

Strategies for the Development of Environmentally Friendly Phosphate Fertilizers Based on Gram-Negative Phosphate Solubilizing Bacteria

VFRC Report 2014/5



Alan Goldstein



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¹biomimetics@hotmail.com

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Washington, D.C., USA

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Suite 800
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Citation

Goldstein, A., 2014.

Strategies for the Development of Environmentally Friendly Phosphate Fertilizers Based on Gram-Negative Phosphate Solubilizing Bacteria. VFRC Report 2014/5. Virtual Fertilizer Research Center, Washington, D.C. 51 pp.; 5 tables; 27 figs.; 45 refs.

Virtual Fertilizer Research Center



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List of acronyms and abbreviations

2-KGA	Arbuscular mycorrhizal fungi
AMF	Direct oxidation
DGGE	Denaturing Gradient Gel Electrophoresis
DO	Environmentally Friendly Phosphate Fertilizer
ELISA	Enzyme Linked Immunosorbent Assay
ELISA	Gluconic acid
GA	Glucose Dehydrogenase
GDH	Glucose oxidase
GOX	2-Ketogluconic acid
IFDC	International Fertilizer Development Center
Mab	Monoclonal antibody
MPS	Mineral Phosphate Solubilization
MPSM	Mineral Phosphate Solubilizing Microbes
NGO	Non-Governmental Organization
PGPR	Plant Growth Promoting Rhizobacteria
Pi	Inorganic phosphate
PQQ	Pyroloquinone quinone
PSM	Phosphate Solubilizing Microbes
qPCR	Quantitative Polymerase Chain Reaction
RPO	Rock Phosphate Ore
RT-PCR	Real-Time Polymerase Chain Reaction
SSF	Solid State Fermentation
VFRC	Virtual Fertilizer Research Center

Abstract

The use of rock phosphate ore (RPO) as an environmentally friendly phosphate fertilizer (EFPF) requires the development of sustainable systems capable of generating soluble phosphate (Pi) from this material in a manner that can support crop production. Mineral phosphate solubilizing micro-organisms (MPSM) have routinely demonstrated the ability to drive agronomically viable levels of RPO dissolution and are obvious candidates for the development of EFPFs. The ubiquitous presence of MPS bacteria and fungi in agricultural and native soils throughout the world has fueled a global research effort by agricultural scientists to identify these microbes and to use them to achieve P fertilization. To date, this effort has failed to produce a sustainable fertilizer technology as evidenced by the simple fact that virtually all P fertilizers are still manufactured via sulfuric acid-based “wet processes.” Why have efforts to produce P fertilizers based on MPS microbes been a failure? The answer is, in point of fact, that there has never been a coherent development effort of sufficient duration and intensity to support biotechnology-based strategy for P fertilization of field crops. What has been witnessed in the past 50 years is an uncoordinated, unsophisticated, poorly funded, often redundant cycle of abortive short-term studies mainly related to the identification of P-solubilizing microbes, their biochemical characterization for this property and the evaluation of their ability to enhance plant performance. In contrast to these basic efforts, there have been no investments in the resources necessary to engage in the prolonged, tortuous process of product development. Thus, the fault lies not with the microbes but with the scientific effort, or lack thereof. This report is designed to strategize a way forward for the development of EFPFs based on the Gram-negative MPS bacteria that dissolve RPO and other poorly soluble mineral phosphates via the extracellular generation of powerful organic acids, specifically the solubilization of RPO and other poorly soluble mineral phosphates via the extracellular generation of gluconic and 2-ketogluconic acids using the enzymes of the direct oxidation (DO) pathway.

1 Introduction

Gram-negative bacteria are a major class of bacteria present in significant numbers in virtually all soils and are among the most important and useful of all rhizosphere bacteria. More than 20 years ago, Goldstein and co-workers, using the power of molecular biology, demonstrated that the direct oxidation (DO) pathway was responsible for high-efficiency mineral phosphate solubilization in Gram-negative bacteria (1). Since then, virtually every worker who has isolated and characterized a Gram-negative MPS bacterial strain has found that the DO pathway is responsible for the high level of MPS activity that distinguished this strain during initial screening. Importantly, all of the genes and enzymes involved in this pathway are now completely characterized at the molecular level (see for example 1-4), which means the full spectrum of modern biotechnology tools are available *either* for product development or to facilitate conventional applied development strategies.

In order to show a way forward for the development of EFPFs based on MPS bacteria, this report must necessarily discuss enabling technologies such as rhizosphere management, biocontrol, rhizoremediation and plant probiotics. It is essential to recognize that the separation of technologies discussed in this report from interrelated (but unanalyzed) fields – such as plant phosphate use efficiency and plant-microbe interactions – is entirely specious. The most efficacious P fertilizer biosystems will utilize a plant-microbial platform possibly involving genetic engineering.

It is also essential to recognize that first-generation commercial fertilizer products based on MPS microbes (MPSMs) are already available (e.g., 5, and also <http://www.bioag.novozymes.com/en/products/Pages/default.aspx>). The challenge moving forward is to sort through the enormous number of possibilities generated by decades of what essentially amounts to preliminary screening and to choose biosystems that offer the highest probability for success in creating EFPFs capable of widespread application. Gram-negative MPS bacteria are one such high-probability target because of their intrinsic ability to efficiently process RPO to Pi *and* because this ability may be enhanced and leveraged by a wide array of powerful biotechnology tools. Three specific paths to EFPFs are recommended based on (i) rhizosphere-competent MPSMs, (ii) free-living soil MPSMs and (iii) MPSM-bioactivated RPO.

Goldstein (6) provided an overview of global R&D into phosphate solubilizing micro-organisms (PSMs). The present report will focus on one PSM subcategory: Gram-negative mineral phosphate solubilizing (MPS) bacteria. Therefore, it is appropriate at the outset to identify other important groups of microbes that will not be discussed:

- a. **Gram-positive MPS bacteria.** The other major class of soil bacteria/rhizobacteria, Gram-positive bacteria are also strong candidates for the development of EFPFs. For example *Bacillus subtilis* strains are often selected as experimental EFPFs. *B. subtilis* is ubiquitous in soils and the rhizosphere. This bacterium forms tough endospores that undoubtedly help it compete and survive, a clear advantage with respect to product shelf-life. Many Gram-positive bacteria (e.g., *B. subtilis*) express the DO pathway, and it is probable (but not proven) that – as with Gram-negative bacteria – high levels of MPS activity are the result of the DO pathway. The bioengineering tools available for Gram-positive bacteria are similar in power and sophistication to those available for Gram-negative bacteria.
- b. **Fungi (excluding arbuscular mycorrhizal).** Given that they are among the most prevalent fungi on earth, it is not surprising that numerous members of the genera *Aspergillus* and *Penicillium* have long been identified as important MPS microbes in both agricultural and natural soil ecosystems. While both these genera are capable of DO of glucose to gluconic acid, high levels of MPS activity are generally attributed to the production of citric and/or oxalic acids. It is important to note that Novozymes and Syngenta

announced a global agreement on April 12, 2012, under which Syngenta will work with Novozymes to commercialize *JumpStart*, a seed-applied EFPF based on the MPS fungus *Penicillium bilaii* (5). This is undoubtedly the most successful commercial effort in the microbial EFPF field to date. Fungi are eukaryotes, and the reproductive genetics and biochemistry of these genera are quite complex. As a result, a somewhat restricted (albeit still powerful) set of biotechnology tools exists for *Aspergillus* and *Penicillium*, compared to what is available for bacteria.

- c. **Arbuscular Mycorrhizal Fungi (AMF)**. These unique fungi are intimately co-involved in a broad range of plant root structures and physiological functions. The very complexity of these interactions precludes the commercial development of AMF as widely applied EFPFs within an acceptable time frame.

It is important to note that all bacteria and fungi (as well as plants) have the ability to mineralize organic P by enzymatic cleavage and release of Pi to the soil solution/rhizosphere space. This report is focused on EFPF technologies that may be integrated with the direct application of RPO and, as such, is only concerned with the MPS phenotype. It is likely that any Gram-negative MPS strains selected for EFPF development will retain their organic P mineralizing capabilities and that this trait may be optimized in subsequent development work.

1.1 Report Format

This document will be designed to act as a development template for MPS-based EFPFs. The template, in turn, will be constructed from the “ground up,” starting with the most fundamental genetic/metabolic building blocks of the MPS trait (the DO Pathway) and proceeding logically from genes to the most current work utilizing selected MPS strains as EFPFs in the field. Next, the state-of-the-art key enabling technologies will also be analysed. For example, the current status of our ability to identify, manipulate and deliver bacterial populations to the rhizosphere and soil will be important enabling technologies for the development of MPS-based EFPFs. Finally, analyses of core and enabling technologies must be synthesized in order to propose model systems that may be used for proof-of-concept development work in the near future. Specific development strategies will be presented along with projected timelines and milestones so that a path forward for MPS-based EFPFs may be clearly visualized.

In order to map a path to the development of EFPFs based on MPSMs, decisions must be made with respect to the choice of technology platform. A generalized decision flowchart representing this development activity, shown in Figure 1, is relatively straightforward.

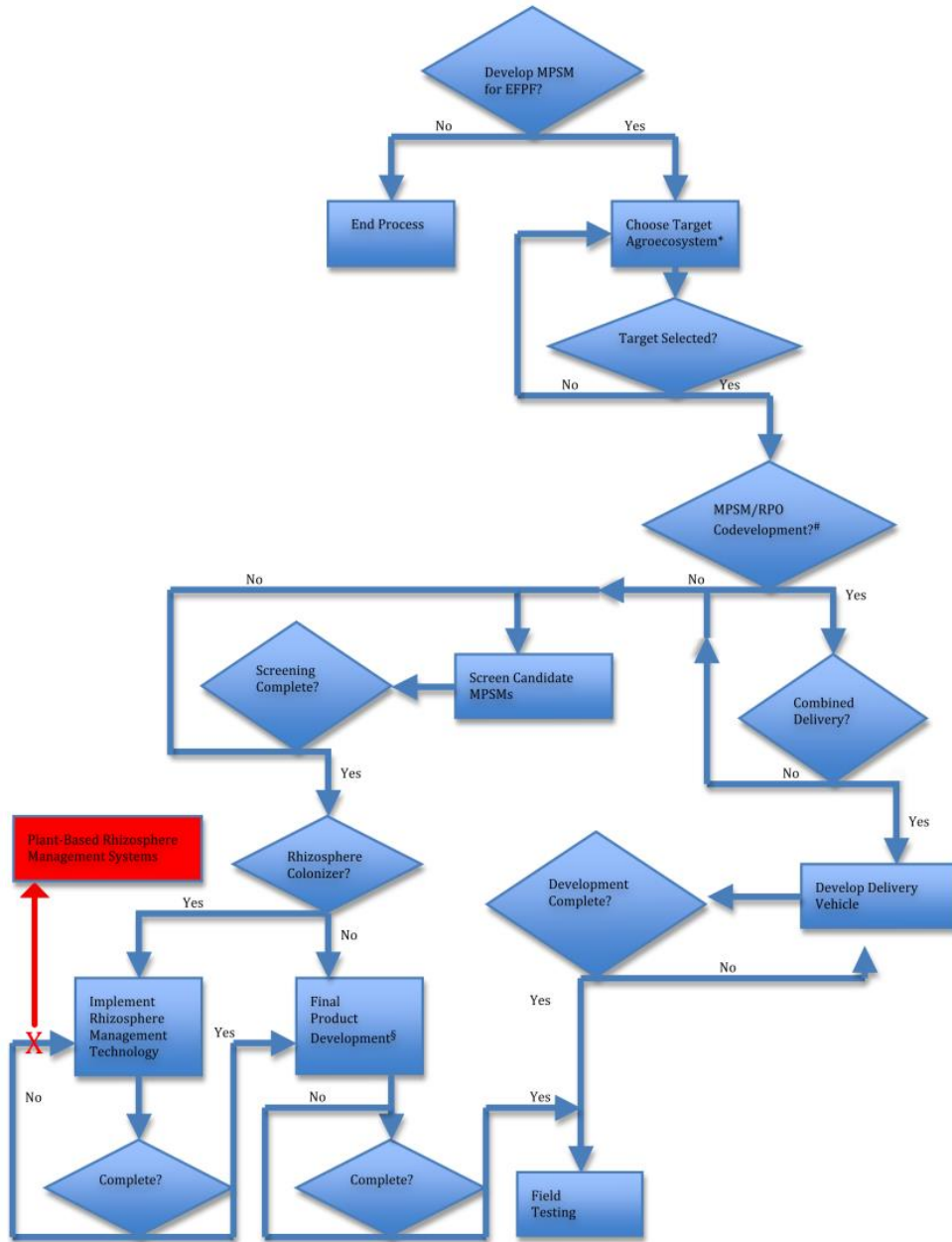


Figure 1. Decision flowchart for development of MPSM-based EFPF.

Superscript annotations: *Multiple target agroecosystems require iterative operation of this algorithm. #MPSM/RPO codevelopment via pelletized or other delivery system which, in turn, will require an entirely different screening system than is covered in this report. Such pelletized biofertilizer systems have been extensively discussed by this author elsewhere. §Lyophilization, formulation, etc. to achieve shelf life, packaging, etc. *Plant-based strategies for EFPF development – including rhizosphere management techniques – not covered by this report.

1.2 Molecular and physiological genetics of the DO Pathway in Gram-negative bacteria: state-of-the-art.

As previously discussed (4), the ability of micro-organisms to solubilize RPO and other poorly soluble calcium phosphates has been exhaustively documented. Literally tens of thousands of MPS micro-organisms (MPSMs) have been isolated from thousands of agricultural and native soil systems. In fact, this prolonged cycle of isolation, screening and characterization has led many to conclude that the EFPF potential of MPSMs had been thoroughly explored and discarded. Nothing could be further from reality. For the purposes of this report, it is assumed that the screening processes shown in Figure 1 have identified a Gram-negative MPS bacterial strain within the targeted agroecosystem. A detailed analysis of the advantages of this biosystem for EFPF development was presented in Goldstein (6). To re-summarize:

- a. Gram-negative MPS bacteria have been identified in virtually every agricultural and natural soil that has been tested. Many strains are highly rhizosphere-competent, and several are currently in commercial use as P biofertilizers, plant growth promoting rhizobacteria (PGPR) and biological disease control (biocontrol) agents.
- b. Gram-negative bacterial strains exhibiting superior MPS activity do so via production of organic acids in their immediate physical environment mediated by the DO metabolic pathway.
- c. The genes and proteins of the DO pathway in Gram-negative bacteria have been sufficiently characterized at the molecular level to allow utilization of the full spectrum of biotechnology tools for EFPF development.

In this section we will take a detailed look at the molecular engine now known to power high-efficacy MPS activity in Gram-negative bacteria, the DO pathway. Direct, non-phosphorylative, bacterial glucose oxidation was discovered over 70 years ago, based on the observed production of gluconic and ketogluconic acids in glucose minimal medium (7). As shown in Figure 2, the first step in this pathway is the oxidation of glucose (G) at the C₁ position to form the intermediate 1,5-gluconolactone, which is hydrolyzed to form gluconic acid (GA). This reaction is catalyzed by the bacterial enzyme Glucose Dehydrogenase (GDH) – not to be confused with the enzyme Glucose Oxidase (GOX), which is responsible for the analogous reaction in fungi (8). Bacterial GDH enzymes can be further subdivided into NAD(P)- and pyrroloquinoline quinone (PQQ)-dependent, depending on the redox cofactor utilized. Most Gram-negative, high-efficacy MPS bacterial strains reported in the literature (e.g., *Pseudomonas* sp., *Pantoea* sp., *Serratia* sp., *Burkholderia* sp., etc.) utilize the PQQ-dependent GDH (PQQGDH, also known as Quinoprotein GDH). From the development standpoint, it makes no difference whether the first enzyme in the DO pathway is an NAD(P)- or PQQ- dependent GDH. For the remainder of this report, the assumption will be made that the DO pathway of interest utilizes a PQQGDH for the first step in the DO pathway.

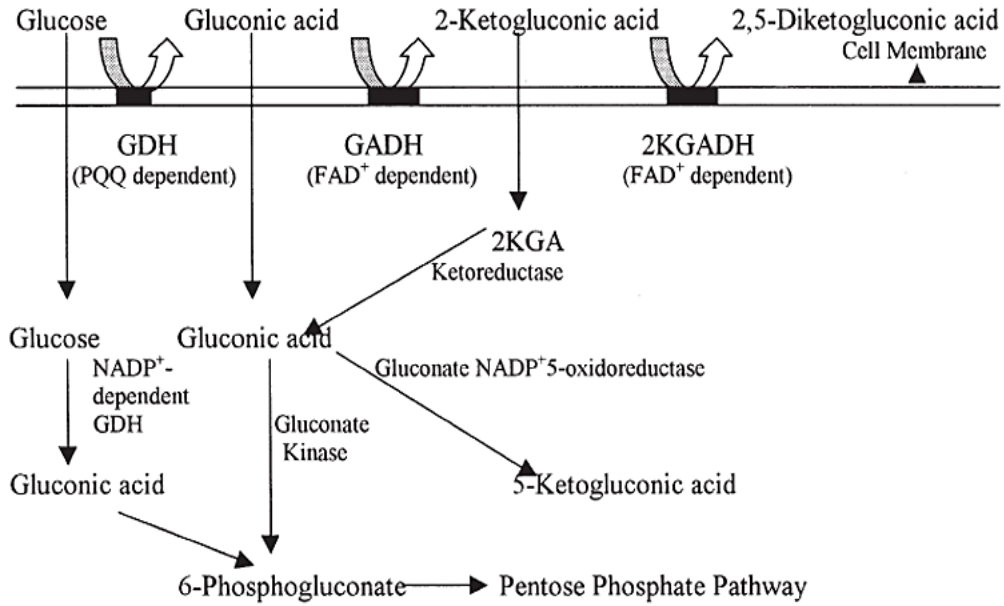


Figure 2. Generalized representation of the DO pathway in Gram-negative bacteria

In order to understand the unique advantages of the DO pathway for mineral phosphate solubilization in Gram-negative bacteria, it is necessary to understand the subcellular location of this metabolic system (Figure 3).

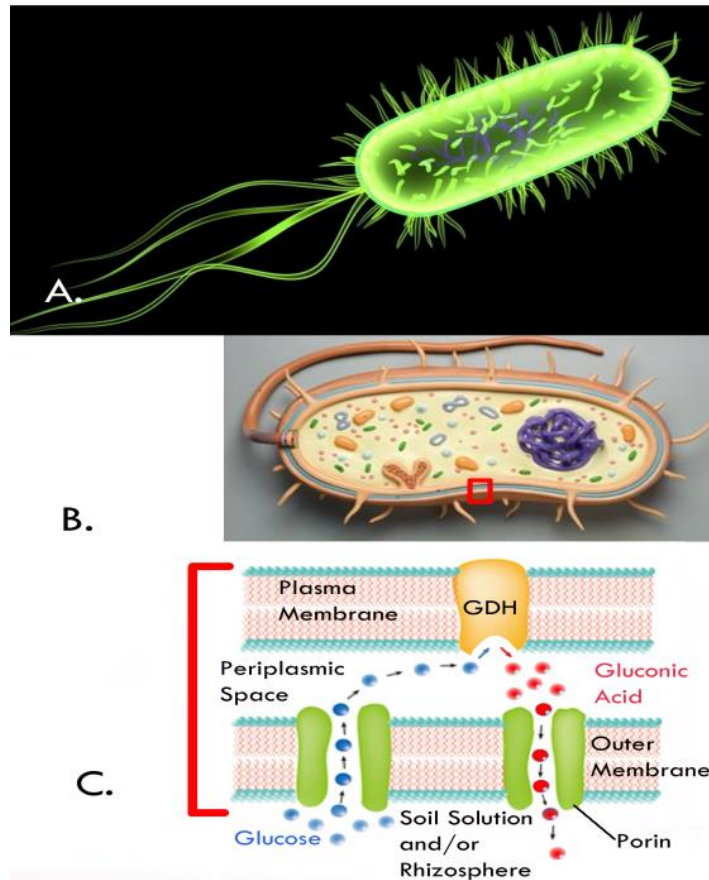


Figure 3. Generalized representation of a Gram-negative bacterium in its free-living (motile) stage.

In general, bacterial dimensions (l x w) are in the 1-5 micron range. B. Cross-sectional schematic shows the characteristic double membrane system separating the internal cytoplasm of Gram-negative bacteria from its environment. C. Exploded cross section from red square shown in 3.B. For simplicity, only GDH is shown, but – in fact – all three DO pathway catalytic sites are located on the “outer” face on the inner (plasma) membrane. As a result, organic acids produced by the DO pathway have a direct path via diffusion to the external environment. which – for EFPFs – will be the soil solution and/or the rhizosphere.

As shown in Figure 3, the DO pathway in Gram-negative bacteria is located on the outer face of the plasma membrane, facing the periplasmic space which, in turn, is in liquid-liquid contact with the external solution via the ubiquitous presence of aqueous protein-lined pores (porins) that permeate the entire sheath of the outer membrane. As a result, the periplasmic space and the external solution constitute a single diffusion free space. The practical result is that DO pathway-mediated acidification of the periplasmic space rapidly results in acidification of the extracellular space as gluconic, 2-ketogluconic and (in some cases) 2,5-diketogluconic acids diffuse outward from their regions of highest concentrations directly adjacent to the active sites of the DO pathway enzymes. Given the dimensions of these subcellular structures, the total diffusional path is generally less than 50 nm, resulting in extremely rapid movement between the periplasm and the extracellular space.

Process bioengineers already understand that evolution has handed them a fully operational, immobilized enzyme system ready for “turn-key” applications in the DO pathway. Gram-negative bacterial strains (e.g., *Gluconobacter* sp.) have been developed for the industrial production of D-gluconic acid, 2-ketogluconic acid, 5-ketogluconic acid, 2,5-diketogluconic acid and L-sorbose from D-glucose and also dihydroxyacetone from glycerol. DO, by what functionally amounts to immobilized enzymes in contact with the industrial bioreactor solution, leads to nearly quantitative yields (e.g., 20% D-glucose to 20% D-gluconic acid). In fact, Figure 4 from Goldstein (4) shows the superior MPS phenotype of an industrial strain of *Gluconobacter oxydans* when compared to an endorhizosphere strain selected for superior mineral phosphate solubilization.

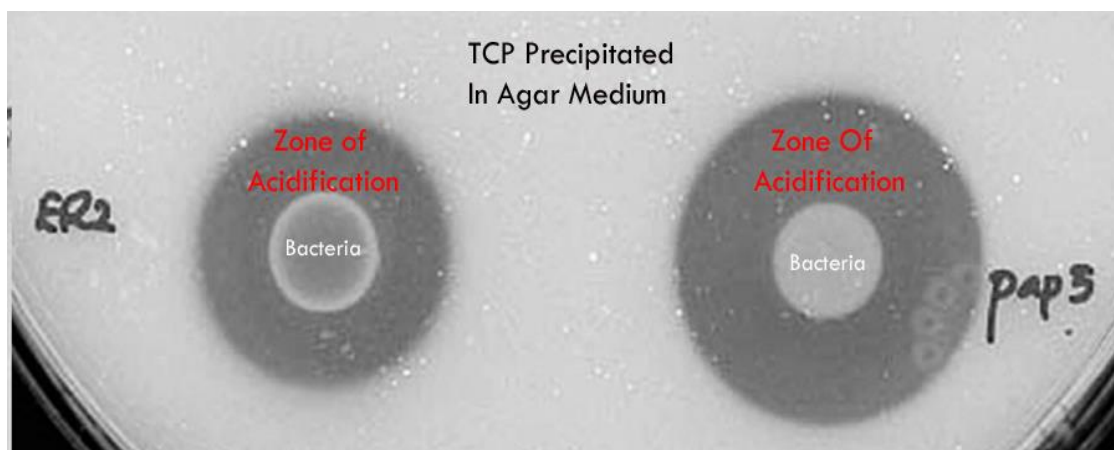


Figure 4. Tricalcium phosphate (TCP) dissolution resulting from the DO pathway-mediated oxidation of glucose to gluconic acid.

ER2 is a *Serratia marcescens* EFPF candidate strain isolated from the endorhizosphere of a plant root. PAP3 is an industrial strain of *Gluconoacetobacter oxydans* used for the commercial production of Gluconic Acid via fermentation. Figure reproduced from Ref 6.

The three components of the DO pathway are not equally distributed among Gram-negative bacteria as shown in Figure 5. Picard et al. (9) screened 506 representative strains of Gram-negative bacteria to determine the relative frequency of each enzymatic component of the DO pathway. It may be seen that ~50% of all tested strains oxidized G to GA, whereas <25% of all strains oxidized GA to 2KGA. Less than 10% of all strains oxidized 2KGA to 2,5DKGA. As discussed above, PQQ is the redox cofactor required to form the functional quinoprotein GDH. Many Gram-negative bacteria (including *E. coli*) do not carry the genes necessary to synthesize their own PQQ. Rather, they absorb it from the external environment as a vitamin, since location of the GDH active site on the outer face of the plasma membrane means that PQQ from the external environment can diffuse into contact with the apoenzyme (i.e., the nonfunctional protein component) and be bound to form the holoenzyme (i.e., the fully functional PQQ:protein supramolecular complex).

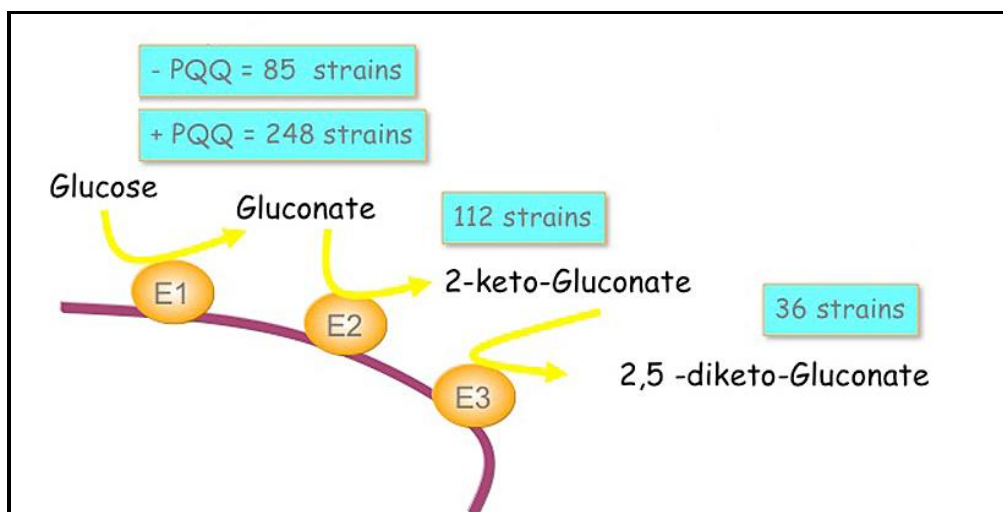


Figure 5. Relative frequency of the different enzymatic components of the DO pathway. Adapted from Picard et al (9).

This report is focused on the solubilization of poorly soluble mineral phosphates (especially RPO and other calcium phosphates) so that we may simplify things by restricting our analysis to the first two steps of the DO pathway (i.e., up to the production of 2KGA) while recognizing that metabolic engineering of the third step (2KGA to 2,5DKGA) may be of utility in later development phases where it may become desirable to engage more sophisticated rhizosphere management techniques involving aspects of bacterial and plant metabolism.

The acidic strengths of Gluconic acid and 2-Ketogluconic acid are shown in Table 1, along with other organic acids commonly cited as solubilizing agents by researchers in the MPSM field. In terms of pKa, GA and 2KGA may be considered as relatively strong organic acids. From the standpoint of MPS activity in the soil, further consideration must be given to the cellular origin of the specific organic acid acting to confer observed MPS activity.

Table 1. Relative pKa of organic acids commonly cited in the relevant literature

Acid	pKa
acetic	4.79
citric	3.09 (pKa ₁), 4.75 (pKa ₂), 6.41 (pKa ₃)
gluconic	3.86
2-ketogluconic	2.66
oxalic	1.25 (pKa ₁), 4.14 (pKa ₂)

Based on pKa values alone, both oxalic acid and citric acid-producing microbes would appear to be favored as MPS agents for EFPF development. However, as shown in Figure 6, both of these acids are produced in the cytoplasm.

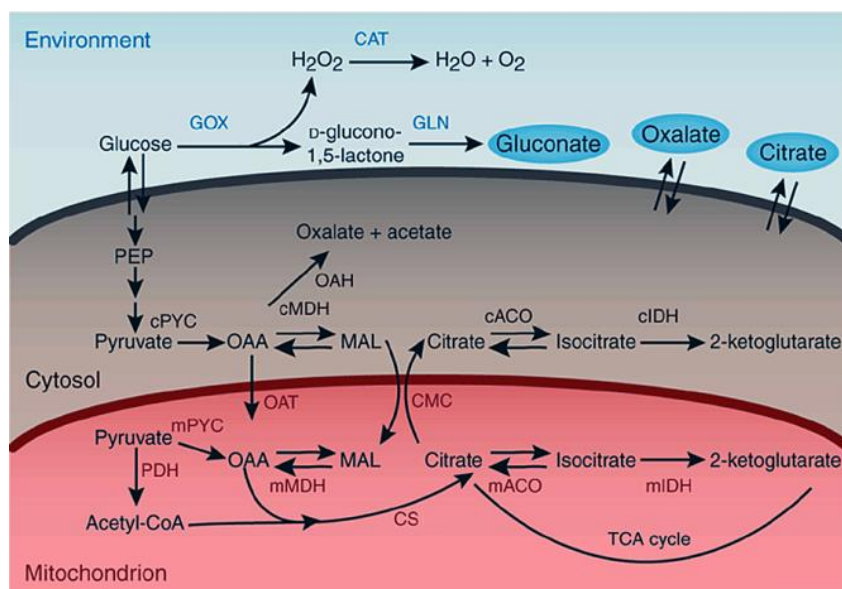


Figure 6. Central pathways and subcellular locations for the synthesis of citric, gluconic and oxalic acids by *A. Niger* (modified from Cullen, 8).

As shown in Figure 6, in *A. niger* (and presumably other related fungi) citric acid is generated in the mitochondrion (a subcellular organelle), while oxalic acid is produced in the cytoplasm. A substantial amount of metabolic engineering has obviously gone into industrial strains of fungi used for production of citric acid, and a thorough review of the technical literature in this field is beyond the scope of this work. However, it is reasonable to suggest that the intracellular pH of these fungal strains will not fall below 6.0, and in fact, a brief review of industrial fermentation methods shows that below a medium pH of 6.0, the production of citric acid falls off dramatically, indicating the inhibition of fungal growth and/or metabolism. Assuming a cytoplasmic pH \geq 6.0, a significant number of the acidic protons of both citric acid and oxalic acid would have dissociated prior to transport into the extracellular medium. The precise ratio of dissociated to associated protons for each acidic group cannot be calculated from standard thermodynamic relationships (e.g., the Henderson-Hasselbach eqn.) without knowledge of the precise physicochemical environment, including the electrochemical activities of all participating organic/inorganic ions/counterions, the water potential (electrochemical potential of intracellular water), etc. This analysis is in no way meant to minimize the MPS capabilities of fungi, especially given the success of *JumpStart*, a *Penicillium bilaii*-based commercial EFPF (5). Rather, the point is to recognize that MPS efficacy will depend on more than the type and theoretical strength of the organic acid produced. The monoprotic acids generated by the DO pathway are produced outside the cell and, as a result, may be more completely available for dissolution of RPO and other poorly soluble mineral phosphates. It is of interest to note that the fungal glucose oxidase (GOX) is also located on the outer face of the cell so that acids may be released directly to the extracellular space (soil solution/rhizosphere). Increased GOX activity in fungal MPS candidates could be produced via bioengineering and/or classic selection and screening methods. This section is designed to provide a functional summary of the molecular and physiological genetics of the DO pathway. The practical implications for these molecular data will be discussed in the following section.

The first thing to note with respect to the basic molecular biology of the DO pathway is that genes from many Gram-negative bacterial genera expected to produce strong EFPF candidates have been cloned and sequenced (1-4). In addition, our ability to “mine” genetic information via DNA sequence homology analysis has evolved to the point where it is reasonable to predict that DO genes may be identified in any Gram-negative bacterium identified as a candidate MPSM. Finally, it is safe to assume that inexpensive, reliable genomic DNA sequence data may be generated from any soil bacterium of interest so that, in terms of biotechnology, MPSM-based EFPF development may be expected to leverage virtually every possible advantage offered by modern molecular methods. For example, the GenBank of the National Center for Biotechnology Information (NCBI) holds 24 separate records for quinoprotein GDH apoenzyme genes for *Pseudomonas* sp., seven records for *Erwinia* sp., six records for *Burkholderia* sp., five records for *Pantoea* sp., five for *Serratia* sp., and so on. Likewise, this database contains numerous entries for both PQQ biosynthesis and GADH genes from Gram-negative bacteria. Therefore, it is reasonable to conclude that molecular genetics of this pathway have been sufficiently elucidated for all conceivable near-term MPSM applications.

What is the value of this pure molecular genetics-based (i.e., DNA sequence-based) information with respect to the development of MPSMs as EFPFs? A complete analysis of DO pathway molecular genetics is beyond the scope of this report, but the most obvious applications can be presented. In addition, it is important to note that additional applications will only become obvious as the details of the project are worked out. But the simple bottom line is that once the molecular genetics of a pathway are known, advanced biotechnology methods, such as metabolic engineering, may be applied. For example, genes for the quinoprotein GDH apoenzyme have been cloned from a large number of Gram-negative bacteria so that the application of biotechnology methods to this system may now be considered routine. What types of data-mining tools are available to add value to a potential MPSM-EFPF system? Even the most rudimentary DNA analysis can provide important insights for engineering a biosystem. As early as 1990, a sufficient number of PQQ-dependent dehydrogenase genes had been cloned, so that Cleton-Jansen et al. (10) were able to translate the raw DNA sequences into primary amino acid sequences and to align these deduced protein sequences in order to identify important mechanistic components of quinoprotein dehydrogenases. The results of a similar analysis conducted in this report are shown in Figure 7.

A significant amount of useful information emerges from this relatively simple analysis. The most obvious piece of preliminary data involves two “highly conserved” regions of primary deduced amino acid sequences. This indicates conserved 3D protein structure, which in turn implies regions essential to enzymatic function. While not necessarily the case, the general rule that linear sequences are conserved in order to maintain essential 3D structures is true often enough so that further data-mining is justified.

			A8	B8	B8-C8 loop
DH1 METEX	511	KIPSGAIGY	PMTY	THKGTQYVAIYY	YGVGGWPGVGLVFDLADPTAGL
DH1 METME	502	KMPSSGGIGS	PMTY	SFKGKQYIGSMY	YGVGGWPGVGLVFDLTDPSAGL
DHG ECOLI	748	RLPAGGQAT	PMTY	EVNKGKQYVVI	SAGGHGSFGT-----
				C8	
DH1 METEX	557	GAVGAFKKLAN	YTMGGGVV	VFSLDGKGPY	DDPNVGEWKSAAK
DH1 METME	548	GAVGAFRELQ	NHTMGGGLM	VFSL-----	-----
DHG ECOLI	781	-----	-----	KMGDIIVAYALP	-----DDVK-----

Figure 8. One of the most highly conserved regions of deduced primary amino acid sequence in PQQ-dependent bacterial dehydrogenases.

Designations, (A8, etc.) refer to regions of 3D protein structure as discussed below. The numbers to the left of the AA sequence refer to linear primary sequence. For example, the PMTY region highlighted in yellow represents amino acids 757-760 in the linear primary AA sequence deduced from the cloned PQQGDH apoenzyme gene of *E.coli*. (Ref 11)

Given that the PQQGDH plays a key role in controlling the MPS behavior of Gram-negative bacteria, Goldstein et al. (11) conducted a series of protein-engineering experiments to determine the role of the highly conserved region in PQQGDH function. Specifically, our analysis of the molecular biology data indicated that this highly conserved region played a role in access of glucose to the catalytic site of the enzyme, which suggested a strategy by which the rate of acid production (and therefore RPO solubilization) could be enhanced. Molecular modeling of the 3D structure of *E. coli* PQQGDH indicated an 8-bladed superbarrel structure similar to other PQQGDHs for which actual crystal structures had been determined. The generalized 8-bladed super-barrel (beta-propeller) structure of membrane-bound quinoprotein dehydrogenases had been demonstrated in at least one case via high-resolution X-ray crystal diffraction (12, 13), and may be deduced from this crystal structure for other proteins via a primary amino acid sequence homology (11, 14). The protein's 3D structure directly determined from X-ray crystallography is shown in Figure 9. As discussed in the legend to Figure 9, the availability of high-resolution 3D protein structural models for PQQGDH opens the door for bioengineering strategies to drastically enhance the MPS in selected strains of Gram-negative bacteria.

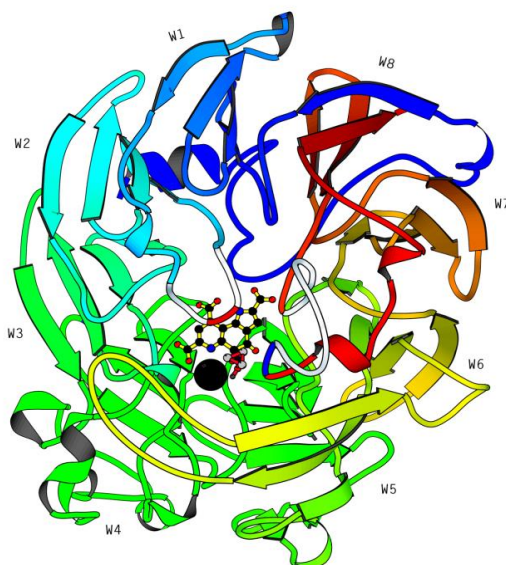


Figure 9. Molecript representation of the secondary structure of the a-subunit of *M. methylotrophus* quinoprotein methanol dehydrogenase.

This 8-bladed super-barrel (beta-propeller) representation was created based on the 1.7 Å crystal structure of this enzyme generated by Ghosh et al. (14). The view shown here looks down the pseudo-eightfold symmetry axis directly “into” the active site of the enzyme. The eight “W” motifs of this structure are visualized in a counter-clockwise manner with the color changes from blue to red moving from the N- to C- termini. PQQ, Ca⁺⁺ (a second essential redox cofactor along with PQQ) and methanol are shown in ball-and-stick motif and are below the apparent surface, (i.e. inside the barrel structure). Goldstein et al. (11) showed that this molecular model could be used to simulate the closely-related PQQGDH enzyme responsible for MPS activity in Gram-negative bacteria. This result allowed Goldstein and coworkers (11), as well as others (reviewed by Sashidhar and Podile; 4), to initiate protein engineering work via site-directed mutagenesis using cloned PQQGDH genes in order to create site-directed mutant proteins with enhanced catalytic rates. This research, which targets increased gluconic acid production in the rhizosphere via genetic and protein engineering, provides an excellent example of how the advanced state-of-the-art – with respect to the molecular and physiological genetics of the DO Pathway in Gram-negative bacteria – can create unique, powerful bioengineering opportunities for the development of MPSMs as EFPFs.

One last example of the prima facie power of genomic analyses in shown is Figure 10. The last decade has witnessed a drop in DNA sequencing costs and an increase in DNA sequencing power. As a result, it is reasonable to suggest that complete genomic data may be generated rapidly and economically for any strain of interest where not already available. An important example is shown in Figure 10, where the primary amino acid sequence of the PQQGDH apoenzyme was deduced directly from the complete genomic DNA sequence of the highly rhizosphere-competent, Gram-negative bacterium *Pseudomonas fluorescens*, biocontrol strain F113. Even without further analyses, it is apparent that a highly conserved region characteristic of PQQ dehydrogenases is present in this potential MPS strain.

The screenshot shows the NCBI GenBank interface for the *Pseudomonas fluorescens* F113 chromosome. The main content includes the following information:

- LOCUS:** NC_016830 2415 bp DNA linear BCT 27-JUN-2012
- DEFINITION:** *Pseudomonas fluorescens* F113 chromosome, complete genome.
- ACCESSION:** NC_016830 REGION: complement(5474963..5477377)
- VERSION:** NC_016830.1 GI:378947941
- PROJECT:** 87037
- KEYWORDS:** *Pseudomonas fluorescens* F113
- SOURCE:** *Pseudomonas fluorescens* F113
- ORGANISM:** Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; *Pseudomonas*.
- REFERENCE:** 1 (bases 1 to 2415)
- AUTHORS:** Redondo-Nieto, M., Barret, M., Morrissey, J.P., Germaine, K., Contreras, M., Moynihan, J.A., Giddens, S.R., Coppolase, E.R., Muriel, C., Stiekema, W.J., Rainey, P.B., Dowling, D., O'Gara, F., Martin, M. and Rivilla, R.
- TITLE:** Genome sequence of the biocontrol strain *Pseudomonas fluorescens* F113
- JOURNAL:** J. Bacteriol. 194 (5), 1273-1274 (2012)
- PUBMED:** 22328765

The partial translation of the DNA sequence is shown below, with a highly conserved region highlighted in yellow:

```

/translacion="MSTEGALRSRLLPSLLGILLMLLGLVLLAGGVKLSLGGSLYY
LLAGIGLALTGVLIMARRAALGLYALVLPASTVWALWEVGLDWWQLVPRLAMLFVLG
IVMLLPWFRRLTGDASATGTRALGAAVVIAGVAALASQFTNPGEIKGQLDRDSVPG
MTNTAPAMPDGDWNSYGRSAHGDRYSPLAQITPENVSCLKVPAWYRTGDLPGPNDPGE
TTAENTPLKANGMLYVCTPHSQVIALEPETGKEIWRFPKLSLSTQKAQNFKGWAHMTCR
GVTYHDDAAYASAEQSPTGTASTTPASSVCPRIIFLPTADTRLIALNADTKMKCEDFG
DKGQVLDLTANIGGFTAGGYYSTSPPAVTONLVVIGGHVTDNVSTDEPSGVIRAYDVHT
GQLVWNWDSGNPDDTTPIAEGKTYTRNSPNMWSMFSVDEKLGMLYLPNGNQTDPDQFGG
FRTPESKEYAAGLTALDIATGKVRWYFQPTHDDLWDMVGGQPTLMDLKTADGVKPAV
LASTKQTSIYVLDLRSNGQPIVPIKEIPVPQGAVEGDHSTPTQPMSDLNFPVPLKESD
MWGVTFFDQMLCRIDFKSLRYDGMFTFPPSLQGSIVYPGNFVDFDGGISVDVPRQLAF
VNPSYMAFKSKLVPAEAVAGGPGPKRSETEGVQPNKGAPYGVILEALLSPMGLPCQAPA
WGYVAAVDLTLNKTTLWKHKNGTVRDSPPVPIPLSMGVPSLGGPFTTASGLAFLSGLTD
QYLRAVDVKNKQLWEGRLPAGAQTTPMTYTKDGKQYVLVAGHGSLGKQGDYVI
AYKLPD"

```

Figure 10. Partial representation of NCBI GenBank.

Partial representation of NCBI GenBank file available for the PQQGDH apoenzyme gene deduced from the complete genomic DNA sequence of the highly rhizosphere-competent biocontrol strain *P. fluorescens* F113. The “PMTY” start of the highly conserved region common to other PQQGDH apoenzymes (Fig. 8) is highlighted in yellow. The presence of this region offers immediate opportunities for direct protein engineering, as well as a number of molecular strategies to “tag” this gene in order to rapidly and effectively develop this rhizosphere colonizer as a MPS bacterium.

In summary, the DO pathway in Gram-negative bacteria offers a mature metabolic and genetic engineering technology platform for the development of MPSM-based EPPFs. Failure to exploit the platform may be attributed to lack of a coherent development effort in this area.

2 Evaluation of the current use and future potential of DO genes as markers for MPS bacteria and MPS activity in vivo, in vitro, and in situ

Given the previous characterization of the global effort into MPSM-based EFPFs as an uncoordinated, unsophisticated, poorly funded, often redundant cycle of abortive short-term experiments, it should come as no surprise that, to date, workers have failed to take advantage of the powerful set of analytical molecular tools that have become available over the past decade. An exhaustive search of the published scientific and technical literature was conducted for this report. This effort has failed to turn up any significant effort to use modern molecular and related biotechnology methods to develop or monitor Gram-negative MPS bacteria as EFPFs and/or as PGPRs. As a result, most of this section will be focused on the future potential of these methods. Figure 11 and Table I are designed to make it obvious that an exhaustive set of molecular tools exist to leverage the development and/or to validate virtually any Gram-negative MPS bacteria selected for development as an EFPF. Simply put, these tools make it possible to unequivocally locate the target organism physically in the soil/rhizosphere system, determine its population, determine whether (and to what level) it is expressing the DO pathway and thus determine to what extent the target MPSM is responsible for any observed RPO solubilization and/or other changes in available Pi in the soil/rhizosphere system. The availability of powerful molecular probes means that questions crucial to EFPF development may be quickly and easily answered, including whether i) the target MPSM survived as a seed coating or soil inoculant, ii) whether its population goes up or down over time, iii) whether the target MPSM is confined only to the rhizosphere, iv) has the MPSM moved into the bulk soil, v) is it associated with RPO particles, and (vi) whether the level of GA and/or 2KGA in the soil solution correlated with changes in expression of the DO pathway in the target MPSM.

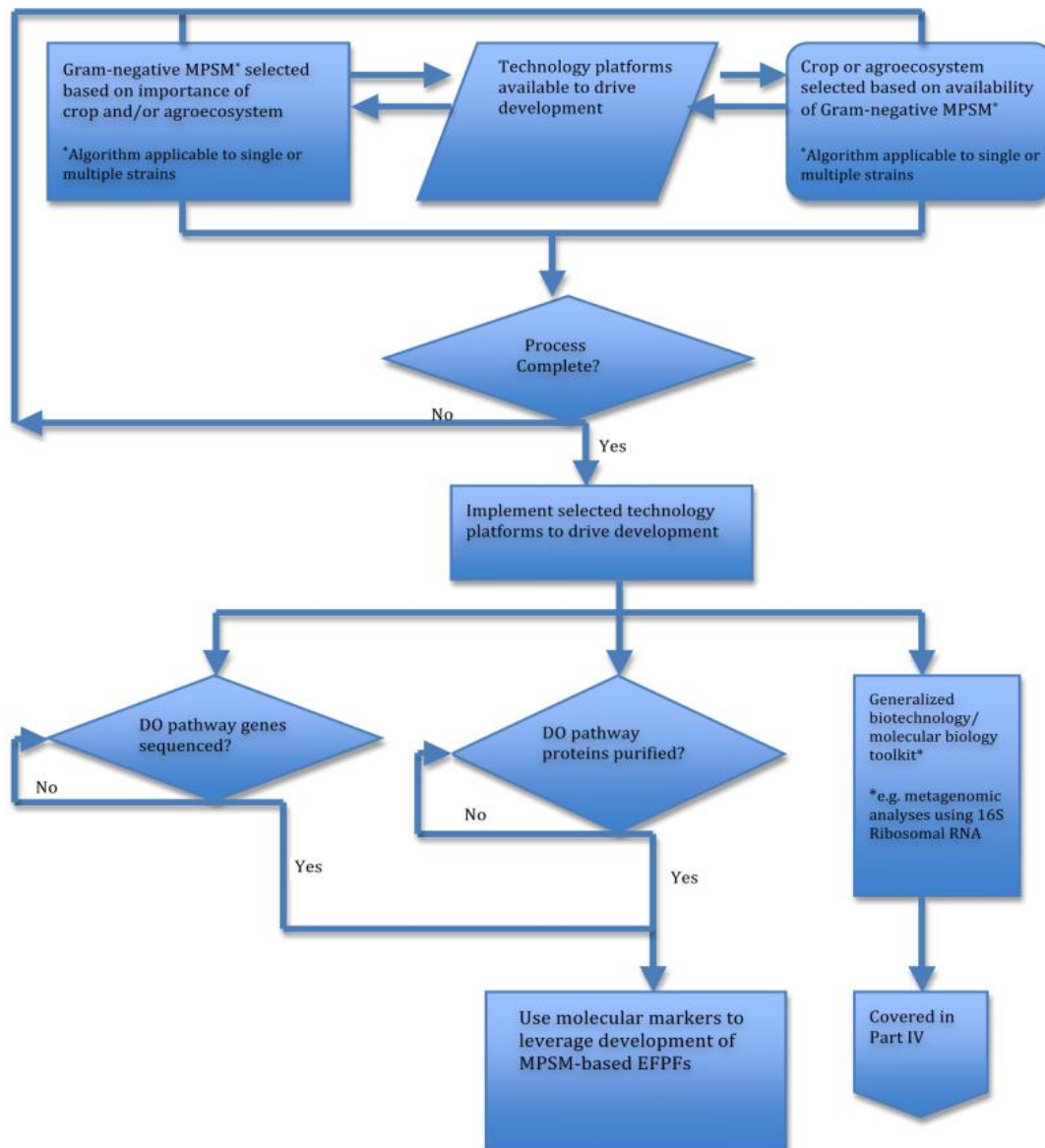


Figure 11. Generalized decision algorithm for use of DO pathway-specific molecular markers to leverage the development of MPSM-based EFPFs.

Table 2. DO pathway-specific markers available for development of MSPM-based EFPFs.

DO pathway marker	Molecular ID	Development role
GDH apoenzyme gene	DNA	Direct real-time PCR analysis of distribution of MSPM-based EFPF agent in soil/rhizosphere system. Validation of survival of selected selected strain(s) in selected agroecosystem.
PQQ biosynthesis gene(s)	DNA	Direct real-time PCR analysis of distribution of MSPM-based EFPF agent in soil/rhizosphere system. Validation of survival of selected selected strain(s) in selected agroecosystem.
GADH apoenzyme gene	DNA	Direct real-time PCR analysis of distribution of MSPM-based EFPF agent in soil/rhizosphere system. Validation of survival of selected selected strain(s) in selected agroecosystem.
GDH apoenzyme mRNA	RNA	RT PCR analysis of DO pathway gene expression.
PQQ biosynthesis gene(s) mRNAs	RNA	RT PCR analysis of DO pathway gene expression.
GADH apoenzyme mRNA	RNA	RT PCR analysis of DO pathway gene expression.
Anti-apoGDH	Protein	ELISA and other direct immunoassays to correlate presence and level of DO pathway enzymes w/ acid production to demonstrate efficacy of MSPM-based EFPF in release of Pi from RPO, etc.
Anti-PQQ*	Protein	ELISA and other direct immunoassays to correlate presence and level of DO pathway enzymes w/ acid production to demonstrate efficacy of MSPM-based EFPF in release of Pi from RPO, etc.
Anti-apoGADH	Protein	ELISA and other direct immunoassays to correlate presence and level of DO pathway enzymes w/ acid production to demonstrate efficacy of MSPM-based EFPF in release of Pi from RPO, etc.

The intentionally redundant nature of this table is meant to exhaustively demonstrate the full spectrum of molecular “tags” and probes available to track and validate the growth and metabolic activity of virtually any Gram-negative MPS bacteria selected for field testing as an EFPF agent. *In order to generate anti-PQQ antisera or monoclonals, derivatization w/carrier molecule would probably be required.

Given this extremely powerful set of molecular tools, the current primitive state of R&D into MPSMs further emphasizes the failure of this field to move beyond its current state. As detailed in Goldstein (6), most of the ongoing work on MPS bacteria involves simply screening for the MPS phenotype in bacteria isolated from “ecosystems of interest” (e.g., 15-18). Initial screening usually involves some variation of the phosphate agar assay that has been detailed previously. Isolation of putative MPS strains is generally followed by routine assays for the presence or absence of the “usual suspects” (organic acids, chelating agents, siderophores, etc.). This type of systematic screening has been going on for more than half a century and has generated an enormous amount of literature. It has led many agronomists and fertilizer engineers to the unfortunate conclusion that this biosystem has already failed as an EFPF (e.g., 19).

In terms of what is state-of-the-art, it is instructive to examine the recent work of Maurhofer’s group (20) at the Swiss Federal Institute of Technology, Zurich. The quality of molecular genetics in this paper is highly sophisticated and beyond reproach. Yet the work falls short in terms of MPS research. For example, these workers discuss P-availability in the soil solely in terms of iron-aluminum oxide complexes. They correctly attribute MPS activity in Gram-negative bacteria to the DO pathway but only in terms of Ca^{++} chelation, inexplicably ignoring the role of direct acidification in DO pathway-mediated CaP solubilization reported by virtually every worker who has ever studied this phenomenon. Unfortunately, these workers do not discuss 2-ketogluconic acid production by pseudomonads. Pseudomonads are known to produce 2KGA, and 2KGA has a significantly lower pKa than GA. This indicates a significantly higher potential efficacy for the solubilization of RPO and other forms of CaP, assuming these workers are even aware that CaP is the major form of poorly soluble mineral P in most of the world’s agricultural soils. Regardless, these workers have published the most recent and sophisticated paper on the use of DO pathway markers to study the MPS trait. More correctly, their work describes the utility of PQQ biosynthetic genes as highly specific and sensitive molecular markers for the deconvolution of natural populations of rhizosphere pseudomonads with the MPS trait acting only as a loosely connected underlying rationale for the study. Their experimental strategy was based on the fact that PQQ biosynthetic genes have been cloned from a wide range of Gram-negative bacteria, including the highly rhizosphere-competent biocontrol species *Pseudomonas fluorescens*, in which the PQQ synthase operon contains 11 genes. They selected the *pqqC* gene because it catalyzes the final step in PQQ biosynthesis and, as such, is the best characterized gene in the PQQ biosynthesis pathway (20, and references therein). New PCR primers were designed to amplify this *Pseudomonas pqqC* gene. These primers were used to amplify and sequence *pqqC* gene fragments from a wheat rhizosphere population using both cultivation-dependent and cultivation-independent approaches. The results of this study clearly demonstrated the power of this DO pathway gene as an effective molecular marker for rhizosphere MPS pseudomonads. These data, in turn, confirm that, above and beyond their direct role in RPO solubilization, DO pathway genes may be used as molecular probes to “tag” and evaluate Gram-negative MPS bacteria during the development process, a claim Goldstein’s proposal made over a decade ago.

Figure 12 is included to show that the power of a DO pathway gene (*pqqC*) as a molecular “tag” compares favorably with standard molecular probes such as *rpoD* and *gyrB*. Based on a detailed review of the literature and a number of personal communications with workers in the field, it is clear that the work of Meyer et al. (20) represents the only comprehensive effort to date targeting DO pathway genes as molecular tags in order to study Gram-negative MPS bacteria. Importantly, this comprehensive study demonstrated the utility of the *pqqC* gene so thoroughly that, in the opinion of these authors, it may be viewed as a proof-of-concept sufficient to validate the use of any gene in the DO pathway for similar application, including product development activities. This assumption is based on the extremely high probability that any other DO pathway gene will also have regions of unique DNA sequence sufficient to support the design of PCR primers for highly specific target amplification.

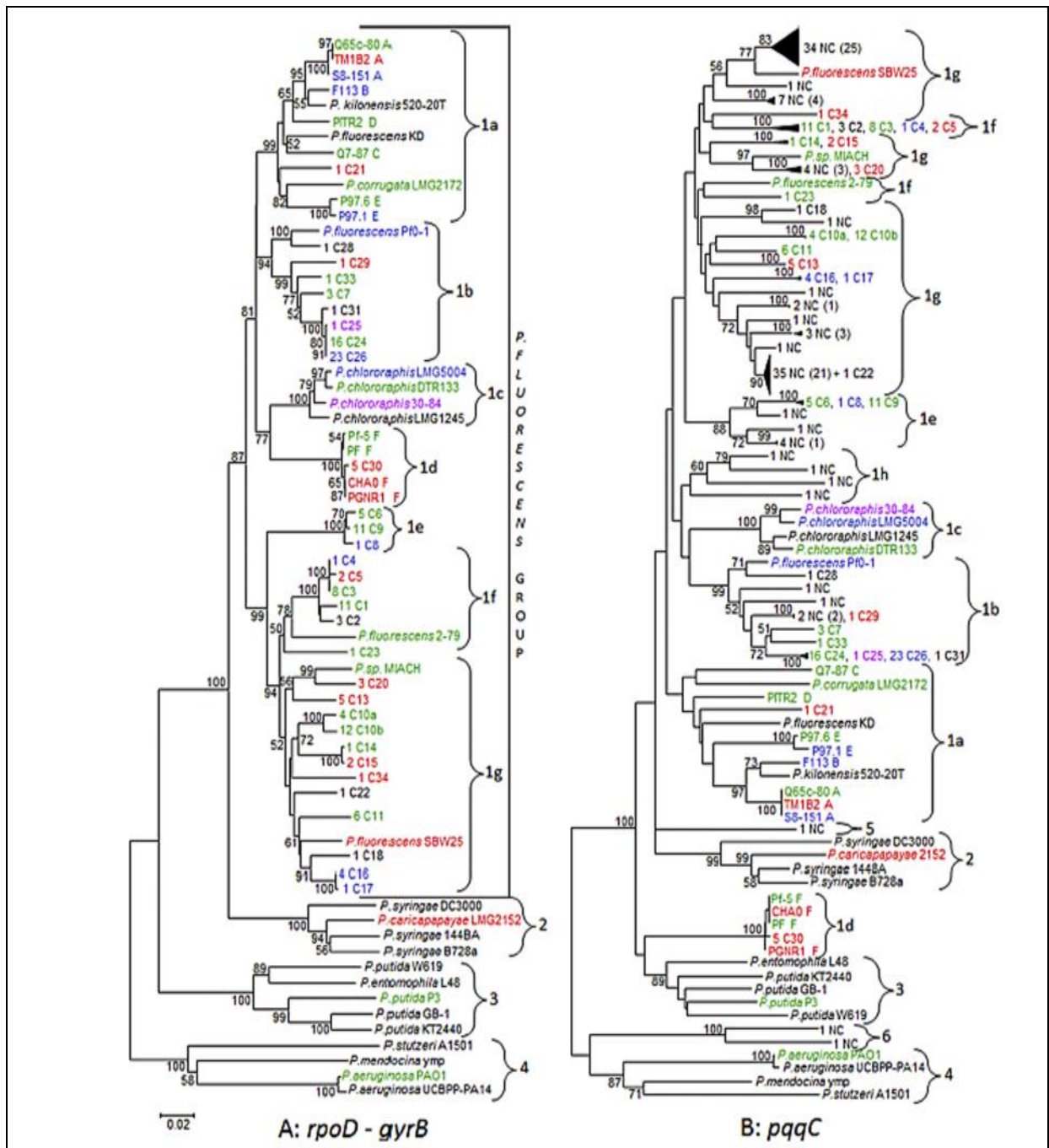


Figure 12. Phylogenetic relationships among *Pseudomonas* reference strains and *pseudomonads* isolated from the rhizosphere of field-grown wheat.

Phylogenetic relationships among *Pseudomonas* reference strains and *pseudomonads* isolated from the rhizosphere of field-grown wheat comparing a DO pathway gene (*pqqC*) as a molecular “tag” with standard molecular probes such as *rpoD* and *gyrB*. Reproduced from Meyer et al. (20).

Given the paucity of work conducted using DO pathway genes as molecular markers or tags to identify and/or follow Gram-negative MPSMs in the soil, it should come as no surprise that neither RNA- or protein-based markers have

ever been used to study these systems. As a result, the concept of using these powerful biotechnology tools for EFPF development is not even on the radar. Regardless of the current failure of MPS researchers to employ even 20th century methods, it would be meaningless to describe a path forward that does not incorporate the most modern molecular tools in any effort to develop EFPFs. Considering that both RNA- and protein-based systems offer extremely powerful molecular markers with which to identify and develop Gram-negative MPS bacteria using the DO pathway as EFPFs, a brief summary of the most obvious applications for these systems in any development effort will be provided below.

Real-Time polymerase chain reaction (quantitative PCR or RT-PCR) is the most powerful, sensitive gene analysis technique available to measure the quantitative level of gene expression *in vivo* or *in vitro*. RT-PCR is also used for direct genotyping, SNP analysis, pathogen detection, drug target validation and measuring RNA interference. Frequently, RT-PCR is combined with reverse transcription to quantify messenger RNA (mRNA) in cells and tissues. Because the genes for the DO pathway have been cloned and sequenced, the entire spectrum of RT-PCR tools automatically becomes available to leverage the development of any EFPFs that utilize this pathway. The work of Maurhofer's group (32) is an example of how RT-PCR may be used for genotyping and detailed phylogeny of MPS pseudomonads from the rhizosphere of a specific wheat cultivar in a specific agroecosystem.

In terms of EFPF development, RT-PCR and other methods such as high-density DNA microarrays (21) could be deployed on both the "front end" and the "back end" in a manner generalized by the flowchart shown in Figure 13. On the "front end," RT-PCR may be used to either identify strong candidate MPS bacteria *ab initio* or validate bacterial strains pre-identified by traditional screening methods (e.g., plate-clearing assays). Identification *ab initio* is based on understanding that is the basis for superior MPS activity in Gram-negative soil/rhizobacteria. Primers based on consensus sequences unique to DO pathway genes (e.g., the *pqqC* gene used by Meyer et al.; 20) will allow direct PCR screening of the soil/rhizosphere DNA (using the metagenomic methods discussed subsequently), which will thus allow identification of the most populous MPS bacteria in the soil/rhizosphere. Conversely, previous traditional sampling/screening methods may have already produced strong candidate MPS bacteria from an agroecosystem of interest. In that case, RT-PCR using the same primers will allow rapid validation that a) the strain(s) of interest is (are) DO pathway positive and 2) the strain(s) of interest is (are) present at a significant level in the soil/rhizosphere. These two alternative "front end" strategies are shown as alternative processes at the top of Figure 13.

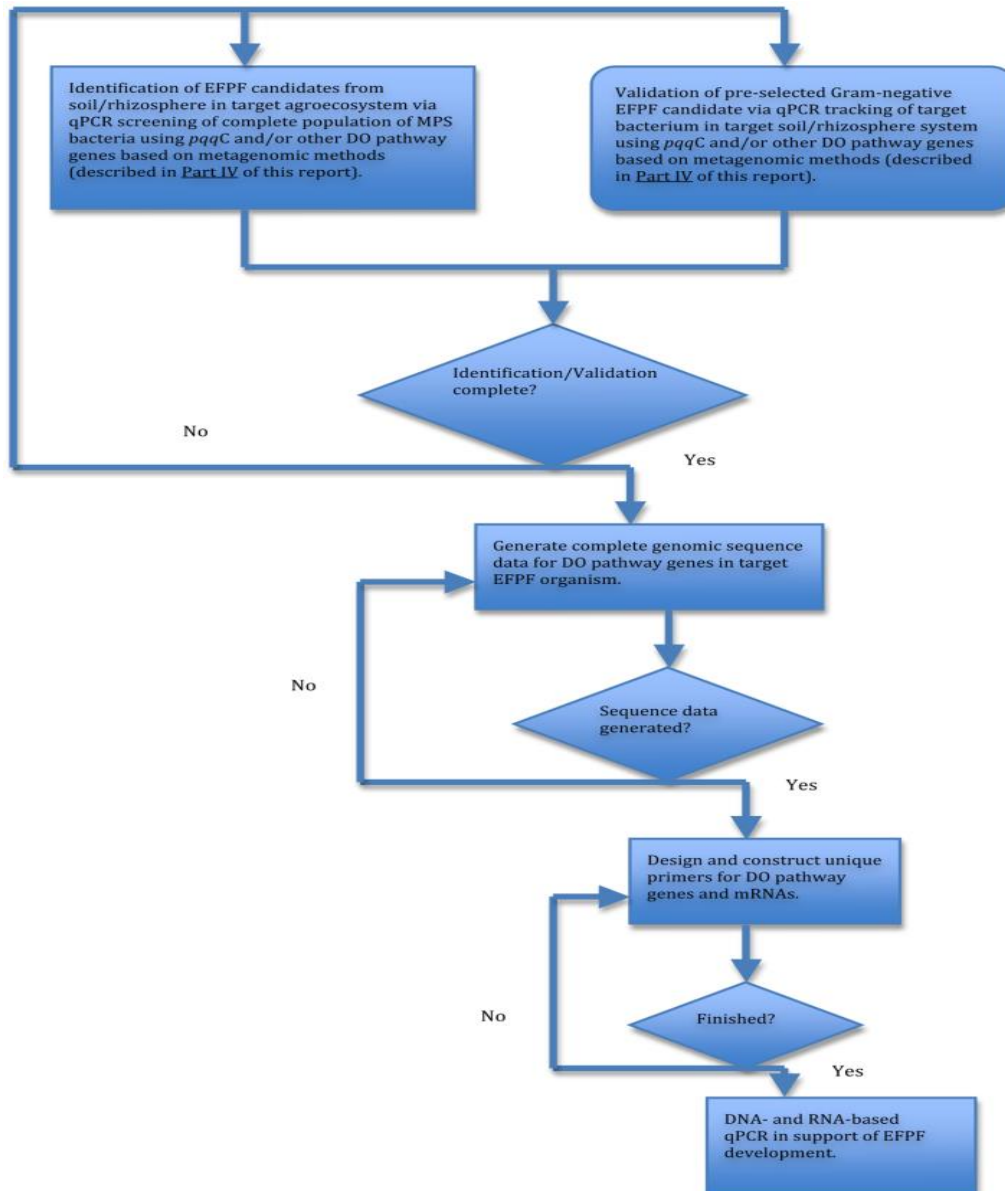


Figure 13. Generalized flowchart for the use of RT-PCR in the development of Gram-negative EFPF system.

Since qPCR enables both detection and quantification, once a MPS organism has been targeted, qPCR will be applied at virtually every EFPF development stage. For example, reverse transcriptase-coupled qPCR may be used to determine the relationship between Pi concentration in the soil/rhizosphere solution phase and the level of DO pathway gene expression. This will allow optimization of the MPS phenotype in further strain selection. Likewise, qPCR can be used to calculate strain viability in various product delivery formulations. Perhaps most importantly, qPCR can be used to verify survival of a selected MPS strain during the course of the growing season in order to verify EFPF efficacy. The complete range of qPCR applications will of course depend on specific development scenarios. The crucial point to be made is that identification of a specific, well-characterized metabolic pathway opens the door for utilization of this extremely powerful biotechnology toolkit in EFPF development.

The final DO pathway-specific molecular marker to be considered here are antibodies, especially, monoclonal antibodies (MAbs). The 'central dogma' of molecular biology provides the foundation of our understanding of physiological genetics. Put most simply: DNA is transcribed into RNA, which is translated into protein. What this means from the standpoint of DO-mediated MPS activity is that any Gram-negative strain that shows efficacious EFPF activity must not only carry the DO pathway genes, it also must express them. As previously discussed, the enzymes responsible for gluconic acid and 2-ketogluconic acid production have been identified and characterized. In addition, purification procedures have been developed for both of these enzymes (i.e. for both the apoenzyme and holoenzyme forms of glucose dehydrogenase and gluconate dehydrogenase). As a result, these proteins may be used to raise both polyclonal and monoclonal antibodies (MAbs). Because other Gram-negative MPS bacteria are expected to have these enzymes as well, it is probable that specific immunological identification of selected strains would require MAbs. Conversely, availability of MAbs against these two enzymes, leaving aside the possibility of raising MAbs against proteins involved in PQQ biosynthesis, creates a myriad of possibilities for EFPF development. Once MAbs are available, simple, routine, and inexpensive assays (e.g., enzyme linked immunosorbent assay; ELISA) can verify the presence of the selected MPS strain in virtually any sample or venue. Slightly more complex (but still standard) assays can provide *in situ* evidence for the presence of the selected MPS strain on (e.g.) the root surface. Finally, MAbs directed against both glucose dehydrogenase and gluconate dehydrogenase, when coupled with a MAb directed against some control target protein that is always expressed by the strain of interest (so-called 'housekeeping enzymes) can provide information about when and how the DO pathway is expressed. Is the strain making glucose dehydrogenase only, or is it making both glucose and gluconate dehydrogenase? Changes in enzyme catalytic activity (as measured by acid production), may be compared to changes in the amount of enzyme present (measured via ELISA) which, in turn, may be compared with changes in DO pathway gene expression (as determined by mRNA levels measured via qPCR), and so on.

The use of these types of molecular markers for the types of applications discussed above (as well as many others) would be considered completely routine for virtually any modern development project involving the use of living cells or cell-based products. In fact, both qPCR and immunological assays (including MAbs) have been commercially available for over 20 years. Why then – with the notable exception of the work of Meyer et al. (20) – are these tools completely lacking from the world of MPS R&D? Or is it more correct to ask whether an ongoing global R&D completely bereft of these modern methods should even be taken seriously? After half a century, it is certainly time for efforts focused simply on isolation and screening of MPSMs to end. Focusing on the DO pathway-mediated MPS phenotype exhibited by Gram negative bacteria is one way to ensure that – moving forward – any future development effort will be enabled by the most powerful biotechnology tools available.

3 Evaluation of current worldwide R&D to identify the most advanced work on DO Pathway-mediated MPS

Given the discussion in previous sections, it should come as no surprise that no 'advanced' work exists with respect to the development of field-applied EFPFs based on MPSMs expressing the DO pathway. However, one area where technology has advanced significantly with respect to P biofertilizers is the direct bioprocessing of RPO. Work supporting these early patents was conducted in the late 1980s and early 1990s, i.e. over twenty years ago. Figure 14 highlights the rate of development in these fields. It may be seen that the work described in this section – i.e. the most advanced to date – is hardly beyond that described in these early patents.

United States Patent [19]
Rogers et al.

US005256544A
[11] Patent Number: **5,256,544**
[45] Date of Patent: **Oct. 26, 1993**

[54] **MICROBIAL SOLUBILIZATION OF PHOSPHATE**
[75] Inventors: **Robert D. Rogers, James H. Wolfram**, both of Idaho Falls, Id.
[73] Assignee: **EG&G Idaho, Inc.**, Idaho Falls, Id.
[21] Appl. No.: **826,563**
[22] Filed: **Jan. 27, 1992**
[51] Int. Cl.⁵ **C12P 1/01; C12R 1/645; C12P 3/00; C12P 39/00**
[52] U.S. Cl. **435/42; 435/168; 435/813; 435/824; 435/911; 435/933**
[58] Field of Search **435/168, 42, 824, 933, 435/911, 813**
[56] **References Cited**
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Kucey Canadian Jour Soil Sci vol. 63 pp. 671-678 (1983).
Singh et al Current Sci vol. 55 pp. 1212-1213 (1984).
Primary Examiner—Herbert J. Lilling
Attorney, Agent, or Firm—Alan D. Kirsch
[57] **ABSTRACT**
A process is provided for solubilizing phosphate from phosphate containing ore by treatment with microorganisms which comprises forming an aqueous mixture of phosphate ore, microorganisms operable for solubilizing phosphate from the phosphate ore and maintaining the aqueous mixture for a period of time and under conditions operable to effect the microbial solubilization process. An aqueous solution containing soluble phosphorus can be separated from the reacted mixture by precipitation, solvent extraction, selective membrane, exchange resin or gravity methods to recover phosphate from the aqueous solution.

11 Claims, 4 Drawing Sheets

United States Patent [19]
Goldstein et al.

US005912398A
[11] Patent Number: **5,912,398**
[45] Date of Patent: **Jun. 15, 1999**

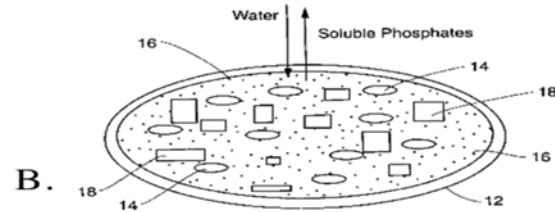
[54] **BIOMEDIATED CONTINUOUS RELEASE PHOSPHATE FERTILIZER**
[75] Inventors: **Alan H. Goldstein**, Beverly Hills, Calif.; **Robert D. Rogers**, Idaho Falls, Id.
[73] Assignee: **Lockheed Idaho Technologies Company**, Idaho Falls, Id.
[21] Appl. No.: **08/490,964**
[22] Filed: **Jun. 15, 1995**
[51] Int. Cl.⁵ **C05F 11/08; A01N 25/00; B09B 3/00**
[52] U.S. Cl. **71/6; 71/64.07; 71/64.11; 71/64.13; 435/262.5**
[58] Field of Search **71/6, 64.07, 64.11, 71/64.13; 435/262.5**
[56] **References Cited**
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5,256,544 10/1993 Rogers et al. 435/42
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Rogers et al., "Research and engineering assessment of biological solubilization of phosphate", 1993, Abstract 122:110025, *Energy Res. Absor.* 1993, 18(9) Absr #26522 no month.
Miahouni et al Effect of five types of organic matter on the microbial solubilization of rock phosphate, Abstract 115:255072, *Rev. Bras. Cienc. Solo* 1991, 15(1), 29-35 no month.
Primary Examiner—Terris Lander
Attorney, Agent, or Firm—Alan D. Kirsch; Theop North & Western
[57] **ABSTRACT**

A composition is disclosed for providing phosphate fertilizer to the root zone of plants. The composition comprises a microorganism capable of producing and secreting a solubilization agent, a carbon source for providing raw material for the microorganism to convert into the solubilization agent, and rock phosphate ore for providing a source of insoluble phosphate that is solubilized by the solubilization agent and released as soluble phosphate. The composition is provided in a physical form, such as a granule, that retains the microorganism, carbon source, and rock phosphate ore, but permits water and soluble phosphate to diffuse into the soil. A method of using the composition for providing phosphate fertilizer to plants is also disclosed.

27 Claims, 8 Drawing Sheets

A.



C.

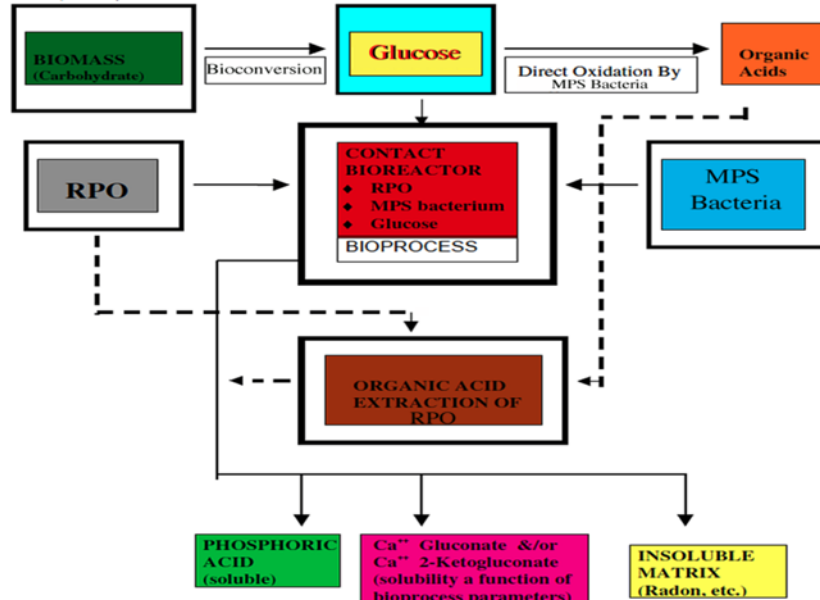


Figure 14. A. Bioprocessing patent. B. Patent for fertilizer pellet containing MPSMs. C. Flowchart present at IFA technical meeting.

A. Bioprocessing patent (Rogers et al., now lapsed) for the extraction of Pi from RPO. B. Patent for fertilizer pellet containing MPSMs, Carbon source, and RPO, i.e. an MPSM-based EPPF (Goldstein et al., now lapsed). C. A

flowchart presented at an IFA technical meeting (circa 2000) summarizing the possibilities inherent in the use of Gram-negative bacteria expressing the DO pathway to bioprocess RPO to Pi as an alternative to the Sulfuric acid-based 'wet process'.

Given that this section is dedicated to the evaluation of current worldwide R&D to identify the most advanced work on DO Pathway-mediated MPS, Figure 15 is included to offer the reader some historical perspective on the current state of progress in this field. The image was originally drawn using one of the first production MacIntosh computers ever sold (purchased in the mid-1980s for its state-of-the-art computer graphics), and printed via similarly state-of-the-art 'diaz' slide method for a presentation to the Idaho National Engineering Laboratory's (INEL's) bioprocess engineering group (long since defunct), all this circa 1990, i.e. well over 20 years ago! The R&D that will be described in the remainder of this section, while 'workman-like' and completely respectable has advanced very little – either in terms of theory or practice - since that time. In fact, the concept represented here has yet to receive any significant development effort. As discussed below, some progress has been made in the direct bioprocessing of RPO.

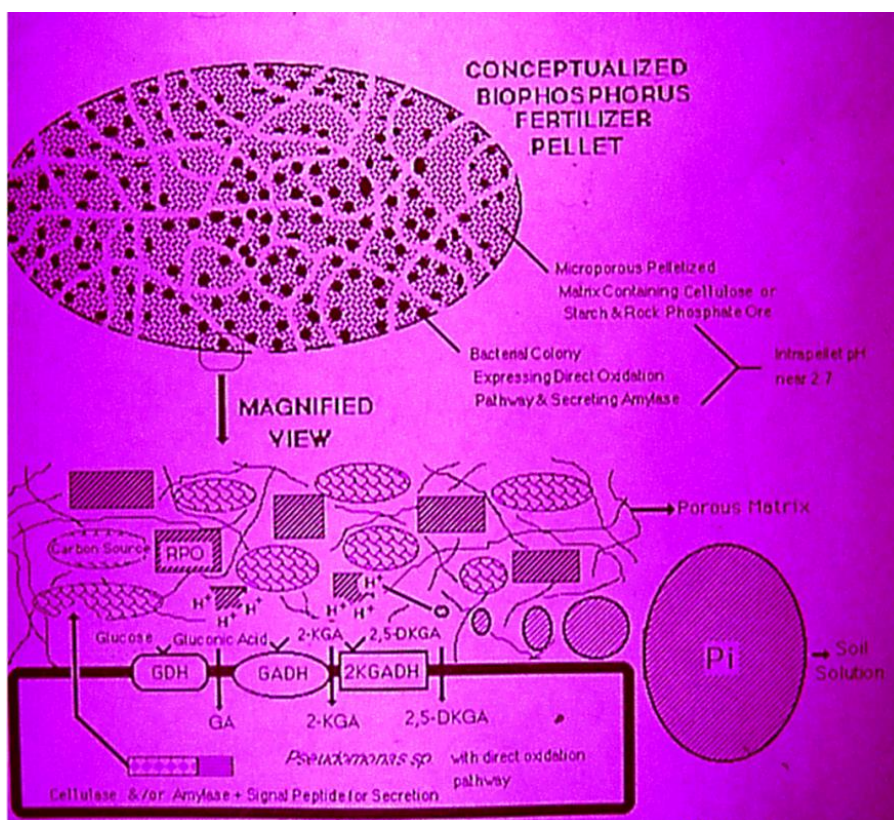


Figure 15. Original 'conceptualized biophosphorus fertilizer pellet'.

The most advanced work on DO Pathway-mediated MPSMs involves an interesting, albeit obvious system that hybridizes the concepts represented in Figure 14 A. & B., i.e. generates a 'bofertilizer' material via the partial bioprocessing of RPO with subsequent application to the soil (22). A number of obvious variations on this development strategy exist one of which is shown in Figure 16.

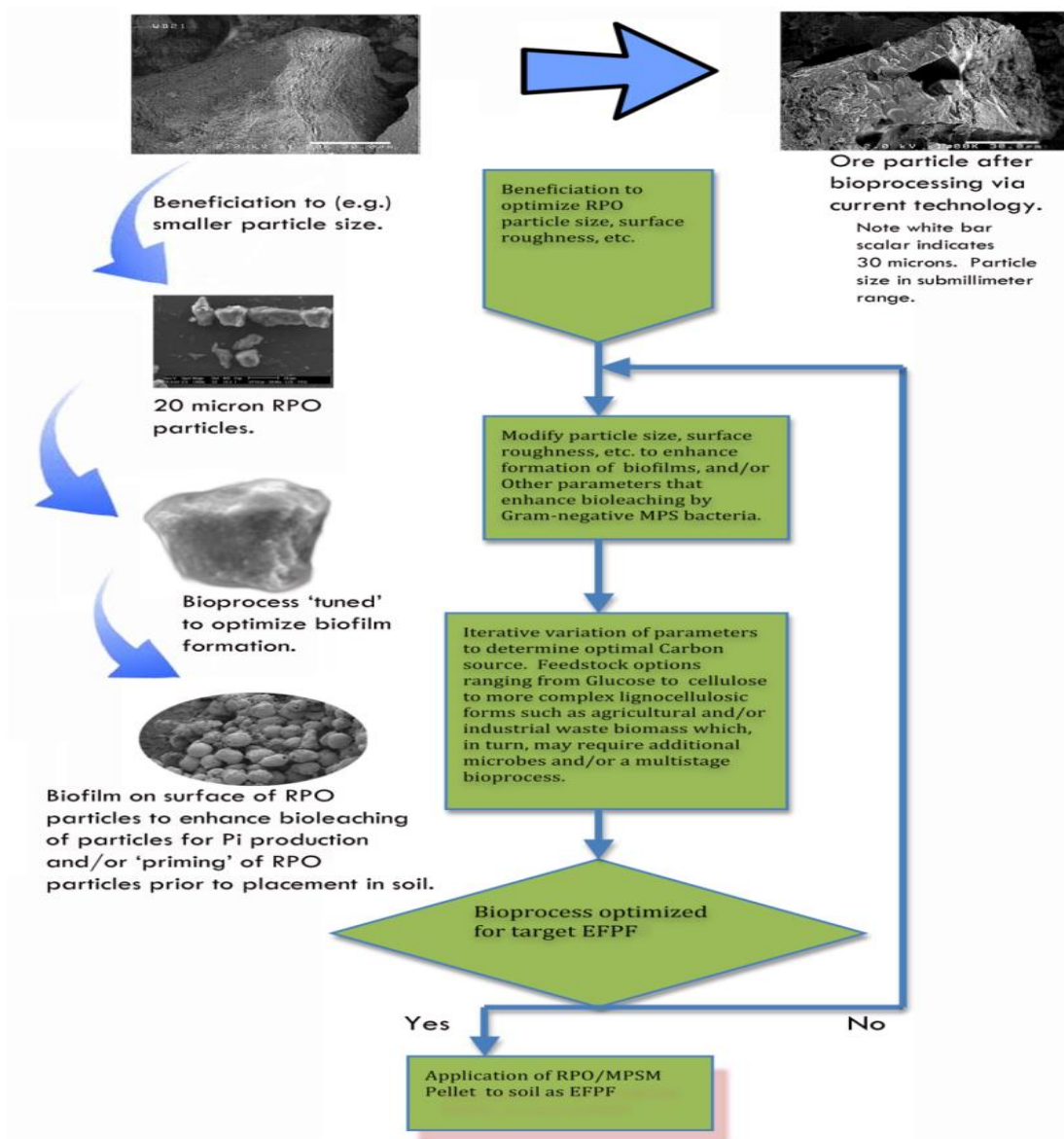


Figure 16. A possible subroutine algorithm generated from the off-screen connector in Figure 14.

RPO ore may be bioprocessed directly as originally proposed in the patents by Rogers et al. and Goldstein et al., etc. Current systems can extract up to 98% of Pi from ore (23 and references therein). The top two pictures show a standard RPO particle (submillimeter size range) before and after bioprocessing (24). This algorithm shows further beneficiation of industry standard finished ore to smaller particles (~20 micron) combined with a solid substrate fermentation-based bioprocessing system 'pre-tuned' to favor formation of the biofilm mode of growth by Gram-negative MPS bacteria using the DO pathway to leach the PRO particle. Finally, the partially leached, biofilm-coated particles are pelletized with the concentrated fermentation supernatant (containing residual glucose, Pi leached from the RPO particles, biomolecules created by the MPSM, free-living MPSM cells, etc.) and applied directly to soil as an EFPF.

Based on the preceding discussion it should be clear that the most advanced work on DO pathway-based EFPFs currently involves a strategy that effectively hybridizes the concepts presented in Figure 14 A. and 14 B. Rather

than attempting to supplant the 'wet process' with industrial-scale bioprocessing of RPO or attempting to create a *de novo* fertilizer system based on MPSMs, this strategy - quite cleverly – leverages decades of research into MPSMs, bioprocess engineering, and industrial-scale fermentation to generate a prototype EFPF composed of partially bioprocessed RPO, MPSMs, and carbon sources (including agro-industrial waste materials). This strategy – which will be called Bioactivated RPO - has obvious advantages with respect to EFPF development, especially in the short term. Bioprocessed RPO need not be fully leached of its Pi as would be the case if the bioprocess was attempting to compete with the sulfuric acid-based wet process (but see closing comments at the end of this section). Likewise, application of partially processed RPO material gives the associated MPSMs a head start (as opposed to a JumpStart®) with respect to colonizing and solubilizing RPO material in the soil. To the extent that a carbon source (residual glucose and/or undigested agro-industrial waste biomass) is included in this EFPF material, a unique microenvironment is created that should carry over through at least part of the growing season. This, in turn, addresses some of the concerns traditionally voiced about MPSM-based EFPFs regarding their inability to compete with native micro-organisms.

Progress with respect to Bioactivated RPO has been recently reviewed by Vassileva et al. (23 and references therein). And, in fact, much of the cutting edge work in this field has been done by Vassilev's group. In discussing this work, the various claims (and associated data) regarding the additional plant growth promoting rhizobacterial-type (PGPR-type) properties claimed by the authors will be ignored, not because these data are in question, but simply because these secondary effects go well beyond an analysis of EFPFs *per se*. This same strategy will be employed in the next session where focus will be on the successful methods that have been developed to deploy PGPRs but will not address any PGPR effects beyond what obviously results from the delivery of nutrient Pi to the plant.

It is important to recognize that advances in bioprocess technology are integral to the successful development of Bioactivated RPO systems. Such advances include immobilized cell-based submerged fermentation and solid state fermentation (SSF), where the substrate itself acts as the carbon source and there is an absence (or near absence) of free water. This method of bioprocessing agro-industrial wastes achieves extremely high substrate (and RPO) concentrations, which, in turn, produces high-efficiency bioprocessing of both these materials. Both fungi and bacteria may be used. To date, fungi have been favored because their mode of growth favored the high surface area and low water potential environment of the SSF. However, recent progress in the engineering and control of bacterial biofilms (discussed in Section 4) could dramatically change this situation. SSF target substrates include all the 'usual agro-industrial suspects' e.g., starch, cellulose, lignocellulose, pectin, etc. Using *A. niger* and *Phanerochaete chrysosporium* (a white-rot fungus known to degrade – among other things - phenolic resins), and MPSMs and SSF substrates such as sugar beet waste and 'olive cake' (from oil processing), Vassilev and coworkers have solubilized up to 75% of Pi from various RPO samples (23, 25 and references therein). The ability to bioleach most, if not all the Pi from RPO samples is not a new development. In the late 1980s at INEL, Rogers and coworkers routinely achieved 80% extraction using continuous flow fermentation systems. While working at Genencor's metabolic engineering facility in Palo Alto (in 2002) with funding from IMC Global, Goldstein used a genetically engineered proprietary strain of *Pantoea citrea* to extract >90% of the Pi from Bartow ore samples in a standard batch fermentation system (26). What is exciting is the use of SSF technology to immobilize MPSMs and formulate this material into a soil inoculant, i.e. an EFPF.

There is little conceptual difference between current formulations and the 'biophosphorus pellet' proposed in Figure 14. But reduction to practice has required the type of dramatic improvements in immobilized cell techniques that can only be accomplished via a sustained, directed development effort that runs precisely counter to the

uncoordinated, unsophisticated, poorly funded, often redundant cycle of abortive short-term experiments characteristic of most research on MPSMs. The difference here is the presence of a significant 'Green' movement in Europe and – in fact – the real driving force behind this development is probably not recognition of the need to develop sustainable P fertilizer technology (i.e. EFPFs), but rather a sustained political will to (and therefore continuous funding for) recycle agro-industrial wastes.

Regardless of the precise reasons, this sustained effort has resulted in significant progress towards the development of EFPFs based on a Bioactivated RPO strategy. SSF products are converted into – what amounts to – an immobilized microbial cell system which acts as a functional EFPF pellet. The immobilized cells are retained in a defined space separate from the soil/rhizosphere system. As visualized in the original patent application of Goldstein et al. (Figure 14B.), this spatial separation offers a number of advantages over EFPF systems involving free-living MPSMs. Essentially, the immobilized cell system offers a microenvironment tailored for the selected MPSM(s) which stabilizes and optimizes the biocatalytic system. When combined with the previously discussed 'fixed enzyme' features of the DO pathway (or fungal GOX), the advantage of such a system becomes obvious. Immobilization strategies are currently being applied combinatorially to several MPSMs (e.g., *P. chrysosporium*, *B. subtilis*), RPO, and SSF agro-industrial waste substrates in various carriers e.g., alginate, crab shell chitin, k-carrageenan). An especially interesting aspect of this work is the consolidation of fermentation and formulation into a single process. A visit to Dr. Vassilev's laboratory is strongly recommended to determine the current state-of-the-art with respect to the use of these associated technologies to develop EFPFs based on Bioactivated RPO.

4 Identification of key enabling technologies and analysis of their utility and/or necessity in MPS-based EFPF development.

It has been more than 20 years since Goldstein and Rogers first patented RPO bioprocessing systems for *both* the industrial-scale production of fertilizer-grade phosphoric acid and for the *in situ* release of Pi from pelletized formulations designed specifically to operate as EFPFs. At the time these patents were granted, it was recognized that successful reduction to practice would require technology infusions from related, enabling fields. In the previous section, Bioactivated RPO systems have been identified as a high probability for EFPF development – enabled by technical advances in SSF and driven by a European political commitment to green technologies in pursuit of sustainability. This section will focus on recent advances in key technologies expected to enable the development of EFPFs utilizing Gram-negative bacteria expressing the DO pathway. These advances and their overlaps are represented in Figure 17, and discussed in detail below.

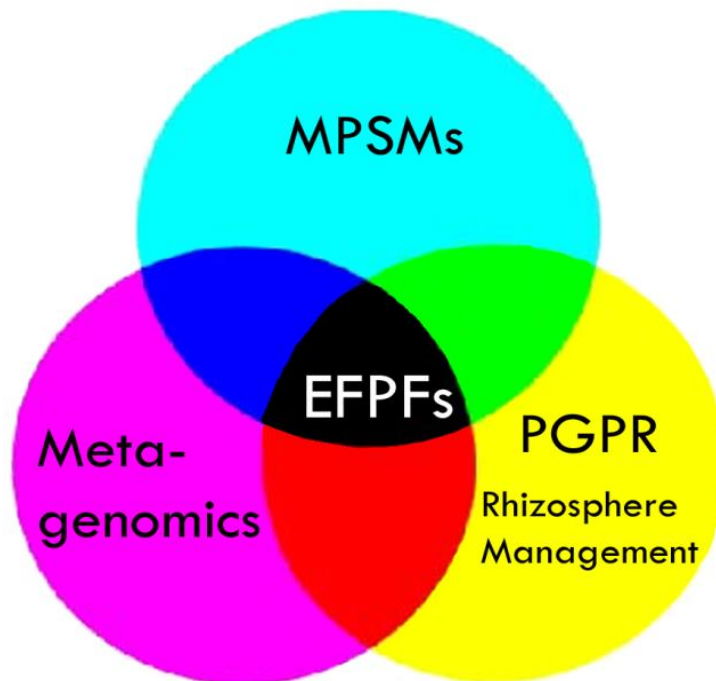


Figure 17. Overlapping spheres of influence

Overlapping spheres of influence generated by technology fields expected to fully enable the development of EFPFs based on the direct application of RPO and Gram-negative bacteria expressing the DO pathway. The MPSM sphere of influence (blue) includes such closely related endeavors as the beneficiation of RPO to optimize biofilm formation, metabolic engineering of the DO pathway, development of MAbs and qPCR primer systems, etc. The PGPR/Rhizosphere Management sphere of influence (yellow) includes highly applied enabling technologies developed over the past 20 years such as the use of root-colonizing and soil-inhabiting micro-organisms for biocontrol as well as areas of basic science such as molecular bases for signaling between root cells and rhizosphere microbes. Metagenomics (purple sphere) is perhaps the most fundamental enabling field of all given

that it offers workers in both other spheres rigorous, highly accurate methods by which an accurate, real-time picture of soil/rhizosphere microbial ecology and population dynamics may be obtained.

Metagenomics: The application of whole genome shotgun sequencing (shotgun metagenomics) to microbial communities represents a major paradigm shift for everyone interested in understanding the population dynamics or physiological ecology of naturally occurring and/or agricultural ecosystems (20, 21, 27-30). Put simply, there is no longer any excuse for considering soil or rhizosphere microbial ecology as a 'black box'. With respect to EFPFs, application of metagenomic principles will allow the unequivocal identification of MPSMs in unstudied agroecosystems and the validation of MPS strains previously identified and selected by traditional methods. The field of metagenomics has emerged from the bioinformatics revolution as a direct result of the perceived economic rewards associated with the rapid, inexpensive generation of whole genome data. In fact, except for the unique strategies associated with DNA sample preparation, the field of metagenomics has been created by entirely from genomic techniques as shown in Figure 18. The advantages of metagenomics techniques are obvious, either for the isolation of candidate MPSMs or for the validation and/or tracking of pre-selected (and pre-DNA sequenced) MPSMs during the product development process.

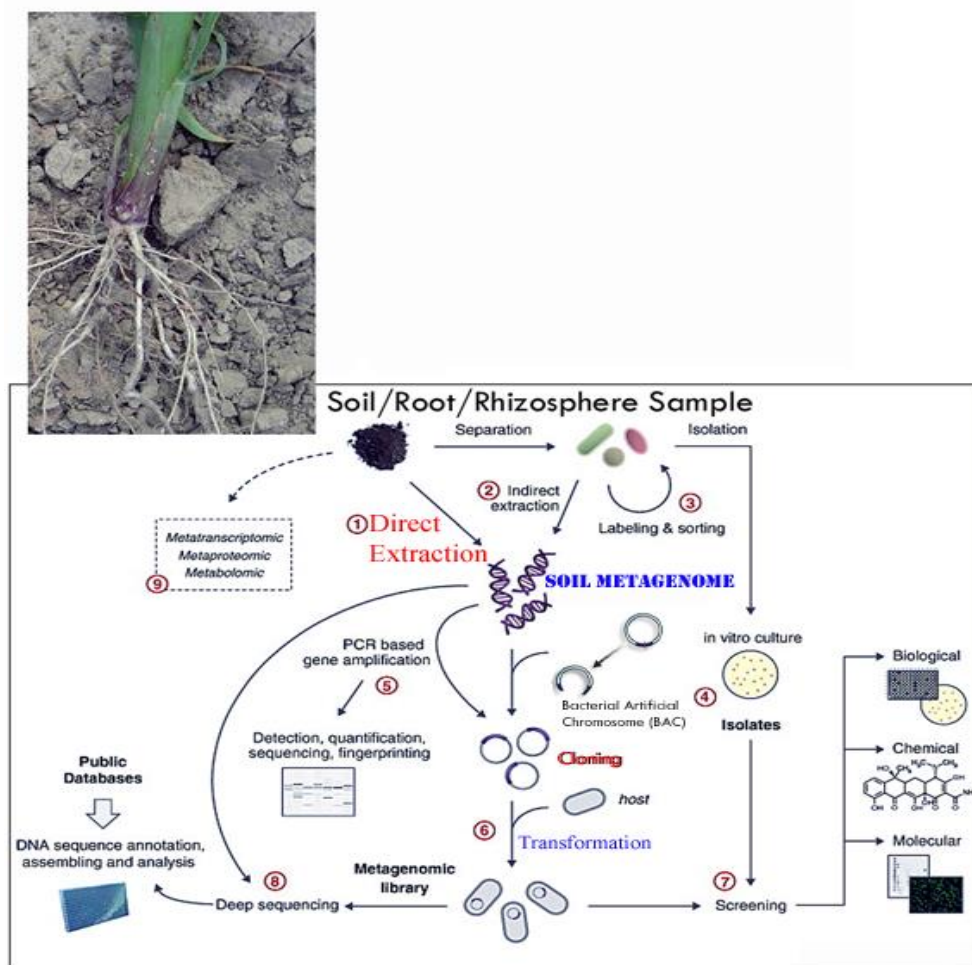


Figure 18. Metagenomic strategies for the complete characterization of a soil or rhizosphere sample.

Direct extraction of DNA from the sample without the bias created by isolation and in vitro culture (i.e. sample analysis pathway step 1) offers the opportunity to obtain an accurate, real-time picture of the numbers and types of microbes present in the sample. Total DNA from the soil or rhizosphere is PCR-amplified and cloned into bacterial artificial chromosome (BAC) vectors and then subjected to high-speed DNA sequencing. Contiguous DNA sequences are assembled *in silico* using standard genomics software and then matched with worldwide genomics libraries to produce an accurate picture of the numbers and types of micro-organisms present in the ecosystem of interest.

PGPRs/Rhizosphere Management: It is not useful, or even possible, to separate the enabling components of plant growth-promoting rhizobacteria (PGPR) from rhizosphere management and biocontrol. Recent progress in PGPR science and technology – especially with respect to molecular methods - has been reviewed by Kamilova et al., Lugtenberg and Kamilova, and others (31-41). These workers make it clear that, after almost half a century of unfulfilled promises, a number of microbes have now been identified, characterized and marketed as successful plant growth--stimulating and/or plant protection (biocontrol) products in the agricultural marketplace. This benchmark is significant for EFPF product development, insofar as it refutes one of the most consistent objections declaimed by the proponents of chemical fertilizers – that, due the inherent variability of agricultural ecosystems, microbial products were not reliable enough to be used for agronomic crop production. Put simply, the criticism on biologicals has been that while they may work consistently in the test tube, and frequently in the greenhouse, all bets are off when they are put in the field. In 2014, that view is no longer tenable. Much remains to be learned about signaling and growth regulation in the rhizosphere. Certainly, we cannot pick winners and losers *a priori*. We cannot isolate a bacterial strain from the root zone, run it through a series of lab tests (or sequence its DNA) and then say with certainty that it will always be found in high populations on the root surface. But just as certainly, we *can* evaluate a given MPSM for rhizosphere competence. We can predict whether it is capable of colonizing the root surface. And, using the various molecular tools described in this report, we *can* follow a candidate MPSM throughout the growing season and establish unequivocally whether it has successfully populated the rhizosphere and/or bulk soil.

While fungi are not part of this report, it must be stated that beneficial, even stimulatory effects have been demonstrated on plant roots and that these effects are reliable enough to support commercial products. And it is well established that arbuscular mycorrhizal fungi must be present in the root zone for many crop plants to achieve even normal growth rates. But, as the eponymous name indicates, a PGPR must, by definition, be a bacterial strain. The rhizosphere is most generally defined as the region of soil that directly influences and is directly influenced by the root. The first and most important cause of this “rhizosphere effect” is the amount of carbon secreted (“sloughed off”) and otherwise made available by plant roots. Depending on the plant and its environment, up to 20% of the total fixed carbon may end up in the rhizosphere. And what does the plant receive in return? There can be no doubt that in many cases rhizosphere microorganisms, especially PGPR, most especially MPS PGPR, help the plant solve the “phosphate paradox” discussed in detail in 18. In order to move forward, it is essential that scientists and engineers interested in developing EFPFs stop treating phenomena involving PGPR and MPSMs as too anomalous and/or hypervariable for product development.

PGP rhizobacteria generally exert their beneficial effects via colonization of the root surface. As a result, factors that regulate root colonization, especially molecular signaling systems, have been the subject of a great deal of study. Obviously, much of what goes on occurs on the “plant side” of this system, while even more involves interactive signaling between the plant and PGP root colonizers. All this is, by agreement, beyond the scope of the current analysis. Regardless, a great deal is now known about the bacterial traits involved in beneficial root

colonization. This knowledge forms the foundation of current “best practices” in rhizosphere management. In their 2009 review, Lugtenberg and Kamilova (32) state clearly that examples of “direct plant-growth promotion” include (a) biofertilization, (b) stimulation of root growth, (c) rhizoremediation and (d) plant stress control. Virtually all these effects have their origin in the ability of PGPR to colonize the root surface. Colonization, in turn, may now be studied by a variety of powerful analytic tools including various advanced forms of microscopy (Environmental S.E.M, scanning confocal, etc.), often coupled with powerful “reporter” proteins (e.g., green fluorescent protein; GFP or mCherry) that can be produced inside bacterial cells of interest via routine genetic engineering methods. An example of these powerful new methods is shown in Figure 19.

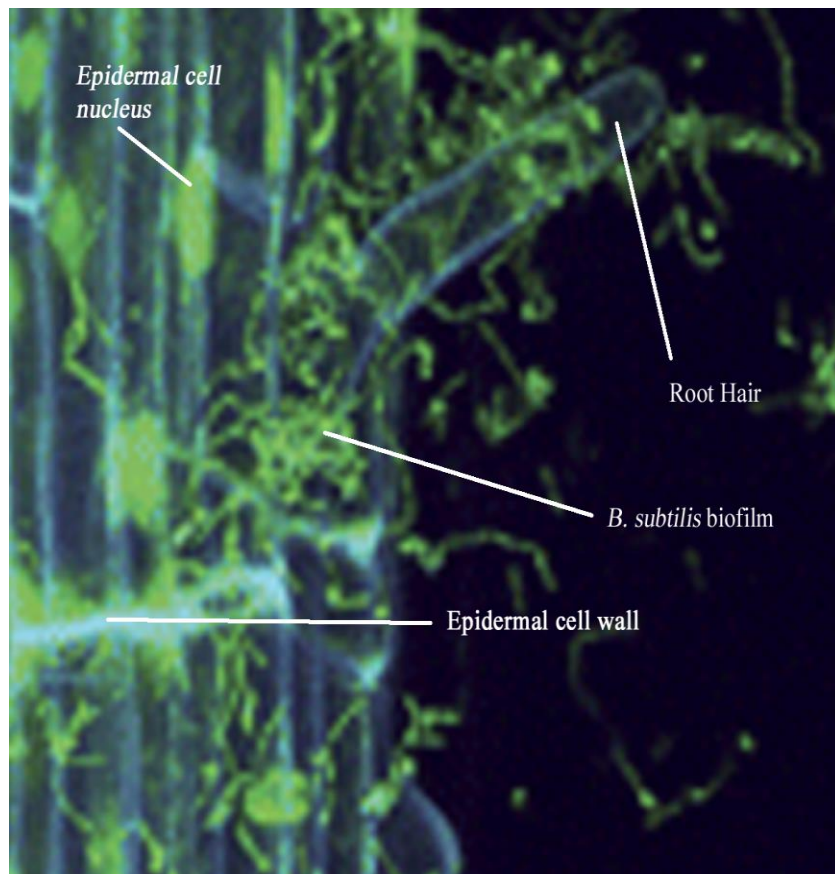


Figure 19. Scanning confocal microscopic image of rhizosphere-competent *B. subtilis* PGPR growing as a biofilm on the roots of *A.thaliana*. Modified from reference 42.

A number of colonization traits and genes have been identified, although the list is far from complete. Antibiotic production, especially among *Pseudomonas* spp. has created an enormous resource for identifying strains that are highly rhizosphere-competent and superior for biological control of plant diseases (43). For example, a great deal of interest has been focused on 2,4-diacetylphloroglucinol (DAPG)-producing *Pseudomonas* strains in the rhizosphere. This compound is now known to have both antibiotic and PGP properties. In addition to antibiotic production, significant progress has been made identifying molecular signaling involved in biofilm formation by root colonizers, including molecular signals involved in the transition from free-living mode to the biofilm mode of growth, so-called quorum sensing. This transition may play a key role in root colonization since many studies suggest that free-living behaviors such as motility and swarming are necessary for rhizosphere-competent bacteria to move to the root tip and other favored niches, whereas once the target area for colonization has been reached, the biofilm

mode of growth becomes dominant (15, 44, 45). Likewise, signaling via exopolysaccharide matrix (and other biomolecular) components is now recognized to play a key role in regulation of biofilm formation for many rhizosphere-competent bacteria. Here, again, the system is bidirectional – it involves the production of biomolecules by both the bacteria and the plant. It is important to note that biofilm formation undoubtedly plays a role in the stability of rhizosphere colonization by imparting stress tolerance (e.g., matrix hydration under water-limiting conditions).

Identification of key genes involved in rhizosphere colonization allows us to leverage the unique, extremely powerful biomolecular “fingerprinting” systems created by the molecular biology revolution to identify, characterize and monitor rhizosphere-competent bacteria. Techniques such as qPCR and MAbs (discussed in Section 2) complement the global strategies of metagenomics to create a fully integrated “diagnostic” system with which to study and validate candidate PGPR strains. For example, Bergsma-Vlami et al. (43) used denaturing gradient gel electrophoresis (DGGE) to track a 350-bp fragment of *phlD* (a key gene involved in DAPG biosynthesis) through a rhizosphere system. DGGE allowed discrimination between genotypically different *phlD*⁺ reference strains and indigenous isolates and enabled detection of specific *phlD*⁺ genotypes directly in rhizosphere samples with a detection limit of approximately 5×10^3 colony forming units per gram of root tissue, an astounding level of resolution. DGGE also allowed simultaneous detection of multiple *phlD*⁺ genotypes present in mixtures in rhizosphere samples. An example of this powerful tracking system is shown in Figure 20 below.

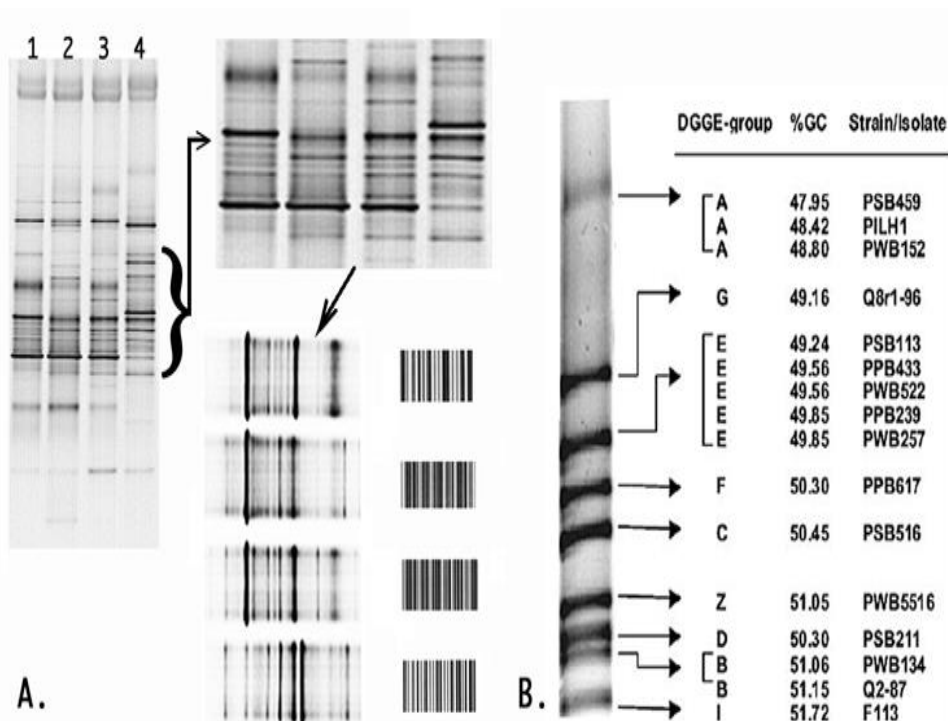


Figure 20. Denaturing Gradient Gel Electrophoresis (DGGE).

One of the powerful molecular tools available for rhizosphere management. A. Characteristic DGGE data. Each lane of the gel shown to the far left provides a different picture of the system of interest (e.g., day 1 to 4, treatments 1 to 4, plants 1 to 4, etc.). Each band in a given lane represents a different amplified DNA product and, therefore, a different bacterial strain so that the population of (e.g.) rhizosphere-competent pseudomonads may be visualized and read by an optical scanner much like a bar code (bottom right). B. Relationship between electrophoretic

mobilities of 350 bp *phlD* *Pseudomonas* gene fragments and their G+C contents in DGGE. Note that in A. DGGE data give information about a population whereas in B. individual *Pseudomonas* strains may be identified (modified from Bergsma-Vlami et al.; 43).

5 Specific paths for proof-of-concept, including timelines and milestones

The first step toward finding a way forward for MPSM-based EFPFs is to recognize that the traditional view of this technology, as a “blue sky” dream of the future (Figure 21), no longer applies. MPSM-based EFPFs are, in fact, already a reality, as shown by Figures 22 and 23 below. The challenge now is to transition to a bioengineering strategy in order to develop highly efficacious systems that – in combination with RPO – can ultimately replace today’s highly soluble fertilizer phosphates which, in turn, are derived from the sulfuric acid-based “wet process.”

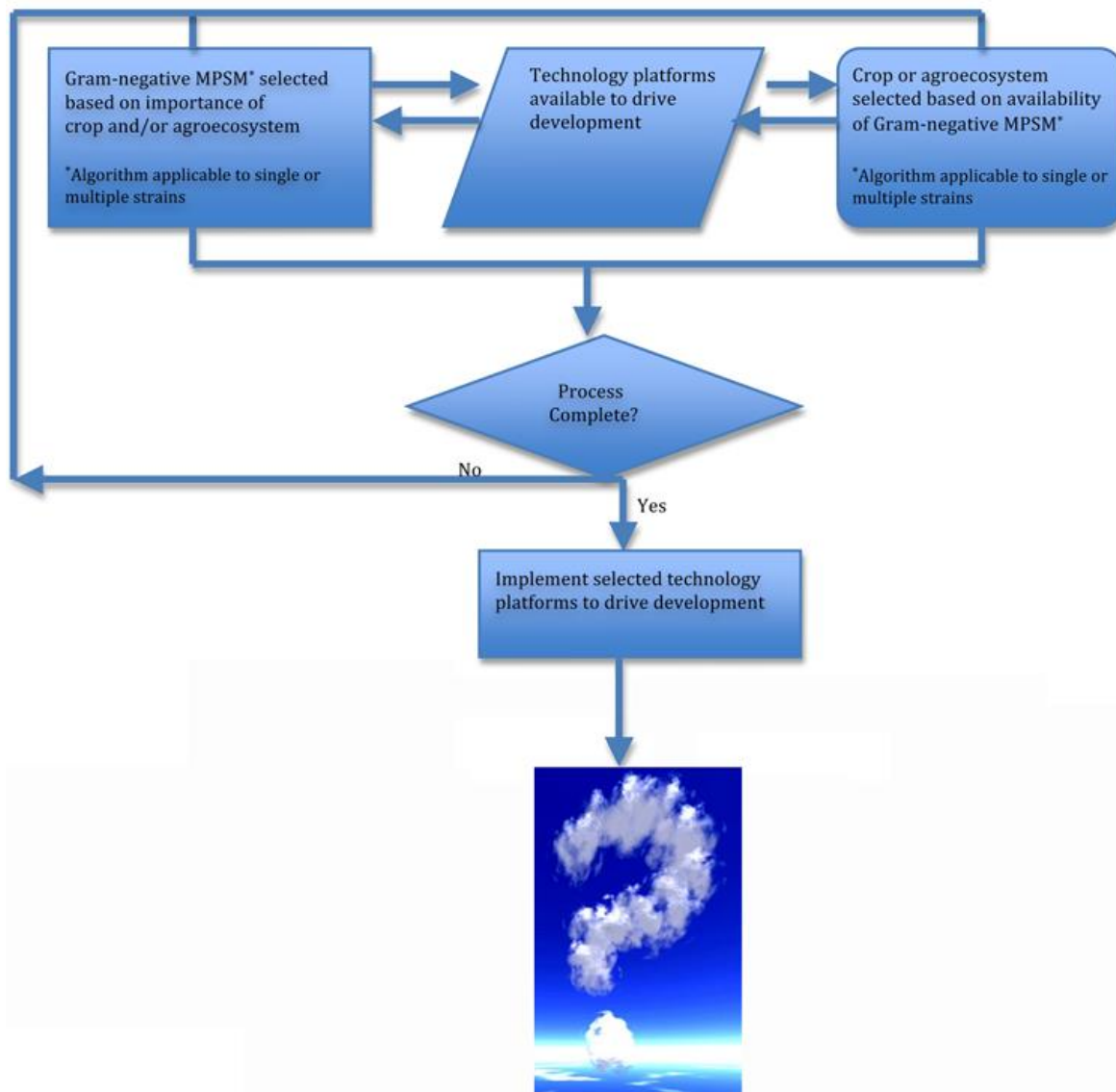


Figure 21. Traditional 'blue sky' view of the path forward for MPSM-based EFPFs.

Novozymes and Syngenta to partner in delivering JumpStart® — For enhanced phosphate fertility within bioagriculture

The agreement provides an exclusive expansion into new markets for JumpStart, which is a biological that enhances phosphate fertility in agriculture, offering growers an integrated and sustainable seed care solution.

25. April 2012

PRODUCTS & SOLUTIONS

Products & solutions > Australia > JumpStart®

WHAT IS JumpStart®?

Better phosphate uptake with inoculation
JumpStart promotes greater phosphate use efficiency, which results in quick emergence, early vigour, greater stress tolerance, and earlier, more even maturity. JumpStart delivers a safe method of supplying phosphate to growing plants, and reduces the need to seed-place high rates of phosphate fertiliser with sensitive seed like canola, pea, and lentil.

How JumpStart works
JumpStart is a phosphate inoculant containing the naturally occurring soil fungus *Penicillium bilaii* (*P. bilaii*), discovered by Agriculture and Agri-Food Canada. It colonises (grows along) plant roots, releasing organic compounds that release the “bound” mineral forms of less available soil and fertiliser phosphate, making it immediately available for the crop to use.

DOWNLOADS

- > Product information
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RESEARCH PAPERS

- > Inorganic phosphate solubilization by two penicillium species in solution culture and...
- > Increased phosphorus uptake by wheat and field beans inoculated with a...
- > Penicillium bilaii inoculation increases root-hair production in the field pea

Figure 22. JumpStart®, the most well-known MPSM-based P fertilizer products of the market (5)

Phosphorus Solubilizing Inoculants
The availability of P to plants is a key constraint to agricultural production across much of southern Australia (3). Depending on geochemical attributes, soils may immobilize P by binding with Ca_2^+ , Mg_2^+ , Fe_2^+ , adsorption to Fe- and Al-hydroxides and Al-silicates, or via precipitation reactions (e.g., Al in acidic soils or Ca in alkaline soils). On such soils the efficiency of P-based fertilizers can be low, with only 10 to 30% of the applied available for plant uptake in the year of application (6). To offset this, approximately AU\$600 million is spent annually on P-based fertilizers. The necessity to repeatedly fertilize soils with P has resulted in a buildup of P in many Australian soils.

Soil microorganisms are responsible for transforming immobilized soil P into plant-available forms (4). Research is underway to determine the potential for exploitation of these microorganisms to increase plant growth on soils high in immobilized P. Most work centers on the utilization of *Penicillium* fungi, as many species solubilize high amounts of inorganic phosphates (10). To elicit plant growth promotion, P solubilization must occur on or adjacent to the plant root. It is essential, therefore, that strains are also selected for the ability to associate closely with plant roots (rhizosphere competence).

Penicillium radicum A. D. Hocking & Whitlaw is an Australian phosphate-solubilizing inoculant for wheat (Fig. 3). The fungus was discovered in 1991 during a survey of wheat rhizosphere fungi for P-solubilizing activity (12) and has the capacity to solubilize a wide range of inorganic phosphates common in soils. The prime mechanism for P solubilization is thought to be secretion of gluconic acid (13), which can mobilize P from insoluble sources by lowering pH or by chelation-based mechanisms. Successful demonstration of plant growth promotion in glasshouse and field trials (11), culminated in the development of *P. radicum* as the inoculant **Pr70Release** (Bio-Care Technology Pty Ltd, Somersby, NSW). In azoo, sufficient inoculum was produced to treat 3000 kg of wheat seed. This production level is high enough to enable extensive testing by farmers so that the best opportunities for its widespread use in Australia can be identified (soil type, agro-ecological conditions etc).

P-mobilization per se does not account for all of the plant growth-promoting effect observed by *P. radicum*. Under field conditions, *P. radicum* increases wheat growth under low and high P-fertilization levels (11). Other possible

BIO-P
Phosphate Solubilizing Bacterium

PRODUCTOS BIOLÓGICOS

NoctinAzo
Producto líquido acuoso para trigo y maíz en base a *Azospirillum brasilense*

RISCL BIO-P - Phosphate Solubilizing Bacterium
RISCL BIO-P is a biofertilizer containing phosphate solubilizing microorganism viz., *Bacillus megaterium*. This bacterium produces organic acids which dissolve the insoluble (fixed) form of phosphorus in the soil and make it available to plant thereby enhancing the plant growth.

ADVANTAGES

- RISCL BIO-P can be used for all crops.
- RISCL BIO-P increases the protein content and quality of proteins in grains.
- RISCL BIO-P helps to increase yield by 10 %.
- RISCL BIO-P helps to reduce or reduce 25-30% of recommended dose of phosphatic fertilizer.

CROPS
All Crops

Trasco
Corporate Profile Products Knowledge Bank Company Activities Link

Bio - Fertilizer

- Organic
- Bio - Fertilizer
- Bio - Pesticides
- Bio - Fungicides
- Bio - Mitiçida

Trasco Phospho-Grow

Super Agro India has brought a new product for the farming community named Trasco Phospho-Grow. This is a product that has been developed indigenously in the Companies own manufacturing facility under the Bio-Pesticide division of Super Agro India

What is Phospho-grow?
Trasco Phospho-grow is a bio-fertilizer comprising of Phosphorus solubilising Bacteria used to release fixed phosphorus in soils for field crops .

PHOSPHATE SOLUBILISING MICRO-ORGANISMS :

Phosphate Solubilizing Bio-Fertilizer, Bacillus Coagulans Bacteria, Phosphate Fertilizer
Chemicals, Plastics, and Raw Materials > Fertilizers, Pesticides, and Agrochemicals > Fertilizers

Contact Supplier **Add to Basket**

- Company Name :** Sundaram Overseas Operation
- Product Name :** Phosphate Solubilizing Bio-Fertilizer, Bacillus Coagulans Bacteria, Phosphate Fertilizer
- Keyword :** nitrogen bacteria nitrogen fertilizer nitrogen fixation ,bio organic fixing nitrogen biological nitrogen ,symbiotic nitrogen fixation lawn fertilizer natural fertilizer,nitrogen plant growth organic grass fertilizer

Figure 23. Some of the other MPSM-based fertilizer products currently on the world market. Product labels from MPSM-based EFPFs available in Argentina, Australia and India.

As Figures 22 and 23 make obvious, MPSM-based EFPFs are already here. The scientific and agricultural communities now accept that MPS bacteria and fungi do exist, (i.e. that MPSMs can solubilize RPO and other poorly soluble mineral phosphates). The question is, can technology harness the MPS phenotype to develop MPSM-based fertilizer products that will reliably solubilize RPO when and where the farmer needs it? This “reliability barrier” is inherent in disclaimers such as the one that appears on the JumpStart® website: “JumpStart does not replace the need for P fertilizer. JumpStart improves the efficiency of phosphate, which can reduce the amount of fertilizer required, depending on levels of available phosphate within the soil.”

In order to break through the “reliability barrier,” it will be necessary to sort through the enormous amount of information available on MPSMs and focus tightly on the most probable path(s) to the creation of systems that may be applied to agricultural systems worldwide. This report identifies Gram-negative bacteria that solubilize RPO via DO pathway-mediated generation of gluconic and 2-ketogluconic acids as a high-probability target for the creation of such EFPFs. A global decision flowchart for achieving this target is presented in Figure 24.

5.1 Milestones and timelines

The decision algorithms and development timelines presented Tables 3, 4, 5, and Figures 25, 26, 27 are all based on the following assumptions:

- A Gram-negative bacteria (MPSM) that uses the DO pathway to accomplish mineral phosphate solubilization.
- Top-down implementation by a single organization (i.e., a company, an NGO, etc.). This avoids timeline complications created by partnering.
- Budgetary sufficiency so that all technical services (e.g., DNA sequencing, production of MAbs, etc.) may be contracted from professionals operating at industry-standard efficiency and capacity.

Three sets of decision algorithms and development timelines are presented, corresponding to the three most probable MPSM-based EFPF strategies identified in this report: (1) Rhizosphere-competent MPS bacteria growing on or near the root surface, (2) free-living soil MPS bacteria and (3) MPS bacteria delivered to soil as part of a Bioactivated fertilizer pellet containing the MPSM + RPO +/- carbon source (e.g., agroindustrial waste) +/- immobilizing agent (e.g., alginate), +/- symbolizing that the Bioactivated pellet may or may not have the additional item. These strategies are presented in descending order of complexity (i.e. most complex to least complex) based on the number of variables associated with the delivery of each EFPF agent. The most complex system is assumed to be based on a rhizosphere-competent EFPF; the least complex is assumed to be the bioactivated pellet. Development of an EFPF system based on free-living soil MPS bacteria is assumed to be intermediate in complexity between the two. These timelines are based on real-time estimates assuming constantly available, fully operational systems. For example, no allowance is made for “down time” based on crop-growing seasons, identification and vetting of qualified vendors, etc. The development timeline does not include iterative secondary activities designed to further optimize the system. For example, it is suggested that the RPO material could be further beneficiated to optimize the biophysical chemistry of Pi leaching (i.e., smaller particle size to create more surface area for bacterial attachment, biofilm formation, etc.). The development timeline does not explicitly include obvious subcategories: greenhouse testing as part of field testing, testing for rhizosphere-competence via DGGE, or qPCR during whole plant greenhouse or field testing, etc.

Table 3. Priority scores of key variable parameters (state variables) for the development of EFPFs based on Gram- negative MPS rhizobacteria.

Rhizosphere-competent EFPF: Key Variable Parameters	Priority Score Max. = 5	Comments
Target Agroecosystem(s)	2.5	MPS bacteria have been isolated from virtually every soil type and/or rhizosphere. In order to focus on CaP solubilization, it is suggested that arid or semiarid cropping system(s) be selected.
Target Crop Plant(s)	2.5	MPS bacteria have been isolated from the rhizosphere of virtually every crop plant. However, in order to focus on CaP solubilization, crop plants from arid/semiarid agroecosystems are recommended.
Previously-identified MPS strain	2.0	MPS bacteria are historically identified based on differential growth in laboratory media. Metagenomic analyses have demonstrated that this type of functional 'pre-screening' can introduce a significant bias into the characterization of natural populations.
Rhizosphere colonizer	4.5	It is clear from analysis of the PGPR literature that rhizosphere colonization is a crucial factor for the successful stimulation of plant growth via biocontrol. Pi generated in the rhizosphere will be most efficacious for plant nutrition.
Established genera	3.5	Common rhizosphere colonizing genera such as <i>Pseudomonas</i> and <i>Bacillus</i> have been well studied and will be easier to 'boot up' as EFPFs.
Gluconic Acid (GA) Producer	5.0	All Gram-negative MPS bacteria are expected to express – at least – the first catalytic step in the DO pathway.
2-KGA Producer	4.0	Production of GA and 2KGA offers enhanced bioengineering options during development.
2,5-diketogluconic acid	1.0	Very few Gram-negative bacteria generate 2KGA.

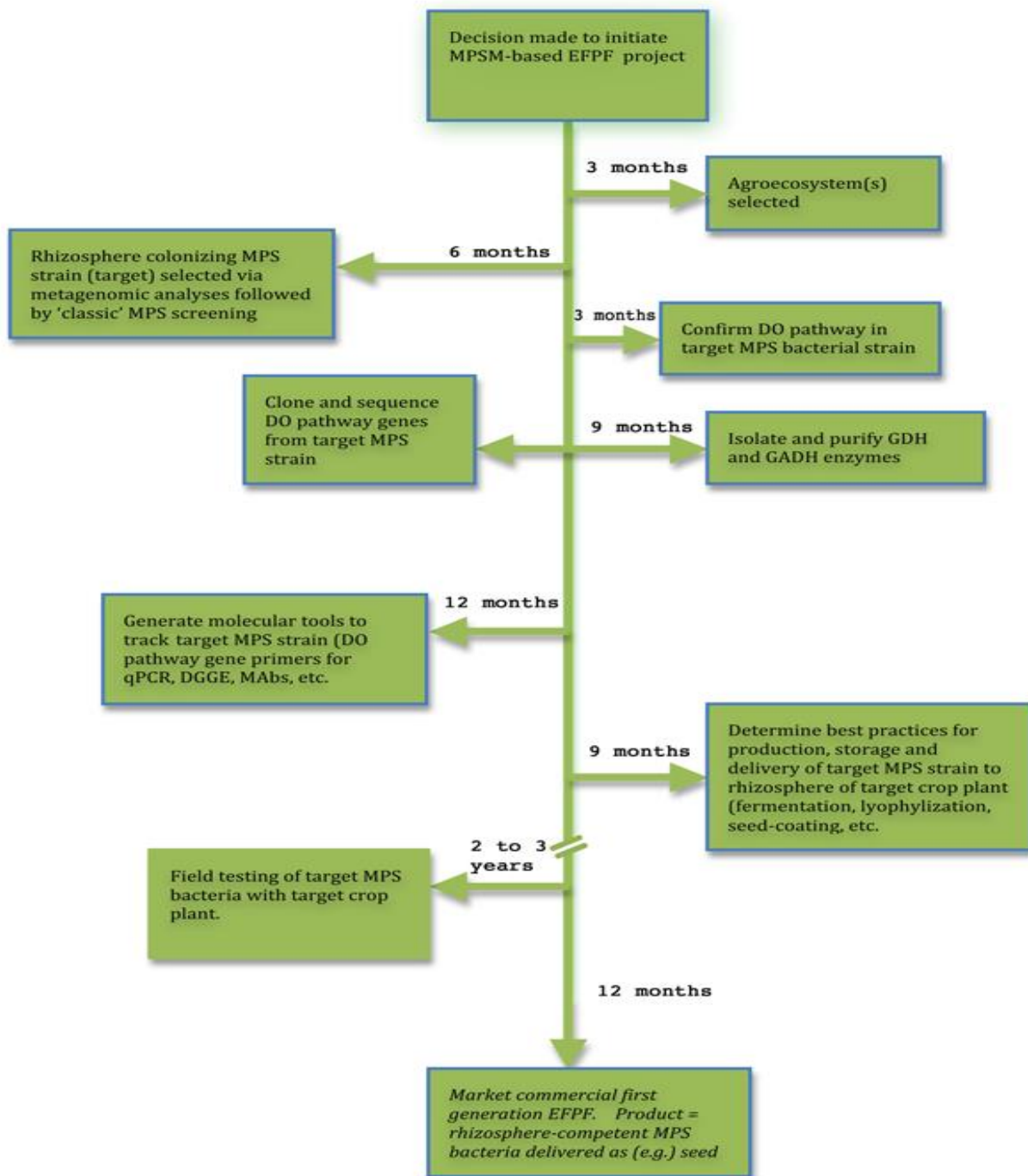


Figure 25. Timeline/milestones for the development of rhizosphere-competent EFPP based on Gram-negative MPS rhizobacteria.

Time to milestone is shown “upstream” (i.e., three months from “Decision made to initiate project” to milestone of “Agroecosystems selected”). The development timeline is designed for a single EFPP agent in a single agroecosystem. Larger versions of timelines are shown in Appendix I.

Table 4. Priority of key variable parameters (state variables) for the development of EFPFs based on free-living Gram-negative MPS soil bacteria.

Free-living soil EFPF: Key Variable Parameters	Priority Score Max. = 5	Comments
Target Agroecosystem(s)	2.5	MPS bacteria have been isolated from virtually every soil type and/or rhizosphere. In order to focus on CaP solubilization, it is suggested that arid or semiarid cropping system(s) be selected.
Target Crop Plant(s)	2.5	MPS bacteria have been isolated from the rhizosphere of virtually every crop plant. However, in order to focus on CaP solubilization, crop plants from arid/semiarid agroecosystems are recommended.
Previously-identified MPS strain	2.0	MPS bacteria are historically identified based on differential growth in laboratory media. Metagenomic analyses have demonstrated that this type of functional 'pre-screening' can introduce a significant bias into the characterization of natural populations.
Capable of competing as free-living soil bacterium	4.5	The ability to compete in soil agroecosystem is a crucial factor for the efficacious solubilization of applied RPO. Metagenomic analyses & other molecular tools can confirm competitive phenotype.
Established genera	3.5	Common rhizosphere colonizing genera such as <i>Pseudomonas</i> and <i>Bacillus</i> have been well studied and will be easier to 'boot up' as EFPFs.
Gluconic Acid (GA) Producer	5.0	All Gram-negative MPS bacteria are expected to express - at least - the first catalytic step in the DO pathway.
2-KGA Producer	4.0	Production of GA and 2KGA offers enhanced bioengineering options during development.
2,5-diketogluconic acid	1.0	Very few Gram-negative bacteria generate 2KGA.

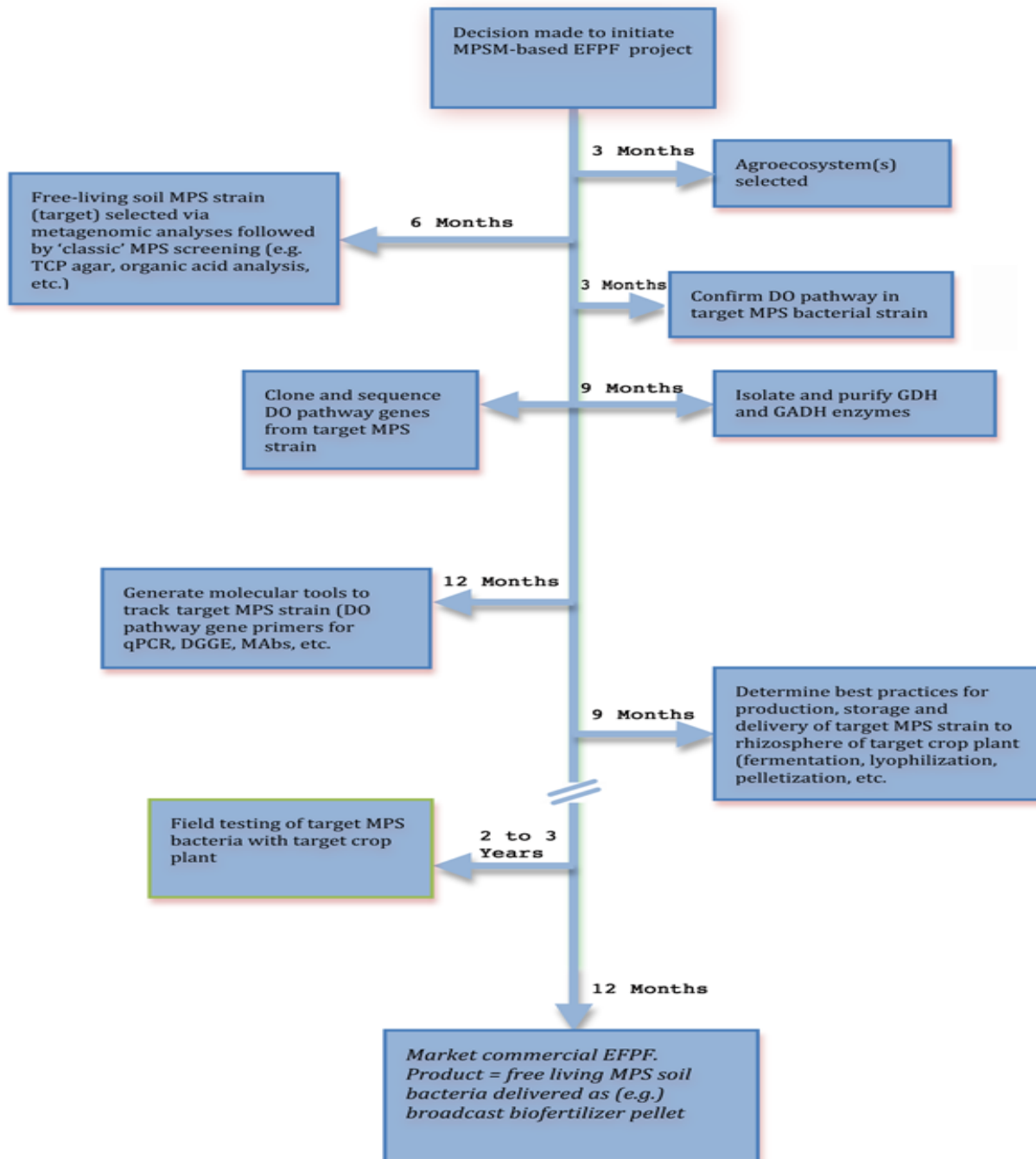


Figure 26. Timeline/milestones for the development of free-living EFPF based on free-living Gram negative MPS soil bacteria.

Time to milestone is shown “upstream” (i.e., three months from “Decision made to initiate project” to milestone of “Agroecosystems selected”). The development timeline is designed for a single EFPF agent in a single agroecosystem.

Table 5. Priority scores of key variable parameters (state variables) for the development of bioactivated RPO fertilizers based on industrial bioprocessing strains of Gram-negative MPS soil bacteria.

Bioactivated RPO EFPE: Key Variable Parameters	Priority Score Max. = 5	Comments
Target Agroecosystem(s)	2.5	MPS bacteria have been isolated from virtually every soil type and/or rhizosphere. In order to focus on CaP solubilization, it is suggested that arid or semiarid cropping system(s) be selected.
Target RPO & agro-industrial waste biomass (C source)	2.5	MPS bacteria capable of bioprocessing RPO have been studied for >20 years. If MPS microbe cannot bioprocess targeted agro-industrial waste then bioprocess can utilize a second microbe in the same fermentation (notobiotic system) and/or sequential bioprocessing.
Previously-identified MPS bioprocessing strain	2.0	Strongly MPS-positive bacteria are historically associated with production of strong organic acids. Specifically, Gram-negative bacteria always produce GA and often produce 2-KGA. Genera commonly used in bioprocessing (e.g. <i>Gluconobacter</i>) will increase rate of progress.
Amenable to Solid State Fermentation (SSF) and/or other advanced bioprocess engineering systems	4.5	It is clear from analysis of the PGPR literature that rhizosphere colonization is a crucial factor for the successful stimulation of plant growth via biocontrol. Pi generated in the rhizosphere will be most efficacious for plant nutrition.
Established genera	3.5	Genera such as <i>Gluconobacter</i> and <i>Enterobacter</i> have been well studied and will be easier to manipulate via genetic/metabolic engineering.
Gluconic Acid (GA) Producer	5.0	All Gram-negative MPS bacteria are expected to express – at least – the first catalytic step in the DO pathway.
2-KGA Producer	4.0	Production of GA and 2KGA offers enhanced bioengineering options during development.
2,5-diketogluconic acid	1.0	Very few Gram-negative bacteria generate 2KGA.

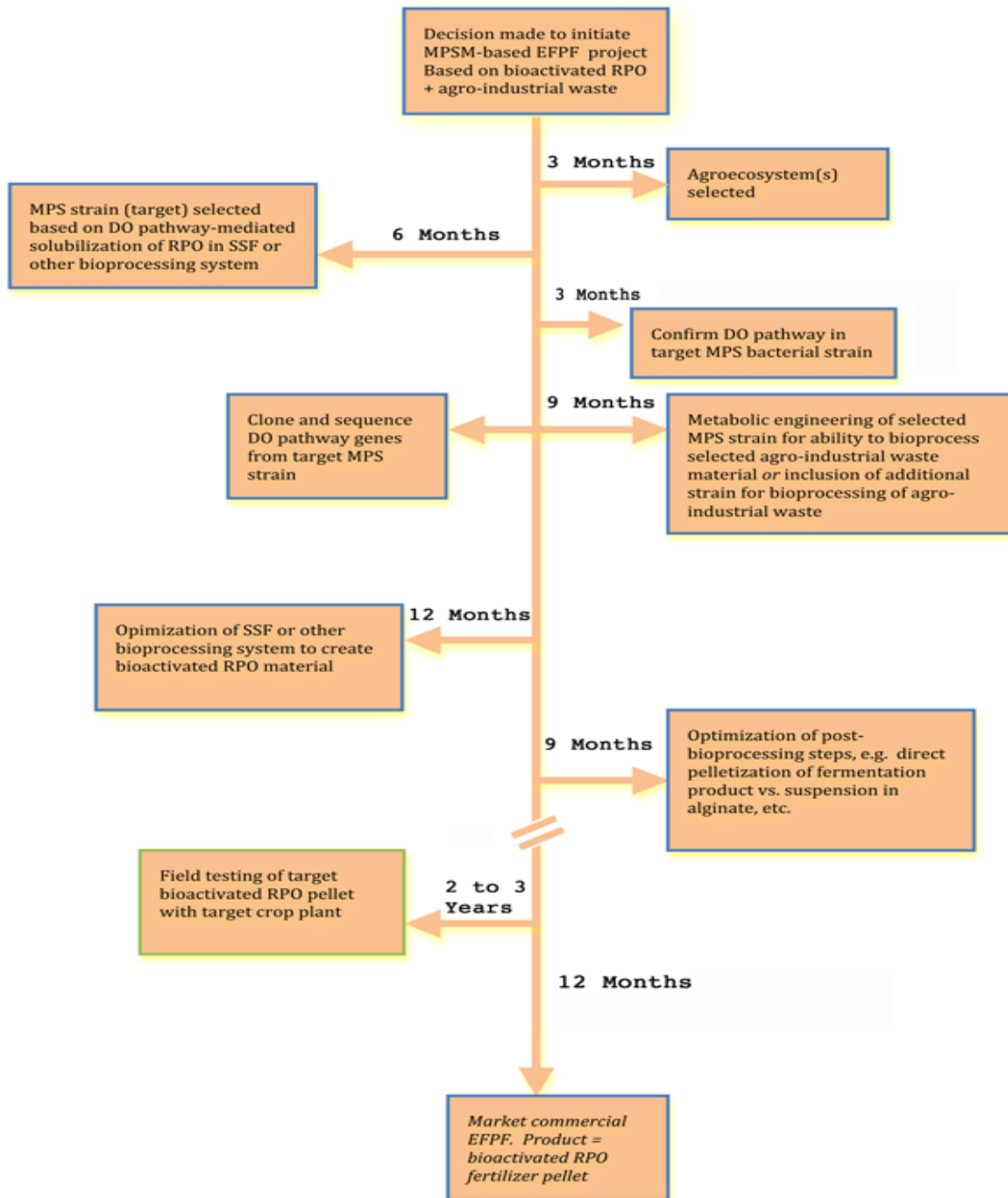


Figure 27. Timeline/milestones for the development of bioactivated RPO fertilizers based on industrial bioprocessing strains of Gram-negative MPS soil bacteria.

Time to milestone is shown “upstream” (i.e., three months from “Decision made to initiate project” to milestone of “Agroecosystems selected”). The development timeline is designed for a single EFPF agent in a single agroecosystem.

6 Acknowledgements

We would like to thank Susan Yippan for editorial assistance.

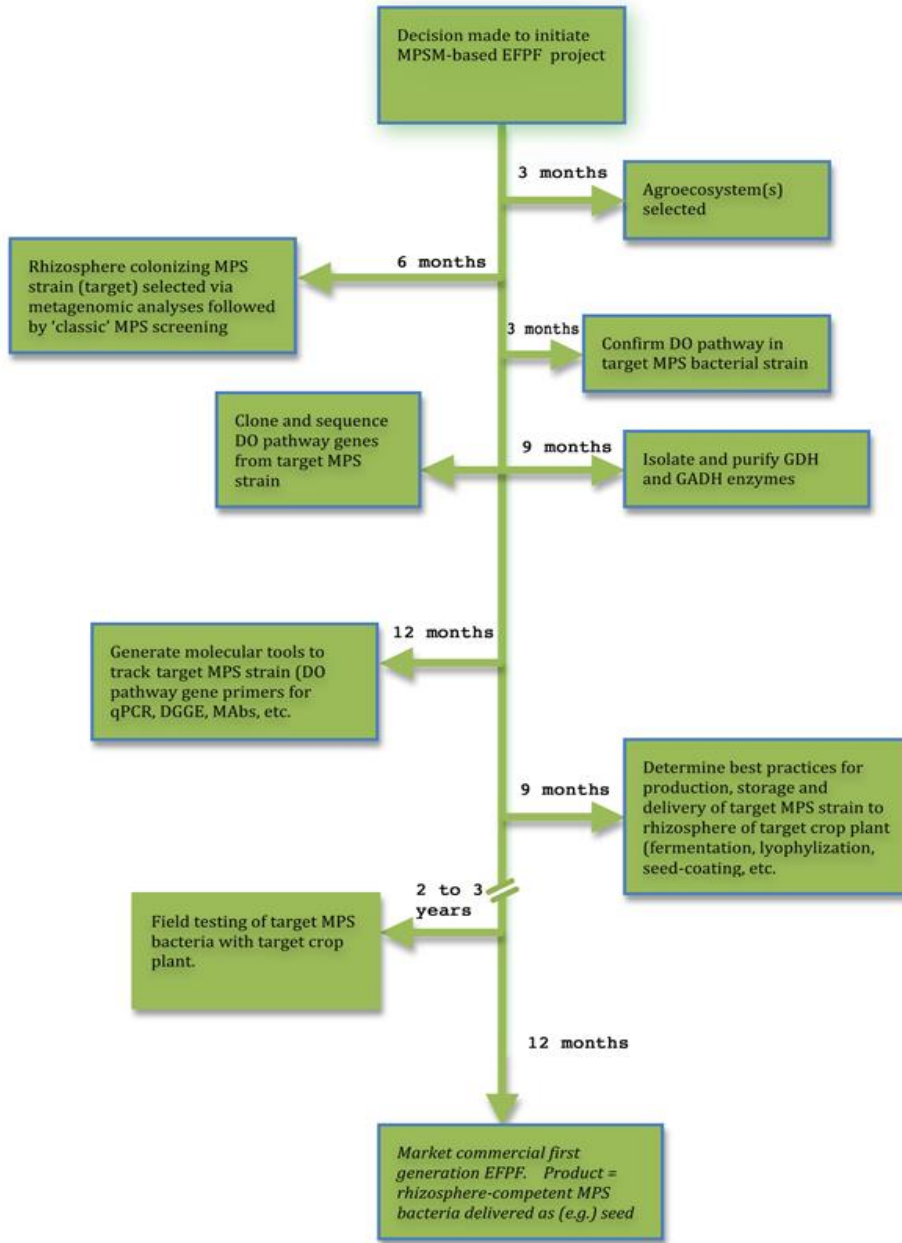
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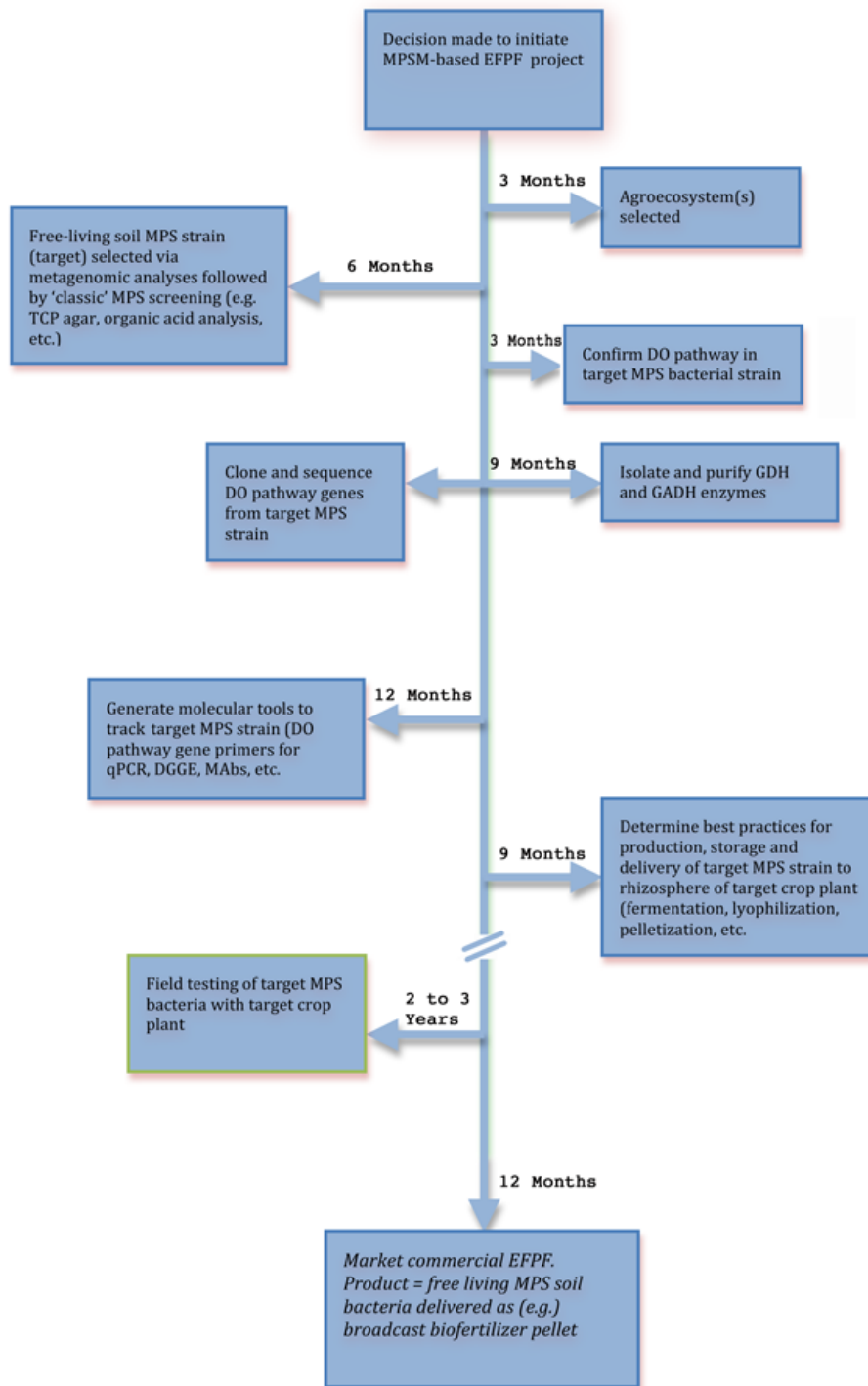
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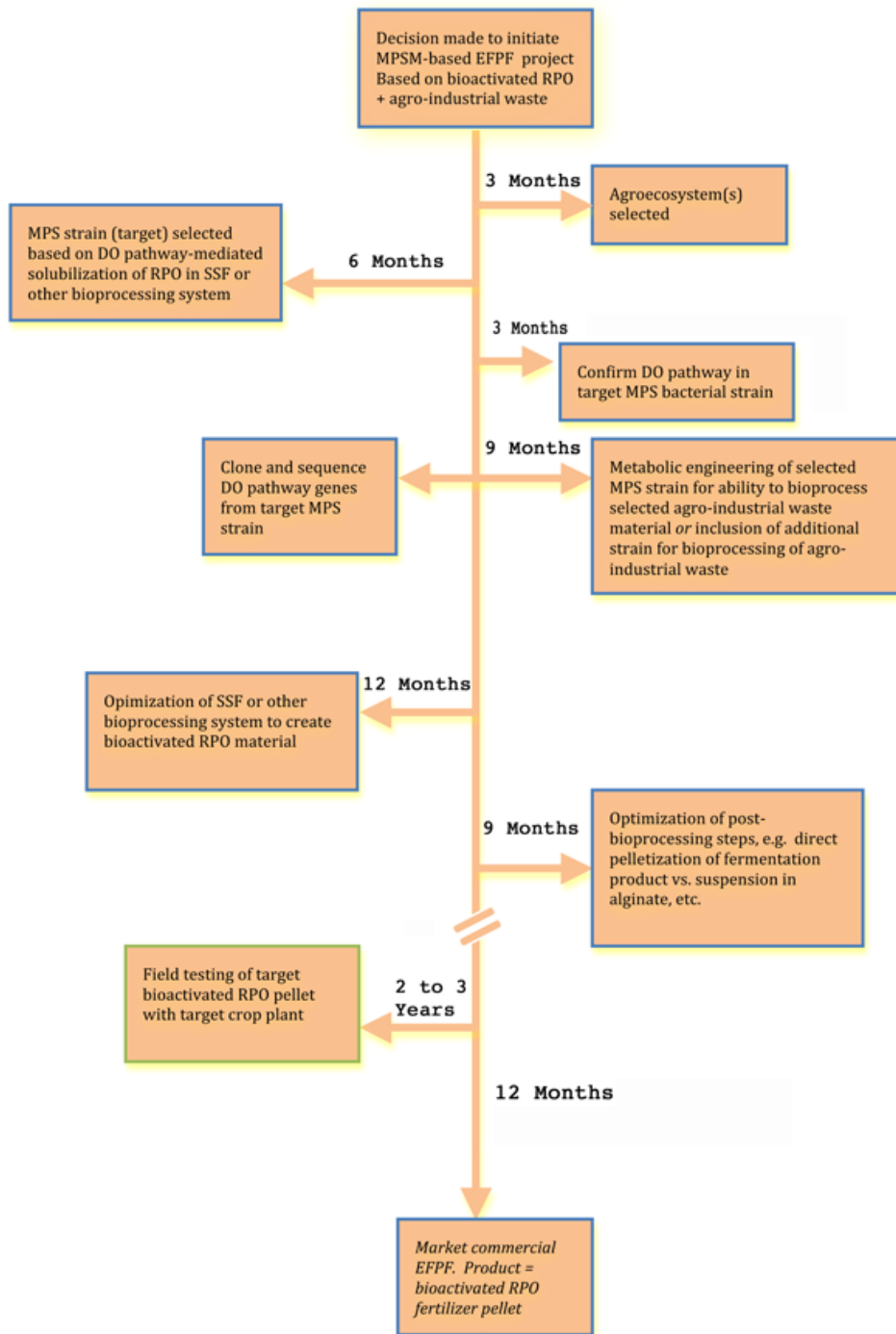
Appendix. Projected development timelines (larger versions)



Timeline 1



Timeline 2



Timeline 3

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Virtual Fertilizer Research Center
1331 H Street, NW
11th Floor
Washington, D.C. 20005
USA
E-mail: contact@vfrc.org

