SUMMARY

Genetic dissection of MPS phenomenon in *Acetobacter diazotrophicus* Pal5 was attempted through analysis of culture supernatant of its growth in TCP broth, development of mutants defective in MPS activity and isolation of genes involved in phosphate metabolism. The results obtained are summarized here.

1. *A diazotrophicus* released 52.88% of Pi in TCP broth after 5 days of incubation. It reduced the pH of the broth from 6.8 to 4.02 during the same period of time. The culture supernatant showed the presence of gluconic acid.

2. *A diazotrophicus* was subjected to mutagenesis using NTG. Of the seven MPS defective mutants obtained six were MPS^- and the other mutant ADM-7 showed reduced MPS activity. These mutants had reduced ability to decrease the pH of the medium. However, the culture supernatant of the mutants contained gluconic acid as observed through TLC and HPLC analysis. The mutants were confirmed as derivatives of Pal5 as they possessed the distinct 411 bp amplicon with AD1440, the species-specific primer.

3. Primers were designed to pick up *pqq, gcd* and *gnd* genes from *A diazotrophicus*. PCR amplification using these primers yielded 800bp, 750 bp and 1200 bp amplicon corresponding to *pqq, gcd* and *gnd* genes respectively. These were cloned into pTZ57R through T/A cloning and transformed into *E coli* XL1Blue.

4. pJKN1containing the *gnd* was custom sequenced and the BLASTn analysis showed 100% homology to *Salmonella enterica gnd* gene, *Salmonella typhimurium gnd* gene and *E coli gnd* gene. The recombinant pJKQ1 containing *pqq* amplicon was transformed into *E coli* BL21 and the transformants showed zone of solubilization indicating that the 800 bp amplicon was sufficient to complement MPS activity in *E coli*. The sequencing of the recombinant plasmid containing *pqq* and *gcd* is in progress.

5. *Azospirillum* IABT1 (MPS^-) showed MPS activity when PQQ was added into MSM. It was however ampicillin resistant. Hence, the construct pJSK15 containing kanamycin marker of pET28 was developed and mobilized into *Azospirillum* IABT1. The resultant tranconjugants however showed no zone of solubilization on MSM indicating the *pqq* gene(s) in pMCG898 was not sufficient to complement MPS activity in *Azospirillum*.

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Appendices

APPENDIX I

Media composition

1. **Nutrient agar + glucose medium (NAG) (Bindu, 2002)**

Peptone	:	5 g
Beef extract	:	3 g
Sodium chloride	:	5 g
Glucose	:	30 g
Agar	:	18 g
Distilled water	:	1000 ml
pH	:	6.8 - 7.0

2. **LGI medium (Cavalcante and Dobereiner, 1988)**

Sucrose	:	100 g
K_2HPO_4	:	0.2 g
KH_2PO_4	:	0.6 g
$MgSO_4.7H_2O$	:	0.2 g
$CaCl_2.7H_2O$	:	0.02 g
$FeCl.6H_2O$	:	0.01 g
Bromothymol Blue(0.5% in 0.2N KOH)	:	5 ml
Agar	:	18 g
Distilled H_2O	:	1000 ml
PH (Adjusted with acetic acid)	:	4.5

3. **Modified Sperber's medium MSM (Krishnaraj, 1996)**

Glucose	:	10 g
Yeast extract	:	0.5 g
$MgSo_4.7H_2O$	:	0.25 g
$CaCl_2$	:	0.1 g
Agar	:	18 g
Distilled water	:	1000 ml
PH	:	6.8

Add 10% $CaCl_2$ @ 3 ml and 10% K_2HPO_4 @ 2 ml/100 ml before pouring the media into the plates.

4 **Tricalcium phosphate medium (TCP) (Pikovskaya, 1948)**

Glucose	:	10 g
$MgSo_4$. 7 H_2O (2.5%)	:	10 ml
$CaCl_2$ (1%)	:	10 ml
Tricalcium phosphate	:	5 g
Agar	:	18 g
Distilled water	:	1000 g
PH	:	7.0

5. **Luria agar (Sambrook *et al.*, 1989)**

Tryptone	:	10 g
Yeast extract	:	5 g
Sodium chloride	:	5 g
Agar	:	18 g
Distilled water	:	1000 ml
pH	:	7.2

6. **M9 Glucose minimal medium (Sambrook *et al.*, 1989)**

Water agar	:	750 ml
M9 salts	:	200 ml
1 M $MgSO_4$	:	2 ml
20% glucose	:	20 ml
1 m $CaCl_2$	:	0.01 ml
M9 salts		
Na_2HPO_4 . $7H_2O$	:	64 g
KH_2PO_4	:	15 g
NaCl	:	2.5 g
NH_4Cl	:	5 g
Distilled water	:	1000 ml

APPENDIX II

Protocol for estimation of available P in the culture

Reagents:

Chloromolybdic acid: Chloromolybdic acid was prepared by dissolving 7.5 g of Ammonium molybdate in 150 ml distilled water to which 162 ml of concentrated Hcl was added. The volume was made upto one litre with distilled water.

Chlorostanous acid: Chlorostanous acid reagent was prepared by dissolving 25 g of $SnCl_2$. $2H_2O$ in 100 ml concentrated HCl and making up the volume of one litre with distilled water.

Both the reagents were stored in amber coloured bottles in a refrigerator.

Preparation of standard curve

Potassium dihydrogen phosphate was dried at 40°C and 0.2195 g of it was dissolved in 400 ml of distilled water. 25 ml of 7N H_2SO_4 was added to it and the volume was made up to 1 litre with distilled water and was mixed thoroughly. 20 ml of this was diluted further to 500 ml with water to obtain 2 ppm solution and used for the preparation of standard curve. At the end of the incubation period, the culture was centrifuged at 10,000 rpm for 20 minutes and the supernatant was separated out. One ml of this supernatant was taken in a 50 ml volumetric flask to which 10ml of chloromolybdic acid was added and mixed thoroughly. The volume was made upto approximately three fourth with distilled water and then 0.25 ml chlorstannous acid was added to it. After 15 minutes, the blue colour developed was read in spectrophotometer at 610 nm using reagent blank. Simultaneously, a standard curve was prepared using various concentrations of standard K_2HPO_4 solution. The amount of phosphorus solubilized was calculated from the standard curve.

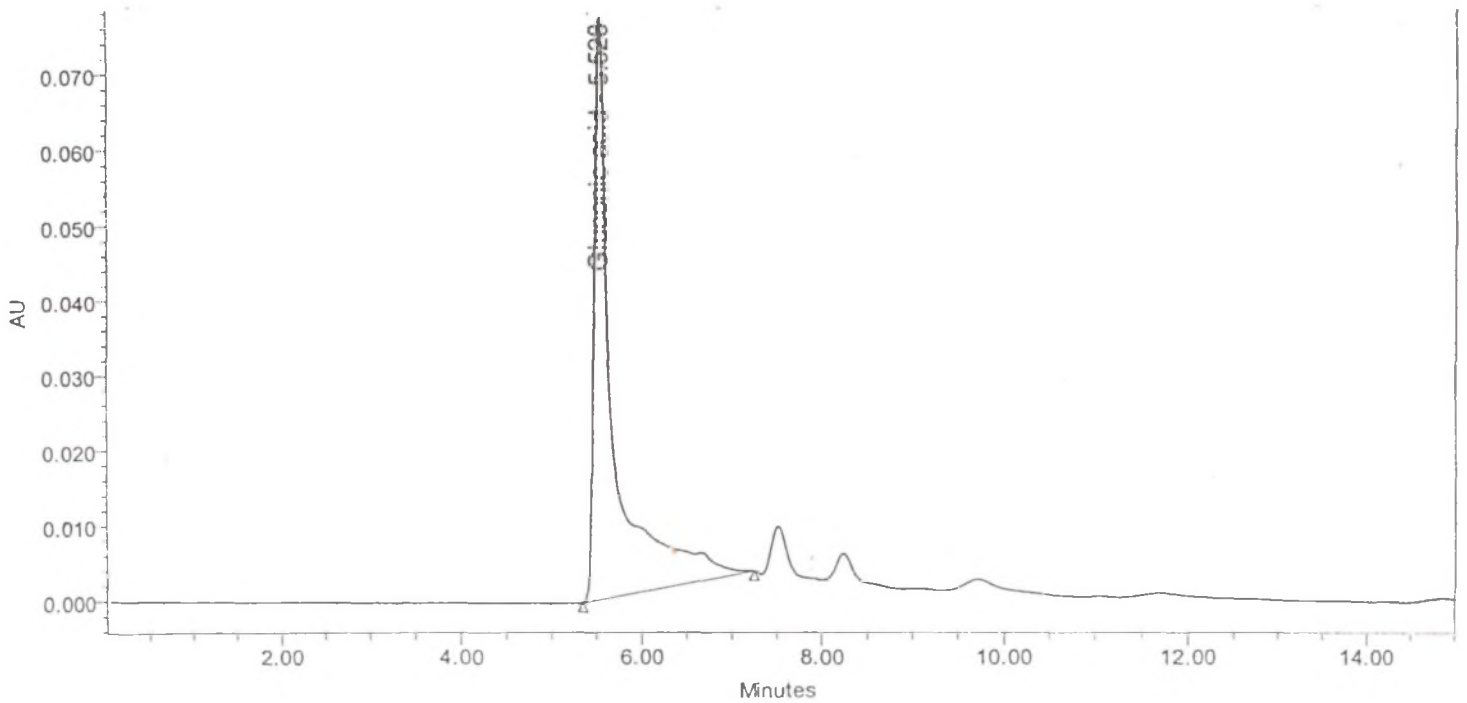

Fig. 7: HPLC analysis of standard gluconic acid

APPENDIX III

Chemicals / solutions used in SDS PAGE

1.	**30% Acrylamide**		
	Acrylamide	:	29.2 g
	Bis-acrylamide	:	0.8 g
	Distilled H_2O	:	Upto 100 ml
2.	**Resolving gel (10%)**	:	**30 ml**
	Water	:	11.9 ml
	30% acrylamide mix	:	10 ml
	1.5 m tris (pH 8.8)	:	7.5 ml
	10% SDS	:	0.3 ml
	10% Ammonium per sulphate	:	0.3 ml
	TEMED	:	0.12 ml
3.	**Stacking gel (5%)**	:	10 ml
	Water	:	6.8 ml
	30% acrylamide mix	:	1.7 ml
	1.5 m tris (pH 8.8)	:	1.25 ml
	10% SDS	:	0.1 ml
	10% Ammonium per sulphate	:	0.1 ml
	TEMED	:	0.1 ml
4.	**2X loading dye**		
	Tris (pH 6.8)	:	100 mM
	SDS	:	5%
	BPB	:	0.2%
	Glycerol	:	20%
	DTT	:	200 mM

5. **Tris glycine tank buffer (5x)**

Tris base	:	15.1 g
Glycine	:	94 g
SDS (10X)	:	50 ml
Distilled water	:	Upto 100 ml

7. **Staining solution**

Coomassie brilliant blue	:	1.5 g
Methanol	:	500 ml
Acetic acid	:	200 ml
Distilled water	:	500 ml

8. **Destaining solution**

Methanol	:	300 ml
Acetic acid	:	100 ml
Distilled water	:	600 ml

APPENDIX IV

Reagents for total DNA isolation

Stock solutions

1M tris(pH 8.0)

Tris	:	121.1 g
Water	:	800 ml
PH (adjusted with concentrated HCl)	:	8.0
Total volume	:	1000 ml

EDTA 0.5 M (pH 8.0)

EDTA	:	18.6 g
Water	:	80 ml
PH (with NaOH)	:	8.0
Total volume	:	100 ml

Sodium chloride 1M

Sodium chloride	:	58.44 g
Water	:	800 ml
Total volume	:	1000 ml

Lysozyme: 50 mg/ml

Dissolve 50 mg of lysozyme in 1 ml of $T_{10}E_1$ (pH: 8.0) store at -20°C

Proteinase K: 20 mg/ml

Dissolve 20 mg of proteinase K in 1 ml of sterile distilled water. Store at -20°C.

Dnase free RNase: 10 mg/ml

Dissolve 10 mg of DNAase free RNAase in 1 ml of sterile distilled water. Store at -20°C.

2% Sarkosyl

Dissolve 2g of Sarcosyl in 100 ml of $T_{50}E_{20}$ (pH : 8.0)

Sodium acetate 3M

Dissolve 408.3 g of sodium acetate in 800 ml of distilled water, adjust pH with glacial acetic acid and make up the volume to 1 litre.

Phenol:Chloroform

Mix equilibriated phenol (pH > 7.8), chloroform and isoamyl alcohol in the ratio 25:24:1 (v/v), store at 4°C.

Appendix IVb:

Ethidium bromide: 10 mg/ml in distilled water. Store at room temperature in dark bottle.

Appendix IVc:

Agarose gel loading buffer (6x)

0.25 % Bromophenol blue

40% (W/V) sucrose in water

Store at 4°C

Appendix IVd:

1% agarose gel (40 ml)

Agarose	:	400 mg
1 x TAE	:	40 ml
EtBr (10 mg/ml)	:	2 µl

Appendix IVe:

50 x TAE composition

Tris base	:	242 g
Glacial acetic acid	:	57.1 ml
0.5 M EDTA (pH 8.0):		100 ml

Total volume 1000 ml with double distilled water

APPENDIX V

Appendix Va: Conversion table for the amount of a PCR fragment required per ligation reaction

Length of DNA fragment	Pmoles of ends per 1 μg of DNA	Quantity of PCR fragments for ligation reaction in μg (0.54 pmol ends)
100	30.0	0.018
300	10.0	0.054
500	6.0	0.090
1000	3.0	0.180
2000	1.5	0.360
3000	1.0	0.540

Appendix Vb: Ligation reaction recipe for *pqq* synthase

1. Plasmid vector pTZ57R/T DNA (0.165 mg, 0.18 pmol ends) : 3.0 μl
2. Purified PCR fragment (Approximately 0.54 pmol ends) : 2.0 μll
3. 10x ligation buffer : 3.0 μl
4. PEG 400 solution : 3.0 μl
5. BSA : 0.75μl
6. Deionised water : 18 μl
7. T_4 DNA ligase (5V) : 1.0 μl

Total volume 30 μl

Ligation reaction recipe for gcd

1. Plasmid vector pTZ57R/T DNA (0.165 μg, 0.18 pmol ends)	:	3.0 µl
2. Purified PCR fragment (Approx. 0.54 pmol ends)	:	4.5 µl
3. 10 x ligation buffer	:	3.0 µl
4. PEG 400 solution	:	3.0 µl
5. Deionised water	:	14.75 µl
6. BSA	:	0.75 µl
7. T_4 DNA ligase (5V)	:	1.0 µl
Total volume		30 µl

Ligation reaction recipe for gnd

1. Plasmid vector pTZ57R/T DNA (0.165 μg, 0.18 pmol ends)	:	3 µl
2. Purified PCR fragment (Approx. 0.54 pmol ends)	:	6 µl
3. 10 x ligation buffer	:	3 µl
4. PEG 4000 solution	:	3 µl
5. Deionised water	:	13.25 µl
6. BSA	:	0.75 µl
7. T_4 DNA ligase	:	1 µl
Total volume		30 µl

Appendix Vc: Control ligation reaction

PTZ57R/T DNA (0.165 μg, 0.18 pmol ends)	:	3.0 µl
Purified PCR fragment, (Approx. 0.54 pmol ends)	:	12.3 µl
10 x ligation buffer	:	3.0 µl
PEG 4000 solution	:	3.0 µl
Deionised water	:	7.7 µl
T_4 DNA ligase	:	1.0 µl
Total volume		30 µl

X-Gal solution (2% W/V) : Dissolve X-Gal at a concentration of 20 mg/ml in water (Sambrook and Russel, 2001)

IPTG : Dissolve 2 g IPTG in 8 ml water and makeup the volume to 10 ml, filter sterilize and store at -20°C.

APPENDIX VI

Restriction reaction recipe

Pqq synthase recombinant

	*Bam*H1	*ECO*R1	*Hind* III
Plasmid DNA	3µl	3 µl	3 µl
Buffer (1 x)	1µl	1 µl	1 µl
Enzyme (2U)	1µl	1 µl	1 µl
BSA (1x)	1 µl	-	-
Sterile H_2O	4 µl	5 µl	5 µl
	10 µl l	10 µl	10 µl

***gcd* recombinant**

	Enzymes		
	*Bam*H1	*ECO*R1	*Hind* III
Plasmid DNA	3 µl	3 µl	3 µl
Buffer (1 x)	1 µl	1µll	1 µl
Enzyme (2U)	1 µl	1 µl	1 µl
BSA (1x)	1 µl	-	-
Sterile H_2O	4 µl	5 µl	5 µl
	10 µl	10 µl	10 µl

	Enzymes		
	*BamH*1	*ECO*R1	*Hind* III
Plasmid DNA	3 µl	3 µl	3 µl
Buffer (1 x)	1 µl	1 µl	1 µlµl
Enzyme (2U)	1 µl	1 µl	1 µl
BSA (1x)	1 µl	-	-
Sterile H_2O	4 µl	5 µl	5 µl
	10 µl	10 µl	10 µl

APPENDIX VII

Appendix VIIa: Reagents for plasmid isolation

STE buffer

Tris-cl (pH. 8.0	:	10 mm
NaCl	:	0.1 M
EDTA (pH 8.0)	:	1.0 mM

Autoclave and store at 4°C

Alkaline lysis solution 1

Glucose	:	50 mM
Tris Cl (pH 8.0)	:	25 mM
EDTA (pH 8.0)	:	10 mm

Alkaline lysis solution II

NaOH	:	0.2 N
SDS	:	1% (w/v)

(Prepare fresh and use at room temperature)

Alkaline lysis solution III

5 M potassium acetate	:	50 ml
Glacial acetic acid	:	11.5 ml
Double distilled water	:	28.5 ml

Autoclave and store at 4°C

Appendix VIIb : Restriction reaction of PET28 and PMCG 898 with *Bam*H1

Plasmid DNA (pET28)	:	5 µl
Enzyme (2U)	:	1µl
Sterile water	:	2 µl
10 x BSA	:	1 µl
10x Buffer	:	1 µl
		10 µl
Plasmid DNA (pMCG 898)	:	5 µl
10x Buffer	:	1 µl
Enzyme (2U)	:	1 µl
10 x BSA	:	1 µl
Sterile water	:	2 µl
		10 µl

Appendix VIIc : Dephosphorylation of pET28

Restricted plasmid (pET28)	15 µl
Enzyme (CIAP) (2U) (Calf intensive alkaline phosphatase)	1 µl
Buffer (10X)	2 µl
Sterile water	2 µl
	20 µl

Appendix VII d: Ligation of pMCG 898 and pET 28

Plasmid DNA (pMCG 898)	5 µl
Plasmid DNA (pET 28)	5 µl
T_4 DNA ligase Enzyme (10U)	1 µl
Buffer(10X)	1 µl
Sterile H_2O	3 µl
	15 µl

Appendix VIIe : Restriction of recombinant plasmid

Plasmid pJSK15	5 µl
*Bam*HI Enzyme (2U)	1 µl
Buffer (10X)	1 µl
BSA (10X)	1 µl
Sterile H_2O	2 µl
	10 µl

APPENDIX VIII

Sequence data of pJKN1M13FP and pJKN1M13RP

Sl. No.	Sequence data	pJKN1M13FP	pJKN1M13RP
1.	Total No. of bases	661	428
2.	Total No. of degenerates	0	0
3.	Per cent GC density	58.5	60.3
4.	Per cent AT density	41.5	39.7
5.	Sense strand molecular weight as DNA	204.4 kDa	131.91 kDa
6.	Antisense strand molecular weight as DNA	204.12 kDa	132.63 kDa
7.	Weight of DNA duplex	408.53 kDa	264.54 kDa
8.	Sense strand molecular weight as RNA	212.93	137.75
9.	Antisense strand molecular weight as RNA	212.9	138.11
10.	Weight of RNA duplex	425.83	275.83
11.	Melting temperature as dsDNA [(K+) = 50 mM]	82.9°C	83.20°C

MOLECULAR DISSECTION OF MINERAL PHOSPHATE SOLUBILIZATION IN *Acetobacter diazotrophicus* Pal5

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ABSTRACT

The genetic dissection of Mineral Phosphate Solubilization (MPS) in *Acetobacter diazotrophicus* Pal5 was attempted through the development and characterization of mutants defective in MPS activity and isolation of genes involved in phosphate metabolism.

A. diazotrophicus released 52.88% of Pi in TCP broth after 5 days of incubation. TLC analysis of the culture filtrate showed the presence of gluconic acid. Mutagenesis of *A. diazotrophicus* using NTG yielded six MPS^- mutants and one leaky mutant. The mutants were confirmed as derivatives of Pal5, since the distinct 411 bp amplicon was obtained on PCR of the genomic DNA with AD-1440, the species - specific primer.

PCR amplified fragments using primers for *pqq, gcd* and *gnd* (800 bp, 750 bp, 1200 bp amplicons respectively) were cloned into pTZ57R through T/A cloning and transformed into *E. coli* XL1Blue. The recombinant pJKN1 containing the *gnd* was sequenced using M13 primers and the BLASTn analysis showed 100% homology with *Salmonella* and *E. coli gnd* gene. The recombinant pJKQ1 containing *pqq* amplicon expressed in *E. coli* BL21 resulting in zone of solubilization on MSM. Hence, the 800 bp amplicon was sufficient to transcomplement apo-*gdh* in *E. coli* to release Pi from mineral phosphates.

Azospirillum IABT-1 (MPS^-) showed MPS activity when PQQ was added into MSM but was Amp^R. Hence, a construct pJSK15 containing *pqq* synthase gene(s) was developed and mobilized into *Azospirillum* IABT-1. The resultant transconjugants however, showed no zone of solubilization on MSM indicating that the *pqq* gene(s) in pMCG898 was not sufficient to complement MPS activity in *Azospirillum* IABT-1.