Chapter 1

Introduction and Review of Literature

1.1 Agriculture and Global demand for food

The world's population is expected to grow to almost 10 billion by 2050, boosting agricultural demand for food, fiber and livestock feeding (FAO UN, 2017). Demand for food and other agricultural products is predicted to increase by 50 percent between 2012 and 2050. The increase in the world's population is expected to be concentrated in Africa and South Asia and in the world's cities with two-thirds of the global population occupying urban areas by 2050. The population will continue to grow in South Asia until mid-century and in sub-Saharan Africa until the end of the 21st century making Asia and Africa together a home of 9 billion people out of the projected 11 billion people on Earth. India is holding a great importance as a global agricultural powerhouse. Currently, India is the world's second most populous country with a population of 1.33 billion. Worth $ 2.94 trillion, India is the world's third largest economy after the US and China. It is the seventh largest country in the world with an area of 3.288 million sq km and is a home to vast agro-ecological diversity. Agriculture, with its allied sectors, is the largest source of livelihoods in India. 70 percent of its rural households still depend primarily on agriculture for their livelihood, with 82 percent of farmers being small and marginal. The country has some 195 m ha under cultivation of which some 63 percent are rain-dependent while 37 % are irrigated (http://www.worldbank.org/en/news/feature/2012/05/17/india-agriculture-issues-rioritie). India is the world's largest producer of milk, pulses and jute, and the second largest producer of rice, wheat, sugarcane, groundnut, vegetables, fruit and cotton. It is also one of the leading producers of spices, fish, poultry, livestock and plantation crops (http://www.fao.org/india/fao-in-india/india-at-a-glance/en/). However, India is still struggling with hunger and malnutrition issues accounting for a quarter of the world's hungry people and a home to over 190 million undernourished people. As per the Global Hunger Index report (2018), 21 and 38.4 percent of children in India are wasting and stunting respectively. According to the Global nutrition report (2018) more than 51 percent of women of reproductive age suffer from anemia.

Globally increasing food demand will pose a stress on agriculture and the grand challenge will be to meet this demand with a sustainable and eco-friendly agricultural approach. Agricultural practices are still majorly dependent upon use of chemical fertilizers, pesticides, herbicides and fungicides, it imposes serious threats to the

environment in many ways. Chemical fertilizers are effective in the soil very initially, they start being trapped inside the soil and a very small proportion of applied fertilizers is used by plants (Liu et al., 2008). It also leads to greenhouse gas emission including carbon dioxide CO_2 and nitrous oxide N_2O and leaching of nitrate (NO_3^-) and phosphorus leaching into the groundwater resulting in groundwater contamination (Good and Beatty 2011; Savci 2012; Dubos et al., 2016; Erbas and Solakoglu 2017). Over usage of fertilizers alters soil properties and reduces its fertility. This can cause alteration in existing microflora in soil (Vitousek et al., 1997; Zhou et al., 2017). Overuse of pesticides, fungicides and herbicides can rapidly lead to evolved resistance and can have deleterious effects on natural beneficial agents which help in keeping notorious pathogens away (Sanchez-Bayo 2011; Ali et al., 2014). Circulation of these hazardous compounds in food chain and crop yields can lead to enormous harm to both ecosystem and human health (Reynolds et al., 2002; Aktar et al., 2009; Sanborn et al., 2007; Jurewicz and Hanke, 2008).

1.2 Biofertilizers – an alternative to Agrochemicals

Under natural conditions, successful plant development and crop yield depend on the type of crop species, the availability of nutrients, the presence of certain beneficial microorganisms and the absence of pathogenic one in the surrounding environment. With the realization of adverse effect of conventional agricultural practices, there is an increasing trend of sustainable and environment friendly approaches in global agriculture. One of these approaches is use of plant growth promoting microorganisms as biofertilizers. Biofertilizers are cost effective; naturally increase soil fertility without causing environmental pollution and health hazards. They include variety of bacteria, arbuscular mycorrhizae and ectomycorrhizae that help to promote the plant growth through various direct and indirect mechanisms (Kloepper and Schroth, 1981; Ryan et al., 2009; Philippot et al., 2013; Mus et al., 2016; Ahkami et al., 2017; Stamenkovic et al., 2018; Adeleke et al., 2021).

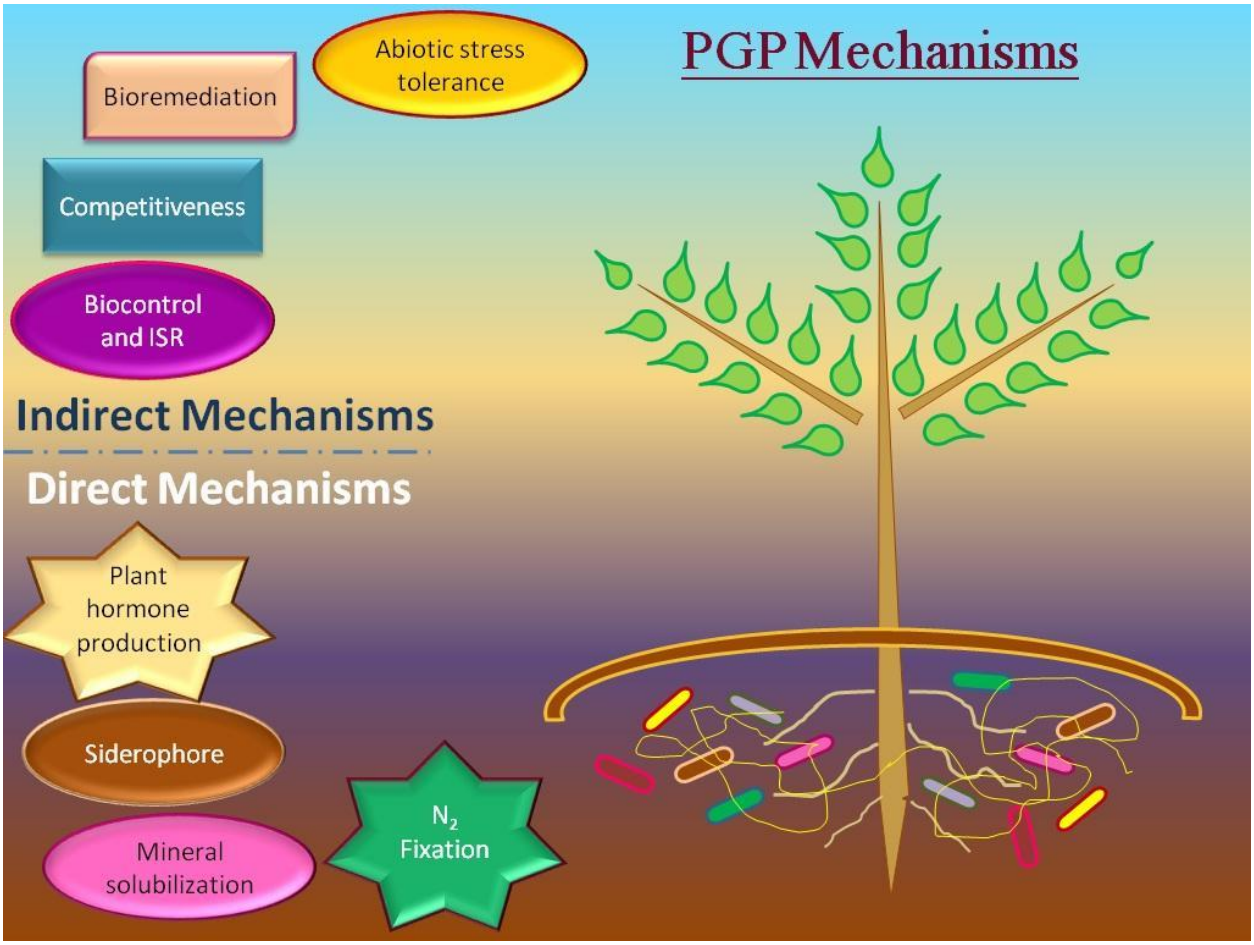

Fig. 1.1: Plant growth promotion mechanisms by Plant Growth Promoting Microorganisms (PGPMs)

1.2.1 Direct Mechanisms

Direct mechanisms involve increased availability of nutrients to plants by nitrogen fixation, mineral phosphate and potassium solubilization along with other minerals, production of siderophores and phytostimulation by production of plant growth hormones (Velivelli et al., 2015; Stemenkovic et al., 2018).

1.2.1.1 Nitrogen fixation

Nitrogen is the most important element for plant growth. In spite of its abundant presence in the atmosphere in the form of N_2 gas, its availability to plants is very limiting because plants can utilize nitrogen only in its reduced form. Atmospheric nitrogen enters soil by lightening and biological nitrogen fixation. Biological nitrogen fixation is

predominantly carried out by *Rhizobium* in a specialized nitrogen fixing apparatus called root nodules by symbiotic association with the host plants. *Frankia* is a genus belonging to actinomycetes that is able to fix nitrogen under both symbiotic and free-living conditions. Some prokaryotes such as *Azospirillum*, *Azotobacter*, *Herbaspirillum*, *Azoarcus*, *Acetobacter* form close association with plants and contribute to nitrogen fixation. In addition to this, *Burkholderia*, *Pseudomonas*, *Bacillus*, *Paenibacillus*, *Citrobacter*, *Enterobacter*, *Klebsiella* represent the group of free living nitrogen fixers (Benson and Silvester, 1993; Steenhoudt and Vanderleyden, 2000; Oldroyd and Downie 2008; Ikeda et al., 2013; Santi et al., 2013; Grady et al., 2016).

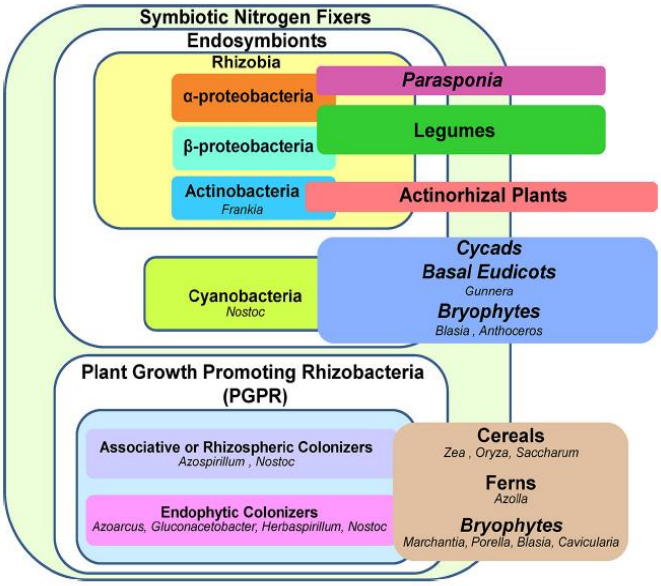

Fig. 1.2: **Schematic representation of the different associations between diazotrophs and plant hosts. Diazotrophs are divided in two main groups: root-nodule bacteria and plant growth-promoting rhizobacteria (PGPR). Root-nodule bacteria include rhizobia and *Frankia*. Rhizobia (alpha- and betaproteobacteria) enter into a symbiotic association with legumes and *Frankia* with actinorhizal plants. Alphaproteobacteria can also nodulate *Parasponia* species. Some plants develop endosymbiotic interactions with nitrogen-fixing cyanobacteria (Nostoc). PGPRs include proteobacteria (alpha-, beta-, and gammaproteobacteria), actinobacteria, bacilli, and cyanobacteria. Many PGPRs develop associative or endophytic associations with cereals. Some cyanobacteria found within plant tissues are classified as endophytes (Mus et al., 2016).**

1.2.1.2 Mineral solubilization/mobilization

Production and secretion of organic acid in the rhizosphere is central to the ability of variety of PGPMs that improve the crop yields. Acidification of soil by organic acids induce/cause the release of phosphorous, potassium, zinc and other minerals from their respective complexes. The focus of this thesis is mineral phosphorous and potassium solubilization.

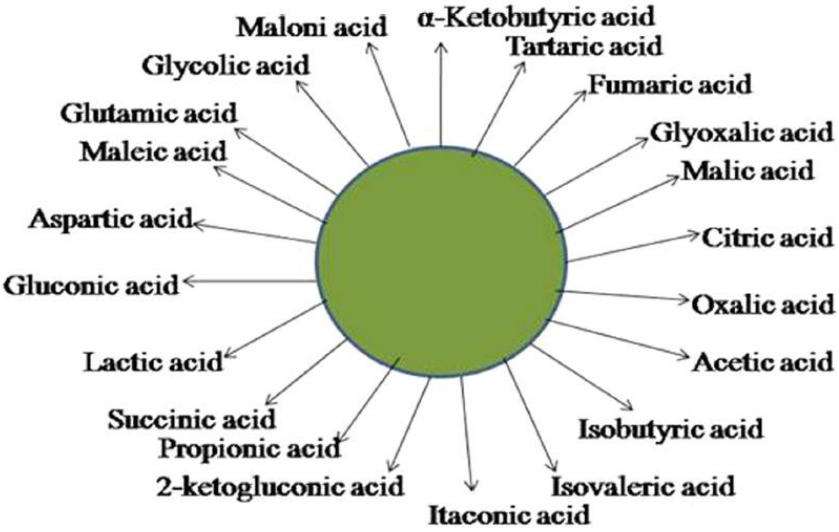

Fig. 1.3: Schematic representation of the organic acids that may be produced by PSM and used to solubilize inorganic forms of phosphate (Alori et al., 2017)

1.2.1.2.1 Phosphate Solubilizing Microorganisms (PSMs).

Content of phosphorus in soil ranges between 300 to 2000 mg kg^{-1} depending on weathering of rock, soil type, environmental factors and fertilizers input (Frossard et al., 1995; Kucey et al., 1989). Soil phosphorus can be present in two forms, organic phosphorous (Po) and inorganic phosphorous (Pi). Pi usually accounts for 35% to 70% of total P in soil while Po accounts for 30% to 65% in mineral soils and up to 90% in organic soils (Owen et al., 2015). Plants can take up phosphorous only in the form of orthophosphates ($H_2PO_4^-$ and HPO_4^{2-}) present in the soil solution. Pi is relatively less mobile due to higher frequency of fixation with soil particles. Pi reacts with Ca^{+2} and Mg^{+2} in alkaline soils and solubility of these complexes depends on the soil pH and concentration of these ions (Richardson, 2001). In acidic soils, Pi is adsorbed on the clay surface by

reaction with Al and Fe oxides. With further reactions Pi may be occluded in the void spaces of aluminosilicates and becomes unavailable to plants (Stevenson and Cole, 1999; Arai and Sparks, 2007). Release of Pi from various Ca^{+2} complexes increases with the decrease in the soil pH therefore local acidification mediated by microorganisms in rhizosphere is a good strategy for increasing the plant available phosphorus. Low molecular weight organic acid ligands help to chelate Al^{+3} and Fe^{+3} in acidic soils which prevent adsorption of free Pi onto these mineral surfaces. Po is present in the form of phytates, phospholipids and nucleotide phosphates, organic polyphosphates in the soil. Mobilization of phosphorus from these compounds is assisted by combined activities of phosphate hydrolyzing enzymes such as alkaline phosphatases, acidic phosphatases and phytases secreted by microorganisms and plants roots (Owen et al., 2015).

Phosphorous is provided in the form of chemical fertilizers derived from the chemical process of acidification of rock phosphates. These rock phosphate reserves are very limited and are unevenly distributed across the globe with 70% of deposits located in China, Russia, Morocco and the US (Parnell et al., 2016). Use of phosphate solubilizing microorganisms has provided a solution to make these unavailable forms of phosphorous to plant usable forms which can also lead to efficient use of applied chemical fertilizers.

Genetic and biochemical characterization of phosphate solubilization traits of PSMs revealed production and secretion of organic acid as a principle mechanism responsible for this trait (Alori et al., 2017). Exudation of organic acids from plant roots has also been observed as a mechanism to cope with either phosphate starvation or heavy metal toxicity (Wenzl et al., 2002; Almeida et al., 2020). Previous studies demonstrated the phosphate solubilization efficacy of different organic acids in vertisols and alfisols (Gyaneshwar et al., 1998; Srivastava et al., 2006). Acidification of soil by organic acids depends on both the nature and quantity of the organic acid for e.g. acetic, lactic and succinic at 100 mM bring about a drop in pH of a soil solution from around 9.0 to about 6.0; a similar drop is brought about by only 20 mM of gluconic acid and lesser amount of oxalic, citric and tartaric acids. Oxalic acid and citric acid were the most efficient in solubilizing inorganic phosphate in both types of soils. 5 mM of oxalic acid was sufficient to release 217–440 μM phosphorous when 10-30 mg rock phosphate was added per gram of alfisol and 10 mM oxalic acid could release 120 μM phosphorous from 2mg rock

phosphate amended alfisol. *Penicillium billai* secretes 10 mM each of citric and oxalic acids (Cunningham and Kuiack, 1992) and has been shown to be effective in releasing P in the field conditions (Asea et al., 1988). On the other hand, *C. koseri* and *B. coagulans* were found to secrete various organic acids in the range 1-5 mM whereas as the concentration of these acids required to reduce the pH of the soil was 20-50 times more (Gyaneshwar et al., 1998). Goldstein (1996) proposed direct glucose oxidation to gluconic acid (GA) as a major mechanism for mineral phosphate solubilization (MPS) in Gram-negative bacteria. GA biosynthesis is carried out by the periplasmic glucose dehydrogenase (GDH) enzyme and the co-factor, pyrroloquinoline quinone (PQQ). This gluconic acid is then converted into 2-keto gluconate via another membrane bound enzyme gluconate dehydrogenase (GADH). It has been established that the organism producing 2-keto-gluconic acid in combination with succinic acid or citric acid are very efficient in solubilizing insoluble inorganic phosphate (Banik and Dey 1981). Yadav et al., (2015) reported efficient phosphate solubilization by *P. fluorescence* 13525 harboring artificial oxalate operon along with *Vitreoscilla* hemoglobin gene that increased P content in *V. radiata* (L.) grown in alfisol soil.

Application of PSMs in the field has resulted in improved crop yield (Pradhan and Sukla, 2005; Khan et al., 2007). After *Rhizobium*, strains of *Pseudomonas* and *Bacillus* are widely used as biofertilizers for their contribution as efficient phosphate solubilizing bacteria (PSBs) and as biocontrol agents (BCAs) (Shafi et al., 2017; Radhakrishnan et al., 2017; David et al., 2018 Santoyo et al., 2012).

1.2.1.2.2 Potassium solubilizing Microorganisms

Potash denotes a variety of mined and manufactured salts, which contain the element potassium in water-soluble form. In agriculture, the term potash refers to potassic fertilizers, which are potassium chloride (KCl), potassium sulfate or sulfate of potash (SOP), and potassium magnesium sulfate (SOPM) or langbeinite. Muriate of potash (MOP) is an agriculturally acceptable mix of KCl (95% pure or greater) and sodium chloride for fertilizer use (U.S. Geological Survey, Mineral Commodity Summaries, January 2018).

Canada is the largest producer of potash fertilizer with the production of 12 million MT in 2017. Russia holds the second position followed by Belarus, China, Germany, Israel,

Jordan, Chile, Spain and United States. World potash demand for all uses was projected to increase from 42.0 million tons in 2017 to 45.6 million tons in 2021, with the largest consumption in Asia and South America.

Potassium (K) exists mainly in four forms in soils: solution K, exchangeable K, non-exchangeable K and mineral K. The order of the forms of K according to their availability to plants and microorganisms is solution >exchangeable >non-exchangeable >mineral (Sparks and Huang 1985; Sparks 1987). Solution K is directly available to plants and it varies from 2 to 5 mg K L^{-1} in soil. Exchangeable K is electrostatically bound to the surfaces of clay minerals. Non-exchangeable K accounts for 1 - 10% of the total potassium content. It is not bonded in the crystal structures of K-bearing minerals but it is entrapped between the adjacent layers of tetrahedral structure of mica, feldspar or biotite. Release of potassium from its non-exchangeable form to exchangeable form takes place when concentration of solution and exchangeable K is depleted by crop removal, surface run-off, weathering/leaching or microbial activity. Mineral K constitutes 90-98% of the total soil potassium. Mineral K is very slowly available to plants and its conversion to other forms mainly depends on the weathering process. Secretion of organic acid by potassium solubilizing bacteria (KSB) dissolve K^+ ions from potassium bearing minerals such as mica, feldspar, illite either directly from the surfaces or by chelating Si^{+4} and Al^{+3} thereby releasing K^+ entrapped in the void spaces of mineral structure (Parmar and Sindhu 2013; Setiawati and Mutmainnah, 2016). Exopolysaccharide secreted by several *Bacillus* spp. facilitated organic acid mediated dissolution of potassium from mica and feldspar mainly by providing microenvironment for bacterium-mineral interactions (Malinovskaya et al., 1990; Liu et al., 2006; Binbin and Bin 2011; Anjanadevi et al., 2015).

1.2.1.2.3 Zinc solubilizing Microorganisms

Zinc is an essential micronutrient for crops as it serves as a co-factor for various enzymatic reactions. In addition to this, Zn plays a vital role in the development of flowering, fertilization, fruiting and production of plant growth hormones. Plants can uptake zinc as divalent cation (Kabata-Pendias and Pendias, 2001) but very less amount of total zinc is present as soluble form in soil solution. Rest of the zinc is precipitated or adsorbed in the form of insoluble complexes and minerals (Alloway, 2008). Due to

unavailability of zinc in soil, zinc deficiency occurs which in plants leads to retarded shoot growth, chlorosis, reduced leaf size (Alloway, 2004), susceptibility to heat, light and fungal infections, affects grain yield, pollen formation, root development, water uptake and transport (Tavallali et al., 2010). Zinc is provided in the form of inorganic fertilizers but it becomes unavailable within seven days of application (Rattan and Shukla 1991; Saravanan et al., 2004). Zinc solubilizing microorganisms utilize various mechanisms such as acid secretion, production of siderophore and protons, oxido-reductive complexes on cell membrane and production of metal chelating ligands for soIubilization of zinc (Kamran et al., 2017). Inoculation of Zinc solubilizing bacteria (ZSB) have resulted in enhanced zinc fortification and plant growth. These include *Rhizobium, Bacillus* and *Pseudomonas* strains.

1.2.1.3 Iron acquisition

Iron is the fourth most common element of earth's crust. It is an essential micronutrient for all life forms, as it is involved in various biological processes such as photosynthesis, chlorophyll synthesis, hemoglobin synthesis, respiration, biological nitrogen fixation etc., (Dixon and Kahn, 2004; Kobayashi and Nishizawa 2012; Saha et al., 2015). In spite of its abundance in nature, it is sparingly available to all life forms because it occurs predominantly as Fe^{+3} and forms insoluble complexes such as ferric oxyhydroxide (Andrews et al., 2003; Gamalero and Glick 2011; Saha et al., 2013). PGPB devise a mechanism to cope with iron limitation by production of siderophore. Siderophore are low molecular weight, high affinity, iron-chelating compounds. Bacteria secrete siderophores to sequester Fe^{+3} and the Fe^{+3}-siderophore complex is taken up by iron-limitation dependent receptors present on the bacterial membrane (Lugtenberg and Kamilova, 2009). There are four types of microbial siderophores according to their chemical structures: Hydroxamate, catecholate, carboxymate and mixed type (Ahmed and Holstrom, 2014). Plants can utilize microbial Fe-siderophore complexes for iron uptake. Two possible mechanisms were suggested by which plants could obtain Fe from microbial siderophores: (1) Microbial siderophores with high redox potential can be reduced to donate Fe^{+2} to the transport system of the plant. In this mechanism, it has been hypothesized that the microbial Fe^{+3}–siderophore complexes are transported to the apoplast of the plant root where

siderophore reduction may occur (Mengel, 1995). Consequently, Fe^{+2} is trapped in the apoplast, which leads to high Fe concentrations in the root (Kosegarten et al., 1999). (2) Microbial siderophores can chelate Fe from soils and then do a ligand exchange with phytosiderophores (Masalha et al., 2000). Certain plants from Graminaceae family also secrete phytosiderophore such as mugineic acid that can bind Fe^{+3} which is taken up by roots (Bulgarelli et al. 2013). Apart from being an iron carrier, siderophores play and important role of mediator of different interactions among microorganisms in a particular niche (Kramer et al., 2020).

1.2.1.4 Phytohormone production

Phytohormones are the chemical compounds which are required for normal development of plants throughout their lifecycle. The major phytohormones are auxins, cytokinins, gibberellins (GAs), abscisic acid (ABA), ethylene (ET), brassinosteroids (BRs), jasmonic acid (JA), salicylic acid (SA) and strigolactones (SLs) (Glick, 2020). Auxin induces root development which is necessary for water and nutrient uptake. Cytokinin stimulates shoot differentiation and Gibberellins are involved in cellular differentiation and elongation. Abscisic acid helps in seed germination, stomatal movement and environmental stress tolerance. Ethylene is a gaseous plant hormone necessary for flowering, fruit ripening and seed germination. Majority of PGPMs are able to produce plant hormones that stimulate plant growth and the phenomenon is called phytostimulation. 90 % of PGPMs are able to produce auxins and cytokinins (Velivelli 2015; Stamenkovic et al., 2018). Inoculation of *Pseudomonas fluorescens* WCS365 *Burkholderia phytofirmans* PsJN and *Bacillus amyloliquefaciens* FZB42 resulted in the growth promotion in radish, maize and duckweed (Kamilova et al. 2006; Idris et al., 2007; Naveed et al.2014). *Bacillus* spp., *Pseudomonas* spp., *Paenibacillus* spp., *Azospirillum* spp. and *Bradyrhizobium* spp. are known to produce cytokinins (Arkhipova et al. 2007; Perrig et al., 2007; Cassán et al., 2009). Similarly, secretion of other growth hormone by PGPBs is known to promote plant growth. Ethylene is produced in response to several biotic and abiotic stresses but excess amounts of ethylene inhibits root development. Some rhizobacteria produce ACC-deaminase enzyme that cleaves 1-aminocyclopropane-1-carboxylate (ACC) – ethylene precursor and helps to overcome ethylene mediated negative effects (Glick 2012).

1.2.2 Indirect mechanisms

Indirect mechanisms include control of plant pathogens by production of variety of antimicrobial compounds, hydrolytic enzymes, induction of systemic resistance in host plants, increased tolerance to abiotic stresses, bioremediation and competition for nutrient resources and niches (Barea et al., 2005; Glick 2012; Vejan et al., 2016; Stamenkovic et al., 2018).

1.2.2.1 Competition

Colonization of rhizospheric zone and various plant surfaces by plant benefitting non-pathogenic microorganisms poses competition to notorious plant pathogens for all the available resources such as nutrients and space (Glick 2012). *Bacillus amyloliquefaciens* SQR9, is reported to protect cucumber plant from infection by *Fusarium oxysporum f.sp. cucumerinum* J. H. Owen (FOC) through competition for nutrients and space (Liu et al., 2014).

1.2.2.2 Biocontrol

Plant growth promoting bacteria can promote plant growth by inhibiting phytopathogens. Variety of PGPBs are known to secrete antimicrobial compounds having antibacterial and antifungal activities. Two of the most common biocontrol agents are members of the *Bacillus* and *Pseudomonas* genera. Both bacterial genera have important traits such as plant growth-promoting (PGP) properties. *Pseudomonas* strains produce wide array of compounds such as antibiotics, siderophores and hydrogen cyanide which are mainly responsible for their biocontrol activity. On the other hand, biocontrol activity of *Bacillus* strains is attributed to their production of fungal cell wall degrading enzymes and lipopeptides. Analysis of whole genome of several *Bacillus* spp. and *Pseudomonas* spp. has revealed that a considerable part of their genome is dedicated for antimicrobial compound production. For example, more than 9% of the genome is devoted to synthesize antimicrobial metabolites in *Bacillus amyloliquefaciens* FZB42 (Chen et al., 2009a; Borriss, 2013), while 6% of the *Pseudomonas fluorescens* Pf-5 genome is devoted to the production of secondary metabolites through nine gene clusters (Loper et al., 2007). PGPR

members of the genus *Bacillus* have the effective formulation for commercialization due to their ability to form heat and desiccation-resistant spores (Emmert and Handelsman 1999; Kloepper et al., 2004). On the other hand, *Pseudomonas* may be preferred for the synthesis of siderophores and non-peptide antibiotics. It is also interesting to note that these bacterial genera, which have been used as biocontrol agents in different studies, have shown very few non-target effects on plant beneficial mycorrhizal fungi during pathogen suppression (Winding et al., 2004), compared with agrochemicals, which pollute the environment and usually generate resistance in pathogens.

1.2.2.3 Induced Systemic Resistance

Induced systemic resistance (ISR) is physiological state of improved defensive capacity elicited in response to a particular environmental stimulus. ISR is nonspecific for pathogens but it primes plants' defense system for subsequent pathogen encounter. PGPR induce systemic resistance in many plants against pathogens (Prathap and Ranjitha, 2015). ISR in plants operates through ethylene and jasmonate dependent pathways (Choudhary et al., 2007). It does not require any direct interaction between the resistance-inducing PGPB and the pathogen (Bakker, 2007). A variety of individual bacterial components induce ISR, such as lipopolysaccharides, cyclic lipopeptides, siderophores, 2, 4-diacetylphloroglucinol, homoserine lactones, and volatiles, like 2, 3-butanediol and acetoin (Berendsen et al., 2015). Various strains from both the genera *Pseudomonas* and *Bacillus* are known to elicit ISR in different plants (Santoyo et al., 2012).

1.2.2.4 Abiotic Stress Tolerance

Abiotic stresses such as drought, salinity, floods and high temperatures are the major factors that negatively affect agricultural production. Drought stress negatively influences nutrient diffusion and availability to plants (Barber, 1995; Selvakumar et al., 2012). It also leads to generation of Reactive Oxygen Species (ROS) such as superoxide radicals, hydrogen peroxide and hydroxyl radicals resulting in oxidative stress (Vurukonda et al., 2016). Salt stress has multiple negative effects on plant physiology such as osmotic imbalance, iron toxicity and oxidative stress (Liang et al., 2018). Flooding creates hypoxic condition in the vicinity of roots. Ethylene is a stress regulator hormone as it is produced

in response to several biotic and abiotic stresses but excess amount of ethylene inhibits root development. Some rhizobacteria produce ACC-deaminase enzyme that cleaves 1-aminocyclopropane-1-carboxylate (ACC) – ethylene precursor and helps to overcome ethylene mediated negative effects (Glick, 2014). PGPR mediated drought resistance has been reported in several crops, including soybean, chickpea, and wheat (Ngumbi and Kloepper, 2016). Secretion of osmoprotectants such as proline and trehalose by PGPRs helps in increased tolerance to drought and salinity. Moreover, inoculation of PGPRs is known to induce synthesis and accumulation of different stress metabolites such as proline, glycine betain, ascorbic acid and enzymatic and non-enzymatic antioxidants in the host plants that collectively strengthens the idea of using PGPRs to alleviate abiotic stress in plants.

1.2.2.5 Bioremediation

Presence of organic pollutants and heavy metal contamination in soil has deleterious effects on agro-ecosystem. Organic pollutants such as petroleum hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated terphenyls (PCTs), halogenated compounds like perchloroethylene (PCE), trichloroethylene (TCE), pesticides and herbicides persist for longer periods in the environment and pose a great risk to all life-forms. Heavy metals such as cadmium, chromium, lead, mercury, nickel, arsenic etc alters plant physiology by generation of ROS (Mithofer et al., 2004; Srivastava et al., 2004; Zhuang et al., 2007). Bioremediation is a technique which uses living microorganisms to detoxify such contaminants by converting them to simpler and less-toxic forms (Gouda et al., 2018). Bioremediation techniques can be categorized in two types; ex situ and in situ. Ex situ technique involves translocation of contaminated material and treated elsewhere while in situ technique bioremediation takes place at the polluted site itself. Various bioremediation techniques are available, including bio-pile, landfarming, phytoremediation, bio-slurry, and bioventing, and all of them can be used to degrade pollutants at contaminated sites. Combined use of plants and their rhizospheric microbial community to degrade environmental pollutants is called rhizoremediation (Kuiper et al., 2004). *Bacillus* and *Pseudomonas* spp. are predominantly

used for rhizoremediation. Several plants and PGPRs have been genetically engineered for enhanced bioremediation.

There are a myriad of products containing bacterial bioinoculants either as single strains or consortia in the market. However, their field efficacy varies with different agro-climatic zones, crop species and existing microbial communities (Owen et al., 2015). It is advisable to utilize the members of indigenous micobiome and to improve their plant beneficial traits rather than applying bioinoculants actually belonging to different soil-plant and climate type (Geddes et al., 2015; Finkel et al., 2017; Manfredini et al., 2021).

1.3 Rhizosphere Engineering

The concept of rhizospheric engineering has emerged as a promising approach to exploit these naturally occurring plant-microbe interactions towards enhanced beneficial effects on plants (Ryan et al., 2009; Quiza et al., 2015; Dessaux et al., 2016; Ahkami et al., 2017; Hakim et al., 2021). After long standing research, rapid advancement in genetic engineering tools, availability of a wide array of genomic data and better understanding of mechanisms underlying plant growth promotion by microbes have opened up new horizons in the field of sustainable agriculture. Since plants are considered as holobionts comprising plants and associated microbiota, rhizosphere engineering constitutes engineering of both the partners - plants and associated rhizospheric microbial community (Fig.1.4).

Plants can be engineered to alter their root exudates composition in such a way to increase nutrient acquisition and proliferation of beneficial microbes, increased release of several organic acid can result in improved availability of phosphorous and iron and increased tolerance to Al^{+3} toxicity. Another successful plant engineering approach includes tolerance to a variety of plant pathogens. Overexpression of *Trichoderma harzianum* endochitinase in tobacco and potato led to increased tolerance to fungal pathogens such as *Alternaria alternata*, *A. solani*, *Botrytis cinerea* or *Rhizoctonia solani*. Genetic modification of plants for modification of plant cell wall components such as pectin and polysaccharide also resulted in increased resistance to fungal pathogens.

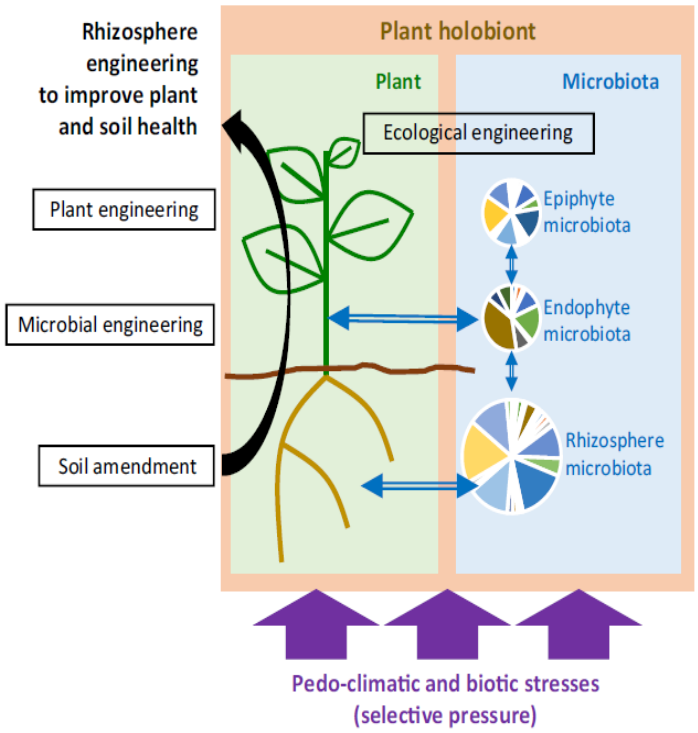

Fig. 1.4: Rhizosphere Engineering Permits Improvement of Plant and Soil Health. Plants are stressed (violet arrows) by pedo-climatic (e.g., high salt or metal concentrations as a result of drought or deforestation) and biotic agents (pathogens). Modification of soil para- meters (soil amendments), as well as microbial or plant engineering, are strate- gies developed to engineer the rhizo- sphere (blue arrows). Recent approaches involve microbial population (soil, plant, rhi- zosphere, and endophyte microbiota) engineering rather than single strain engineering, and engineering of the interactions (ecological engineering) between the plant and its associated microbiota (blue arrows). Both the plant (green box) and the associated microbiota (blue box) are now considered as a superorganism, a holobiont (beige box), that is the unit on which the selective pressure applies (Dessaux et al., 2016).

Currently increasing efforts are being made in direction of transferring nitrogen fixation process in cereals by engineering perception of rhizobia and subsequent nodule formation by cereals, engineering expression of nitrogenase in organelles of plants. However, Plant genetic engineering is a cumbersome task requiring great deal of efforts to achieve a successful and consistent outcome. Complex metabolic regulation, post-transcriptional and post-translational modification and some unanticipated pleiotropic

effects of plant genes all need to be considered. Although plant engineering has provided some solutions to agricultural demand but there is a major concern regarding their social acceptance by the consumers and environmentalists due to risks to food security, the environment and human health such as loss of biodiversity; the emergence of superweeds and superpests; the increase of antibiotic resistance, food allergies and other unintended effects (Maghari and Ardekani, 2011; Lucht, 2015; Cui and Shoemaker, 2018). On the other hand engineering the microbial partners seems relatively easy. Single strain and population engineering both can be employed to selectively enhance plant growth promotion by microbes. Introduction of different microbial inoculants each having different PGP activity as a consortium has resulted in the synergistic outcomes (Akhtar and Siddiqui, 2008; Figueiredo, 208; Dutta and Thakur, 2017). Incorporation of multiple PGP traits in single rhizospheric competent strains without altering their normal functioning could result in better performance as compared to wildtype counterpart. Heterologous expression of gene encoding siderophore receptor into a *Pseudomonas fluorescens* strain was carried out to increase its rhizospheric competence (Raaijmakers et al. 1995). Incorporation of genes encoding either a proline dehydrogenase or ACC deaminase in *Sinorhizobium meliloti* increased its ability to nodulate legumes (Dillewijn et al., 2001; Ma et al., 2004). Recently, a chitinase gene from *Bacillus subtilis*, introduced into the *Burkholderia vietnamiensis* PGPR strain P418, led to a significantly enhanced suppression of wheat sheath blight, cotton *Fusarium* wilt, and tomato grey mold (Zhang et al., 2012). *Ensifer medicae* strain was genetically modified to tolerate higher copper concentrations to improve the nodulation in *Medicago truncatula* in copper contaminated soil (Delgadillo et al., 2015). *Bacillus subtilis* 168 was genetically engineered to express the arsenite S-adenosylmethionine methyltransferase gene from the thermophilic alga *Cyanidioschyzon merolae*. The transformants converted most of the inorganic As in the medium into dimethylarsenate and trimethylarsine oxide within 48 h and volatilized substantial amounts of dimethylarsine and trimethylarsine (Huang et al., 2015). Successful transfer of the nitrogen fixation island from *P. stutzeri* to *P. protogens* Pf5 showed significant growth enhancement effects for plants grown in nitrogen-limited conditions (Setten et al., 2013). Use of synthetic biology either to enhance nitrogen fixation capacity of existing endosymbiont and associative nitrogen fixers or transferring N_2-fixation gene cluster to

non-nitrogen fixers is of renewed interest to many microbiologists working in the field (Temme et al., 2012; Wang et al., 2013a; Wang et al., 2013b).

1.4 *Bacillus* spp. as a versatile PGPR

Extensive research in the field of plant-microbe interaction and their plant growth promoting effects has identified diversified population of bacteria and fungi as promising tool for crop improvement (Tonelli et al., 2010; Radhakrishnan et al., 2014). Some of the bacteria belonging to the *Rhizobium, Acetobacter, Azospirillum, Azotobacter, Bacillus, Streptomyces, Burkholderia, Klebsiella, Pseudomonas*, and *Serratia* genera have been reported as plant growth-promoting bacteria (PGPB) (Glick, 1995; Jones et al., 2007; Nadeem et al., 2014). Among this *Rhizobium, Pseudomonas* and *Bacillus* spp. have been extensively studied. Rhizobia hold great importance because of their major contribution in biological nitrogen fixation while *Bacillus* and *Pseudomonas* are predominantly found in rhizosphere (Podile and Kishore, 2006; Kang et al., 2015a; Vandana et al, 2018), and some of the PGPB have been commercialized due to their survival within a diverse range of biotic and abiotic environments. However, *Bacillus* based biofertilizers possesses an advantage of increased shelf life and cell viability over *Pseudomonas* based products due to their ability to form heat and desiccation resistant spores and excellent capacity to secrete proteins at higher concentration (Van Dijl and Hecker 2013; Radhakrishnan et al., 2017) The first commercial bacterial fertilizer, Alinit, was developed from *Bacillus* spp. and resulted in a 40% increase in crop yield (Kilian et al., 2000). Other *Bacillus* spp.-based products, such as Kodiak (*Bacillus subtilis* GB03), Quantum-400 (*B. subtilis* GB03), Rhizovital (*Bacillus amyloliquefaciens* FZB42), Serenade (*B. subtilis* QST713), and YIB (*Bacillus* spp.), have been commercialized for improving crop production (Brannen and Kenney, 1997; Ngugi et al., 2005; Cawoy et al., 2011).

1.4.1 Metabolic engineering in *Bacillus* spp.

Microorganisms have been exploited by humans long before they were visualized under microscope. Ancient practices of making curd, wine and bread by fermentation are the best elucidations. Microbes serve as tiny cell factories for production of variety of products that are valuable for many purposes. Antibiotics, industrially important enzymes,

nutraceuticals, food additives, biodegradable polymers are few of them (Gupta, 2007; Gurung et al., 2013; Sun et al., 2015; Wang et al., 2016). In addition to industrial production, microorganisms serve as important tools for sustainable agriculture and human health (Bhardwaj et al., 2014; Markowiak and Slizewska, 2017). Metabolic engineering (ME) is a process involving knowledge of metabolism and genetic engineering approaches in order to maximize the desired output. It is a targeted and willful manipulation of metabolic pathways found in an organism in order to better understand physiological processes and utilize these pathways for product formation (Lessard, 1996). Microbial metabolic engineering is a multidisciplinary field implying integration of principles from microbiology, chemical transformations, computational biology, biochemistry, biotechnology and molecular biology.

Initially, ME tools were confined to classical random mutagenesis followed by screening of genetically stable mutants with efficient product formation but development of recombinant DNA technology allowed targeted pathway engineering by overexpression of native genes or by heterologous expression of foreign genes (Thykaer and Nielsen, 2003). Lots of new opportunities emerged for metabolic engineering in the post genomic era with the knowledge of whole genome sequences of some reference organisms (Alper and Stephanopoulos, 2004). Better understanding of metabolic pathways and genetic circuits governing their regulation facilitated precise identification of multiple targets that could be altered to achieve significant yield improvement. Integrated –omics approach by convergence of genomics, transcriptomics, proteomics, metabolomics, interactomics, fluxomics not only expanded the knowledge base for metabolic engineering but also changed the focus from modification of individual pathways to systematic programming of whole cell and gave rise to system biology approach for metabolic engineering. Since the advent of synthetic biology, high throughput, cost effective chemical synthesis of DNA enabled the construction of biobricks as well as artificial chromosomes (Vemuri and Aristidou, 2005; Yadav et al., 2012; Stephanopoulos, 2012; Fong, 2014; Nielsen and Keasling, 2016). Thus, combination of synthetic biology and system biology, optimization output prediction softwares and improvements in experimental and analytical techniques will contribute tremendous progress in this field.

Bacillus subtilis has achieved a unique importance as industrial work horse and it

has been used for production of a variety of useful products (Perkins et al., 2009; Cui et al., 2018). *B. subtilis* belongs to the generally regarded as safe (GRAS) category for protein production. Moreover, natural competence for DNA transformation, availability of numerous genetic tools, such as vectors and gene expression systems, makes it an easy-to-handle organism (Dong and Zhang 2014; Liu et al., 2017a). It has been considered as one of the most efficient bacterial chassis mainly due to efficient protein secretion system that enables the transport of proteins into the culture medium to reach concentrations in the range of grams per litre, thus reducing purification and recovery costs (van Dijl and Hecker, 2013). The best-studied protein secretory systems in *B. subtilis* are the Sec and Tat pathways (Hohmann et al., 2017). However, *B. subtilis* naturally produces a number of proteases and, although some of them might have an interest for the industry on their own, their activity usually limits the overall efficiency of heterologous protein production. Some protease defective mutants such as WB600 and WB800 have been constructed to overcome this problem (Wu et al., 1991; Wu et al., 2002). Several super-competent cells have been generated to facilitate standard competence protocol and to achieve higher transformation frequencies (Zhang and Zhang 2011; Konkol et al., 2013; Rahmer et al., 2015). Engineering of cell surface components to reduce electrostatic interaction between cell envelope and the target protein alpha-amylase increased secretion efficiency of host *B. subtilis* DB104 (Cao et al., 2017). Reduced genome version of *B. subtilis* MGB874 in which 20% of the genome was reduced by rationally designed deletions, has been shown to produce higher concentrations of alkaline cellulose and protease as heterologous proteins (Morimoto et al., 2008; Manabe et al., 2013). Dong and Zhang (2014) have described different types of genetic engineering techniques based on inducible promoters and operator-repressor system, multiple sequential deletions by counter selection markers, auxotrophy, site specific recombination and transconjugation. Availability of new tools for controlling metabolic pathways, systems-level research methodologies, and mathematical model based simulation strategies are becoming available to achieve the expected phenotypes and it also enlightens the missing gaps in information to understand the multiple factors affecting the particular behavior of cell under different conditions (Liu et al., 2017a). For example, introduction of glyoxylate shunt in *B. subtilis* by incorporation of aceA and aceB genes from *B. licheniformis*, improved acetate utilization and allow

robust cell growth on excess glucose (Kabisch et al., 2013). On the other hand, analysis of flux, transcripts and metabolites in *B. subtilis* grown under different metabolic states composed of eight combinations of carbon sources provided an insightful observation that transcriptional regulation, substrate concentration and enzyme concentration are sufficient to explain flux changes pinpointing the role of for allosteric regulation and enzyme modification in the control of metabolic fluxes (Chubukov et al., 2013).

1.4.2 Carbon metabolism and regulation in *Bacillus subtilis*

Bacillus subtilis is the most extensively studied Gram-positive bacterium. Like most of the bacteria, glucose is the preferred carbon source for *B. subtilis*. When *B. subtilis* is grown in a medium containing excess glucose, carbon overflow metabolism takes place. Glucose is taken up via phosphotransferase system (PTS) and it is primarily metabolized through glycolysis. Large amount of glucose is converted to pyruvate and Acetyl CoA which are then converted to fermentation products such as lactate, acetate and acetoin and secreted in the medium. Electrons and hydrogen atoms released by substrate oxidation are utilized by NAD^+ to form NADH and conversion of pyruvate to lactate regenerates NAD^+ from NADH. Conversion of Acetyl CoA to acetyl phosphate and subsequently to acetate via phosphotransacetylase and acetate kinase respectively, generates ATP. Thus, these overflow pathways enable the cell to maintain redox balance and generate ATP without using the cytochrome system. When the glucose is exhausted, the cells utilize the fermentation by-products and channelize them into central metabolism (using lactate dehydrogenase, acetoin dehydrogenase and acetyl CoA synthetase) and metabolize them further through the try carboxylic acid (TCA) cycle and generate more ATP and reducing power (Sonenshein 2007).

Carbon catabolite control protein A (CcpA) is a global transcription regulator of carbon metabolism in *B. subtilis* and many other gram positive bacteria. CcpA senses the nutritional status of the cell and acts as either a positive or a negative regulator. In the presence of glucose or other rapidly metabolized carbon source, Hpr and Crh - two cofactors of CcpA, are phosphorylated by Hpr kinase and they independently bind to CcpA and increase its affinity to palindromic operator sequences called *cre* (Catabolite responsive element). cre sequences have been identified for the genes involved in

acquisition and utilization of alternate carbon sources (Fujita 2009). Location of cre sites varies for different genes/operons depending on their positions from the transcription start site (TSS). Some are located upstream of the TSS, some are located downstream of TSS while some are located in the promoter region.

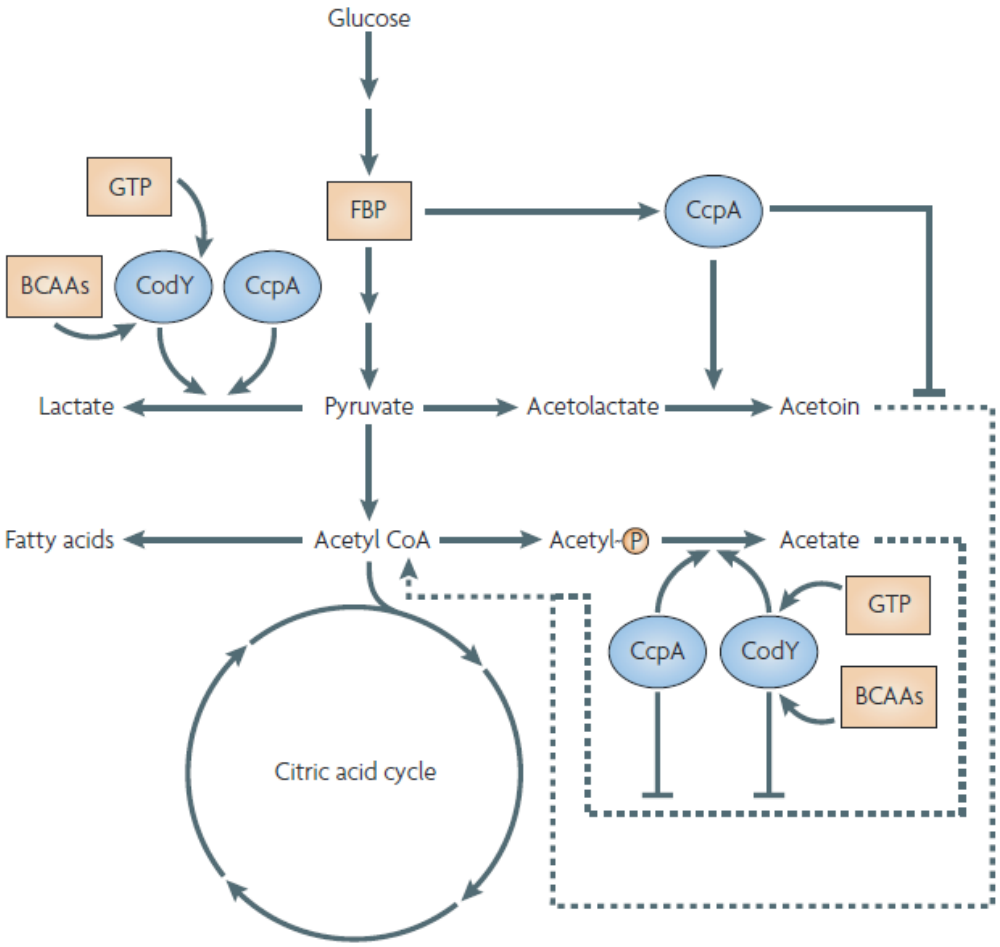

Fig. 1.5: Interactions of the global regulators CcpA and CodY with carbon overflow metabolism in *Bacillus subtilis*. (Sonenshein 2007)

The strength of CcpA-dependent regulation is affected by certain residues at specific positions in the cre box, more palindromic nature of cre sequences and the location of cre in close vicinity to the transcription start site (Marciniak et al., 2012). The regulatory phosphorylation of HPr at Ser46 residue is catalysed by bifunctional HPr kinase/phosphorylase, and its activity is stimulated by ATP and availability of fructose-1,6-bisphosphate as an indicator of high glycolytic activity. By contrast, under nutrient

limitation phosphorylase activity is stimulated by the accumulation of inorganic phosphate in the cell. This way modulation of the phosphorylation states of PTS components contribute to activity of CcpA in response to nutritional conditions and the metabolic state of the cell (Gorke and Stulke 2008). CcpA activates the expression of the genes that are required for the synthesis of acetate, lactate and acetoin and it represses citrate synthase gene *citZ*. CcpA binds to cre site located at the position +89, downstream of the promoter and create a roadblock for transcription elongation. CcpC is another transcriptional regulator that represses *citZ* and *citB* (aconitase) genes in response to citrate availability. When citrate is used as sole carbon source it causes derepression of citZ and citB transcription (Rosenkrantz et al., 1985; Jourlin-Castelli et al., 2000). Moreover, Citrate synthase is subject to feedback inhibition by 2-oxoglutarate, which acts competitively with respect to the substrate oxaloacetate. CcpC also acts as a negative regulator for its own synthesis (Kim et al., 2002). CcpC is transcribed from two different promoters P1 and P2. CcpC binds to CcpC box located downstream of the -10 region of P1 promoter and Expression from the ccpC-specific promoter (P1) was negatively regulated by CcpC but independent of the carbon source in the medium. CcpC is also produced from P2 promoter located within or upstream of the neighboring gene *YkuL*. Read through transcription from the P2 promoter is repressed by CcpA in a glucose dependent manner. CcpA binds to the cre site located upstream of the –35 region of the P1 promoter and about 331 bp downstream of the P2 promoter and reduces ccpC expression from P2, perhaps by acting as a road-block to read through transcription. When *B. subtilis* cells are grown in a minimal medium containing glucose and glutamine, *ccpC* expression is maintained at a very low level by CcpA and CcpC. However, the amount of CcpC that accumulates is sufficient to repress target genes. When citrate is the carbon source, *ccpC* expression increases, but the protein is inactive as a repressor of Krebs cycle genes. This higher expression of *ccpC* may allow rapid restoration of repression when citrate is exhausted or glucose becomes available. Thus, *B. subtilis* has evolved an unusually intricate mechanism to regulate the synthesis of CcpC to balance the need to activate the TCA branch genes when glucose is limiting and shut them down quickly when glucose is in excess, in order not to waste energy. Although CcpC is the specific regulator of TCA cycle, there exists two other mode of regulation overlapping this primary regulation. CcpA can directly repress Mg-citrate

transporter gene and *citZCH* operon therefore CcpA and CcpC both are required to repress *citZ* completely in presence of glucose. CodY acts as a repressor of *citB* gene.

CodY regulates nearly 224 genes in *B. subtilis* (Zhu and Stulke, 2018). It acts a repressor of genes involved in chemotaxis, motility, degradation of extracellular polymers and transport of the breakdown products, development of genetic competence and sporulation initiation in nutrient rich conditions (Bergara et al., 2003; Sonenshein, 2007). It senses nutritional status mainly by intracellular concentration of GTP and branch chained amino acid (BCAA). The interaction of CodY with GTP or a BCAA (particularly valine or isoleucine) increases its DNA-binding activity. CodY is a positive regulator of *ackA* and a negative regulator of f *ilv-leu, acsA, citB* and the lactate transporter gene *lutP* (Kim et al., 2003; Shiver and Sonenshein 2005; Shiver et al., 2006; Belitsky and Sonenshein 2013). Thus, in *B. subtilis*, three regulatory proteins (CcpC, CcpA and CodY) and four metabolites (citrate, FBP, GTP and isoleucine or valine) together, potentially control the flow of carbon from glycolysis to overflow metabolism to the citric acid cycle. In toto, during the growth of the cells in a rich medium containing glucose and a mixture of amino acids glucose generates FBP, which activates CcpA, and therefore contributes to repression of the *citZCH* operon. Any synthesis of citrate synthase will be nullified by the presence of a pool of 2-oxoglutarate, which is produced by the catabolism of amino acids. As a result, the pool of citrate will be low, CcpC will be highly active as a repressor and *citZ* transcription will be severely limited. Moreover, *citB* will be repressed strongly by the combined activities of CcpC and CodY; in this case the richness of the medium will allow efficient synthesis of GTP, and the presence of isoleucine and valine in the medium will help to keep CodY highly active.

Generally glucose is considered to be the preferred carbon source for *B. subtilis* but several findings of the last decade suggest that malate is an exception. Co-utilization experiments with eight different carbon sources revealed that Malate is the second preferred carbon source for *B. subtilis* and it is rapidly metabolized with glucose allowing similar doubling time as with glucose (Doan et al., 2003; Kleijn et al., 2010). Transport of organic acids such as citrate, succinate, and fumarate is repressed in presence of glucose by binding of CcpA on , cre sites located upstream of the genes encoding their transporters (Asai et al., 2000; Warner et al., 2000) but no such cre sites have been found upstream of

the malate-Na^+ symporter encoding *maeN* gene (Wei et al., 2000). Moreover, expression of *dctP*, encoding the generic C4-dicarboxylic transporter for succinate and fumarate, is repressed in presence of Malate.

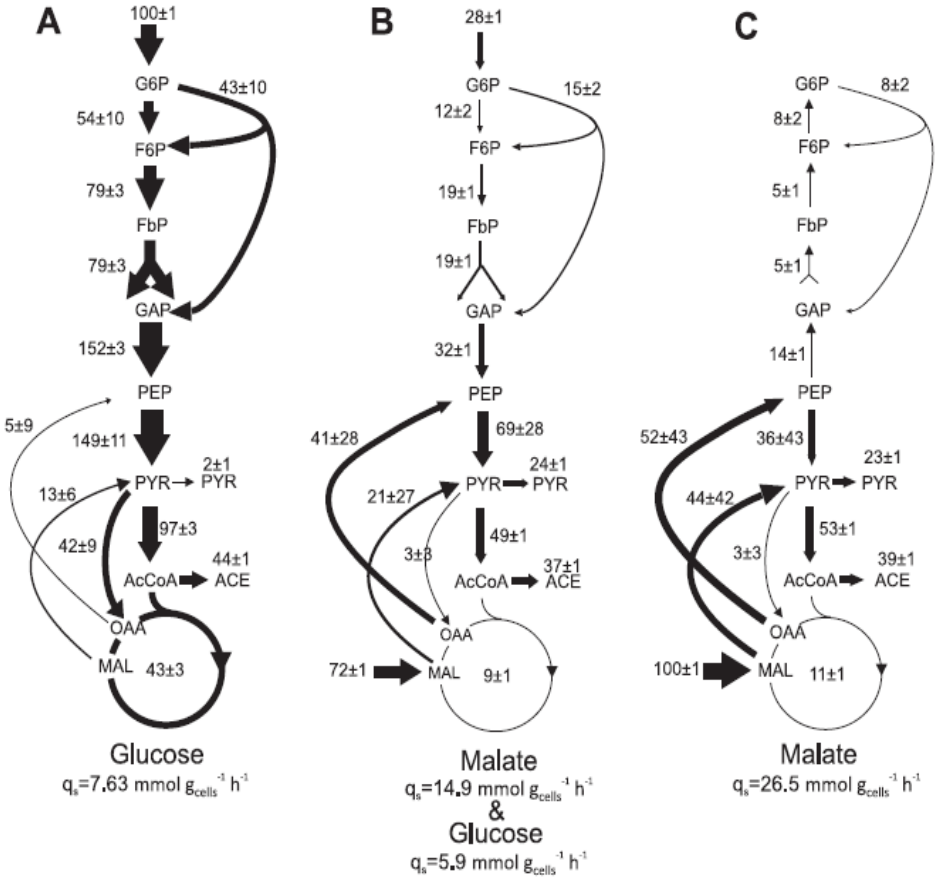

Fig. 1.6: Relative distributions of intracellular fluxes in *B. subtilis* grown on glucose (*A*), a mixture of glucose plus malate (*B*), and malate (*C*) (Kleijn et al., 2010).

Co-utilization experiments with other carbon sources viz. succinate, glycerol, pyruvate, arabinose, gluconate and fructose demonstrated that malate almost completely repressed the uptake of succinate, glycerol and pyruvate and least repressive effect was found with fructose whose uptake was reduced by 50%. Malate was consumed at unaltered rate and except succinate and glycerol all the above mentioned carbon sources were slowly co-utilized with malate, but at strongly reduced rates (Kleijn et al., 2010).

Carbon flux analysis in *B. subtilis* grown in glucose, malate and a mixture of glucose plus malate revealed differential distribution of carbon in different pathways. In presence of glucose alone, glucose is primarily metabolized via glycolysis and then

secreted as acetate in overflow pathways or completely oxidized via TCA. During growth on malate, large portion of malate was converted to PEP and pyruvate resulting in high gluconeogenic fluxes and overflow metabolism of pyruvate and acetate (Fig. 6.). Only 10% of the malate is respired via the TCA cycle, whereas the majority was secreted as overflow metabolites. Co-utilization of glucose and malate also led to high gluconeogenic fluxes and overflow metabolism. However, glucose is directed downward toward PEP synthesis. In both cases, malate flux through the pentose phosphate pathway and TCA cycle remains low. Malate feeding increases the amount of four carbon organic acids therefore only the four-carbon branch and not the entire TCA cycle is predominantly used. Expression of *citZ* and *citC* remains repressed during growth on glucose plus malate. Malate metabolism involves PEP–pyruvate–oxaloacetate node that connects the TCA cycle with glycolysis/gluconeogenesis.

After entering the cell, malate can have different fates according to nutritional and energy requirements of the cell. It can be oxidized either by the malate dehydrogenase (Mdh) to oxaloacetate and oxaloacetate can then be used for gluconeogenesis (phosphoenolpyruvate carboxykinase, PckA), for the synthesis of amino acids (aspartate and asparagine) or can serve as substrate to replenish the TCA cycle. Malate can be converted to pyruvate through oxidative decarboxylation by one of four malic enzymes (MaeA, MalS, MleA, or YtsJ). In contrast to the Mdh-PckA pathway, the conversion to pyruvate catalyzed by the malic enzymes mainly ends in the production of acetyl CoA or in the production of the overflow metabolites lactate and acetate (Kleijn et al., 2010). Both malic enzymes and malate dehydrogenase contribute equally to Malate utilization. Among the four malic enzymes (MaeA, MalS and MleA and YtsJ), YtsJ is NADP-dependent while the rest of three are NAD dependent (Lerondel et al., 2006). YtsJ is the major malic enzyme because only *ytsJ* mutants grow significantly slower than the wild-type on malate or other TCA cycle intermediates, while *maeA* and *malS* single or double mutants are indistinguishable from their parent (Doan et al., 2003). *ytsJ* gene is constitutively expressed during exponential growth on either glucose or Malate. When Malate is used as sole carbon source, the cells do not have the possibility to produce NADPH by the pentose phosphate pathway. Thus, the conversion of malate to pyruvate using the NADPH-forming malic

enzyme YtsJ helps to balance the redox pool of the cell and to allow efficient anabolic reactions.

Phosphoenolpyruvate (PEP)–pyruvate–oxaloacetate anaplerotic node interconnects the major pathways of carbon metabolism and is responsible for the distribution of the carbon flux among catabolism, anabolism and energy supply of the cell. In *B. subtilis*, pyruvate carboxylase (*pycA*) is the sole anaplerotic reaction, thus *B. subtilis* cannot grow on substrates that are metabolized via acetyl-CoA. Pyruvate carboxylase is a constitutively expressed biotin requiring enzyme which is subject to strong allosteric activation by acetyl-CoA (Diesterhaft and Freese, 1973; Doan et al., 2003; Henke and Cronan, 2014). The lack of this allosteric activation appears to cause the low in vivo activity during co-metabolism of glucose and citrate (Dauner et al., 2002). PEP-carboxykinase (PckA) decarboxylates oxaloacetate to PEP and it is required for gluconeogenesis and sporulation (Diesterhaft and Freese, 1973). PckA synthesis is repressed by the presence of glucose in CcpA-independent manner (Servant et al., 2005). Malate dehydrogenase and PEP carboxykinase are very essential for utilization of malate as the sole carbon source (Meyer and Stulke 2013).

B. subtilis is considered as a soil dwelling organism and it is associated with the plant roots. Bacteria adapt themselves according to their surroundings and ability of *Bacillus subtilis* to utilize malate as efficiently as glucose seems to be a part of such adaptations. Malate is commonly found in root exudates of many plants (Bais et al., 2006; Rudrappa et al., 2008). Malic acid secretion by *Pseudomonas syringae* pv *tomato* (Pst DC3000) infected *Arabidopsis thaliana* roots led to recruitment and biofilm formation by a plant beneficial bacterium *B. subtilis* FB17 on *Arabidopsis* roots. Secretion of malate from tomato roots is sensed by KinD histidine kinase and triggers biofilm formation in *B. subtilis* (Chen et al., 2012).

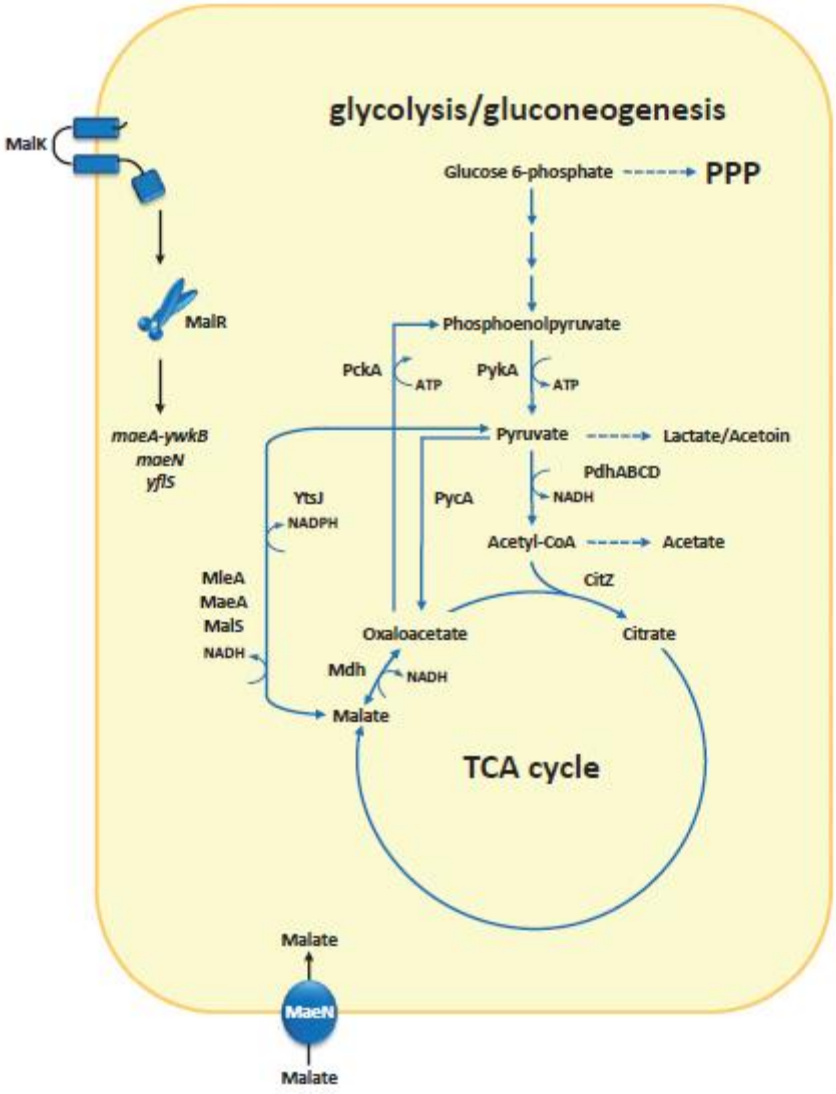

Fig. 1.7: Overview about the PEP–pyruvate–oxaloacetate node, the malate transport, and the MalKR two-component system in *Bacillus subtilis* (Meyer and Stulke 2013).

1.5 *Bacillus subtilis* – a model organism for cell differentiation

If one considers the natural habitat of PGP members of genus *Bacillus*, the growth conditions are entirely different than what is found in laboratory or industrial settings. Contrary to vigorous shaking and other instrumental controls to provide ideal growth conditions, biofilms are the preferred mode of life for many Bacilli (Beauregard et al., 2013; Kasim et al., 2016; Pandin et al., 2017) in the rhizospheric niches. Biofilm formation is an integral part of cell differentiation process in *B. subtilis*. Apart from their importance

as biofertilizer *Bacillus* is considered as a model organism to study cell differentiation events in simple prokaryotes (Vlamakis et al., 2013). All these events are governed by a complex regulatory network that senses both extracellular and intracellular environmental cues. Nutrient availability, Metabolic regulation, progress through different growth stages all contribute to the decision making process. *B. subtilis* adopts different lifestyles viz. motile state, competence state, extracellular matrix (ECM) producing sessile state and stress resistant endospore state in response to environmental stimuli. Each physiological state is described in detail.

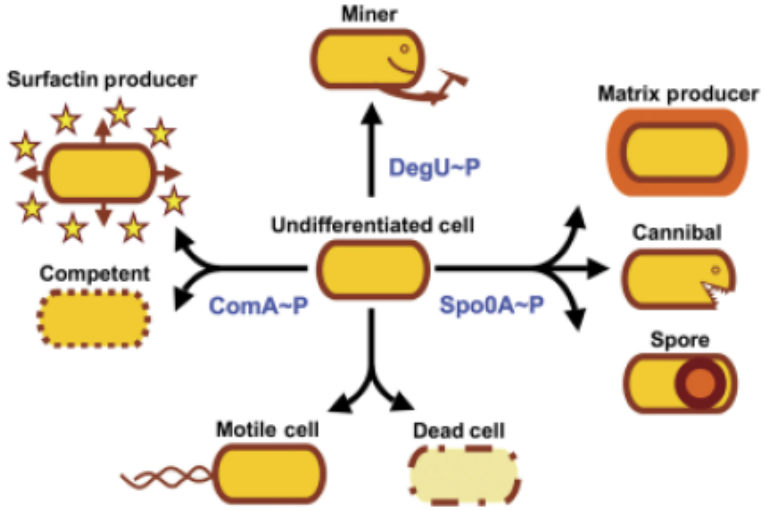

Fig. 1.8: Schematic representation of the distinct cell types that differentiate in the communities of *Bacillus subtilis* (Lopez and Kolter 2010).

1.5.1 Motility

Motility and chemotaxis facilitate swimming and swarming of *B. subtilis*. Bacterial flagella help to explore the surroundings for new sources of nutrients and chemotaxis machinery guides them towards potential attractants and away from the toxic growth inhibiting repellents. When bacterial populations grow in the liquid environment, movement of single planktonic cells powered by rotating flagella is called swimming motility while swarming motility is a rapid multicellular movement of bacteria across the solid surface powered rotating flagella. Another form of motility called sliding motility is surface spreading of bacterial colonies that is flagella independent but require surfactants

to reduce surface tension, enabling the colony to spread away from the origin, driven by the outward pressure of cell growth (Mukherjee and Kearns 2014). Flagellar structure in *B. subtilis* is made up of three components: basal body, hook and the filament. The basal body inserted in the cell envelope, contains the secretion apparatus that exports the more distal flagellar components, and provides the power for flagellar rotation. The hook is a flexible joint connected to the basal body that changes the angle of rotation of the long helical flagellar filament that acts as a propeller.

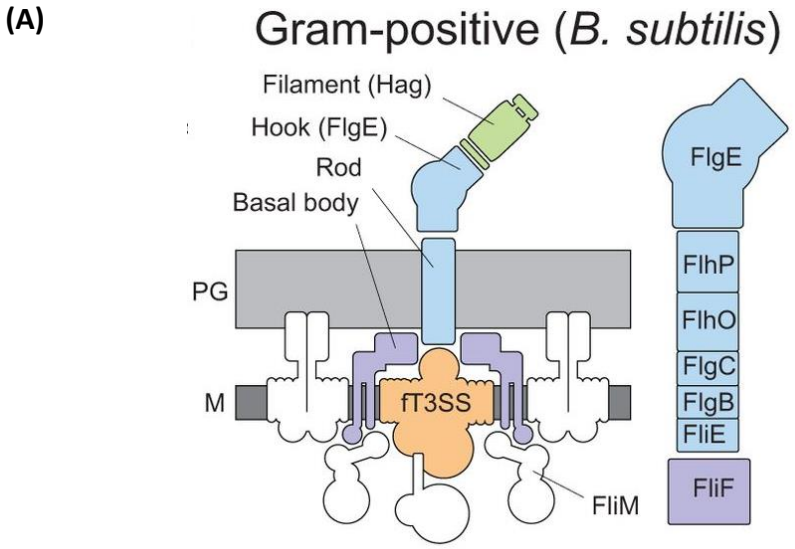

Fig 1.9 (A): Cartoon model of Gram-positive flagellar architectures (Burrage et al., 2018).

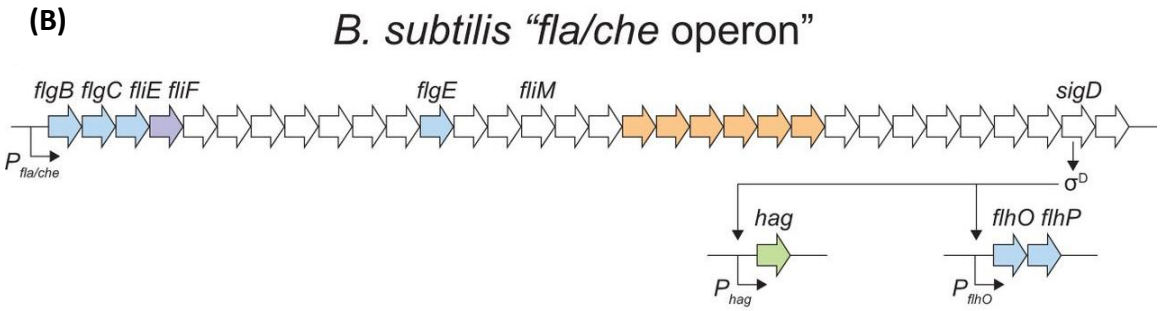

Fig 1.9 (B): *B. subtilis fla/che* operon structure and flagellar genetic hierarchy. Bent arrows indicate promoters, and open arrows indicate genes. Genes are color coded to match the relative locations of their gene products in panel A. (Burrage et al., 2018)

Total thirty-two genes are required for basal body synthesis and they are located in the large 27-kb *fla*/*che* operon. They are expressed via the action of RNA polymerase and the vegetative sigma factor sigma A (σA). Once hook assembly is complete, the alternative sigma factor sigma D (σD) is activated and expression of genes coding for filament assembly and rotation is carried out. Production of flagella and motility is an energetically expensive process in bacteria including *B. subtilis* (Macnab 1996; Smith and Chapman 2010; Holscher et al., 2015). Both hook and helical filament of flagella is made by polymerization of approximately 200 and 20000 monomer subunits respectively, and proton motive force is required to power flagellar rotation. Exponentially growing cells of *B. subtilis* are found in two different states: motile state in which σD is on and sessile state in which σD is off and cells are adhered to each other to form long chains. The formation of motile and sessile cell types by *B. subtilis* is mediated by a bistable switch that locks the activity of σD into an ON or OFF state. In wild (undomesticated) strains, the switch is biased in the direction of the σD-ON state by SwrA, a regulatory protein that stimulates expression of *sigD* by modulating the DNA binding property of unphosphorylated DegU. *B. subtilis* laboratory strains are able to swim in liquid media but they lack swarming ability because they carry a frameshift mutation in *swrA* gene which is required for activation of fla/che operon and *sfp* gene responsible for production of surfactin. The ability to generate a mixed population of sessile and nomadic cells may confer on *B. subtilis* the capacity both to exploit its present location as well as to disperse to new and perhaps more favorable niches. (Kearns et al., 2004; Kearns and Losick 2005; Lopez et al., 2009; Patrick and Kearns, 2009).

1.5.2 Competence

Natural competence is one of the unique attributes of *B. subtilis* which allows the uptake of exogenous DNA molecules from the surroundings and helps to adapt to fluctuating environments. Competent bacterial cells synthesize DNA binding, uptake and recombination machinery and it can incorporate plasmid DNA, phage DNA or chromosomal DNA. Double stranded DNA is converted into single stranded form while uptake by membrane localized nuclease NucA. These fragments are taken up in single-

stranded form. The complementary strands are degraded, and nucleotides are released into the medium. After internalization, the newly acquired single-stranded DNA is integrated into the genome by combined action of DNA-recombination protein RecA and DNA helicase AddAB. The expression of the DNA-binding, -uptake and -recombination genes is controlled by the competence transcription factor ComK. Initiation of ComK expression starts only when exponential growth ceases, and reaches its optimum after 2 h stationary growth. A sufficiently high cell density is very much necessary for competence to develop optimally. Medium constituents are important regulatory factors as well, and the highest ComK expression is obtained in minimal medium with glucose as the sole carbon source (Hamoen et al., 2003). Competence is a transient genetic state, and after several hours the cell switches back to vegetative growth on its path toward sporulation. In *B. subtilis* 168, under stressful conditions ~10% of the cells develop competence and a cell spends ~20 h in this state before switching back into the vegetative state. Competence is considered as an opportunity window to resist the stress encountered with the help of newly acquired DNA and to extend vegetative phase before entering into sporulation (Schultz 2009).

1.5.3 Biofilm formation

Biofilms are aggregates of surface associated microorganisms enclosed within self-produced extracellular matrix. They are formed as a result of complex multicellular behavior of bacteria to combat environmental challenges faced by them (Costerton *et al*., 1995; Mah and O'Toole 2001; Singh et al., 2006). They are ubiquitous in nature and gaining attention of microbiologists globally, mainly for the following reasons. First, they are an excellent example of microbial development and second, they are predominantly beneficial in the environment. Biofilms have been employed for bioremediation purposes (Halan *et al*., 2012), they exhibit the potential to be used as microbial fuel cells and in agricultural settings as biofertilizers to reduce the use of petrochemical derived fertilizers (Bais *et al*., 2004; Logan et al., 2009; Erable et al., 2010). Third reason is that they present a threat to human health when associated with biofouling and pathogenesis (Guttenplan and Kearns 2013; Vlamakis et al., 2013). Biofilm formation and its regulation mechanisms are extensively studied in *B. subtilis*. Isogenic populations of cells exhibit differential expression of genes within the biofilm. In *B. subtilis*, biofilm formation is a very well

orchestrated process involving expression of multicistronic *epsA-O* operon and the *tapA–sipW–tasA* operon coding for exopolysaccharide and amyloid fiber components of the biofilm matrix respectively. In addition to these, a third component is encoded by a single gene bslA. BslA is a small extracellular protein that forms a hydrophobic coat on the biofilm (Cairns et al., 2014).

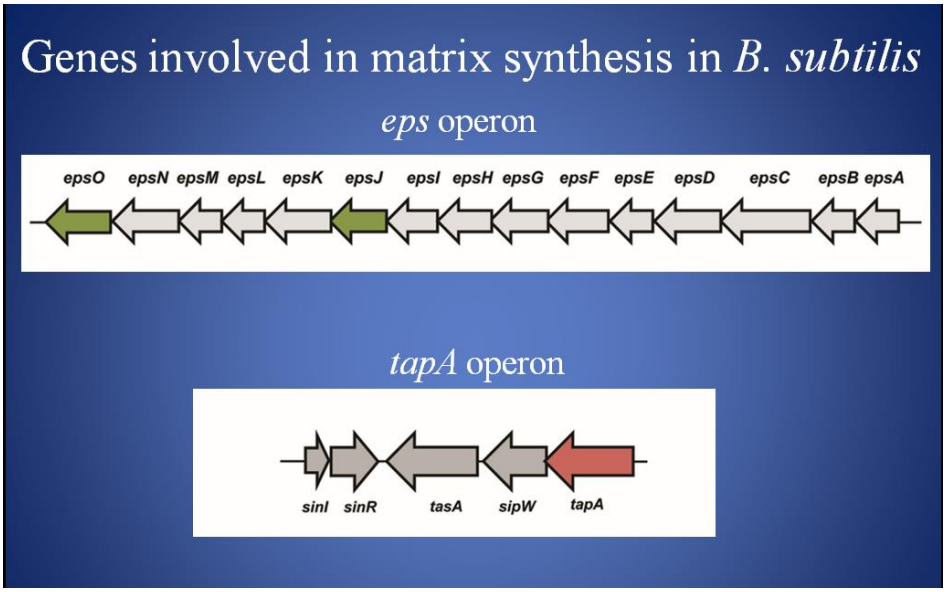

Fig. 1.10: Chromosomal organization of genes involved in matrix synthesis in *B. subtilis* (Luccia t al., 2015).

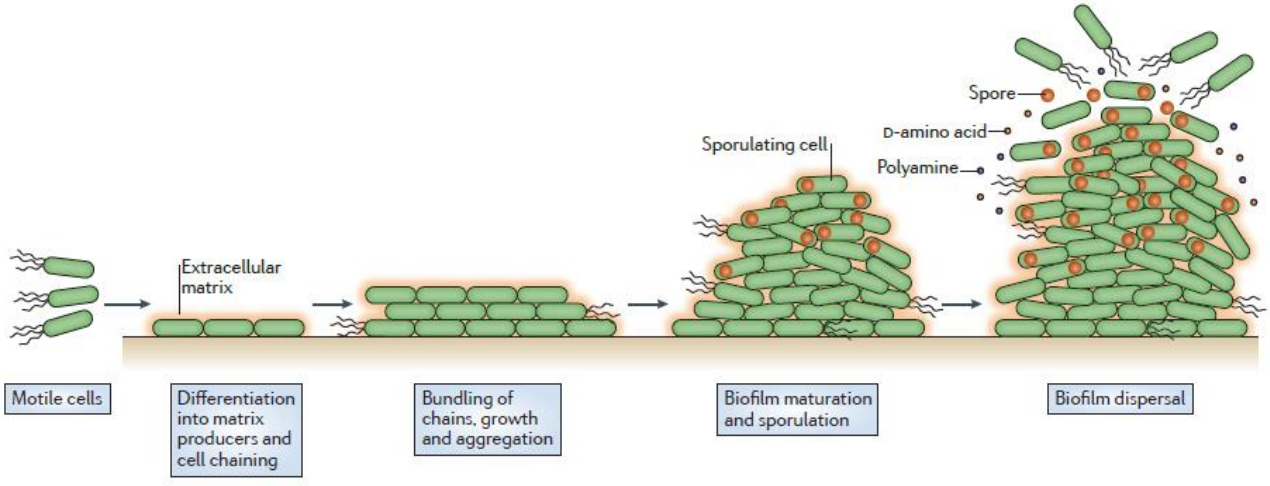

Fig. 1.11: The life cycle of a *Bacillus subtilis* biofilm. (Vlamakis et al., 2013)

Initially, cells are short motile rods, but as the biofilm develops, they become non-motile and form long chains of cells that adhere to each other and to the surface by secreting an extracellular matrix. In addition to matrix producers, motile cells and spores are present and are spatially organized within the maturing biofilm. *B. subtilis* forms different types of biofilms. Colony biofilms are formed on solid surfaces and pellicle biofilms are formed at air-liquid interface. In laboratory, colony biofilms develop complex architectural features after a few days of incubation characterized by wrinkles. Wrinkles are formed as a result of localized dead cells and the rigidity provided by Extracellular Matrix (ECM). Wrinkles are also formed as a consequence of impaired respiration that increases the surface to volume ratio facilitating greater access to oxygen (Kolodkin gal et al., 2013). Spo0A is central to a complex regulatory network that regulates the transcription of genes involved in motility, matrix production and sporulation (Fujita et al. 2005). Expression of matrix producing genes is under negative control of SinR and AbrB. SinR is the master regulator of biofilm formation and AbrB is a transcriptional repressor of early stationary phase genes. Spo0A is activated by phosphorylation via a multicomponent phosphorelay involving five sensory histidine kinases (KinA–E), Spo0F and Spo0B in response to various environmental signals. Concentration of phosphorylated Spo0A (Spo0A~P) determines the gene expression profile of a cell at a given time (Vlamakis et al., 2013; Cairns 2014). Low to intermediate levels of Spo0A~P represses expression of *abrB* and turns on the synthesis of two anti-repressor proteins SinI and AbbA which derepress the genes under the control of SinR and AbrB, respectively. Biofilm formation is an energetically expensive process so it is under tight control of multiple regulatory pathways. Biofim formation and motility are mutually exclusive events. The *slrR* gene is under the transcriptional control of SinR, thus high levels of SinI allows expression of slrR. SlrR binds to SinR with 1:1 stoichiometry and this SlrR-SinR complex binds to promoters responsible for expression of autolysin production and represses genes required for motility and autolysins hence promoting the transition from a motile state to biofilm formation (Chai *et al*., 2010). As SlrR re-directs SinR activity, and cells accumulate high levels of SlrR in a self-reinforcing negative feedback loop (Chai *et al*., 2010). Flagella are numerous and stable, thus, transcriptional inhibition is insufficient to inhibit motility. EpsE, is a bifunctional protein which brings out the short term inhibition of flagellar motility. In addition to the

glycosyltransferase activity that is required for EPS synthesis, EpsE functions as a molecular clutch that inhibits flagellar rotation by interacting with the flagellar motor switch protein, FliG. EpsE-mediated inhibition of motility occurs independently of the glycosyltransferase activity of the protein. Exopolysaccharides play an important role in colonies spreading on solid surfaces. EPS is thought to generate osmotic pressure gradients that allow the colony to spread outwards and thus acquire nutrients. Finally, because flagellar gene expression in *B. subtilis* is heterogeneous, there is a pre-existent nonmotile subpopulation that need not inhibit motility and may proceed to biofilm formation directly (Kearns & Losick, 2005; Chai et al., 2008, 2010; Lopez et al., 2009). In contrast to colony biofilms, formation of pellicles is assisted by flagellar motility as flagellum deficient mutants are delayed in pellicle formation. Despite this delay the mutant strains exhibited wild-type morphology demonstrating that motility is not essential for robust pellicle formation. Motility provides a competitive advantage to reach a favourable niche at air-liquid interface (Kobayashi 2007; Holscher 2015).

Population of matrix producers can be increased by a non-signalling mechanism called cannibalism. Low levels of Spo0A-P induce the expression of two cannibalism operons that encode secreted toxin peptides: sporulation killing factor (SkfA) and sporulation-delaying protein C (SdpC). Moreover, these cannibal cells also express the resistance machinery for the toxins. Thus, the cannibal cells secrete toxins that kill siblings which are not expressing the toxin or resistance genes. Because the cannibalism genes and matrix-producing genes are both activated by low levels of Spo0A-P, most of the matrix-producing cells also secrete toxins as well as resistance factors, and this effectively decreases the population of non-matrix producers. This system ultimately results in a population consisting of an amplified number of matrix-producing cells. The cannibalism toxins produced by *B. subtilis* are not specifically active against only *B. subtilis* siblings. In fact, they selectively kill different species when *B. subtilis* is grown in mixed cultures. Consistent with this idea of non-self-specificity, cannibalism-like toxins secreted by close relatives of *B. subtilis* probably have a role in increasing the *B. subtilis* matrix-producing subpopulation in mixed-species soil communities (Vlamakis et al., 2013). Cannibalism is another survival strategy that allows the subpopulation of matrix producers/cannibals to thrive using the nutrients released from the lysed sibling (or neighbouring bacteria) cells

that may be competing for the same resources. It also helps to delay sporulation in the cells that are not fully committed to sporulation by providing nutrients (Gonzalez-Pastor 2011). Delaying sporulation before commitment might be beneficial in case of temporary nutrient limitation.

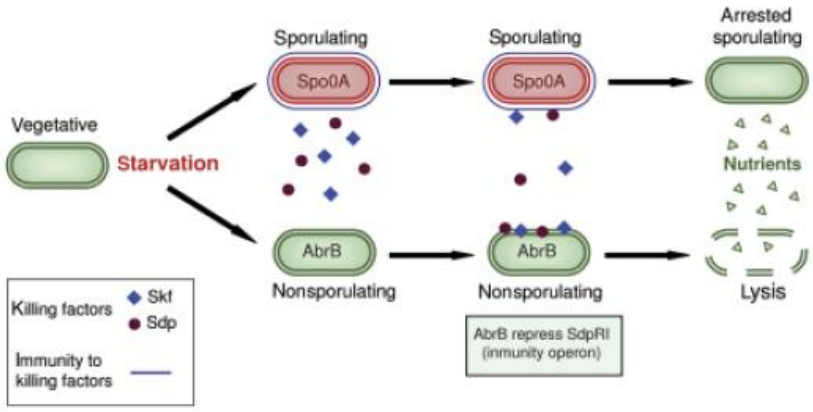

Fig. 1.12: Model for cannibalistic behavior (Gonzalez-pastor 2011)

Matrix producers, motile cells and spores are present and are spatially organized within the maturing biofilm. The percentage and localization of each cell type is dynamic. During initial stages of biofilm formation, motile cells are abundant but as the biofilm matures, many of the motile cells differentiates into matrix producing cells and at later time points the subpopulation of sporulating cells arise from the matrix producing subpopulation (Vlamakis et al. 2008). Spores are preferentially localized in the aerial structures in the colony biofilms. It is noteworthy that the process of differentiation is not terminal especially under environmental conditions, alteration in gene expression takes place according to the need to respond to the environment. However, under laboratory conditions, biofilms have a limited lifespan. As the biofilm matures, resource limitation and waste product accumulation eventually lead to dispersal of biofilms and spores are released from the matrix, giving them the potential to disperse and encounter environmental conditions that are amicable for germination. Release of a mixture of D-amino acids (D-tyrosine, D-leucine, D-tryptophan and D-methionine) is one such mechanism in *B. subtilis* which results in dissolution of the mature biofilm or inhibition of

biofilm formation. Incorporation of D-amino acids into peptidoglycan alters the association of certain proteins, including TapA, with the cell wall and disrupts biofilm formation. Thus, d-amino acids result in the release of the TasA amyloid fibres from the cell. Low, non-toxic concentrations of nitric oxide (NO) induces biofilm dispersal in *B. subtilis* and *B. licheniformis* (Lopez et al., 2010; McDougald et al., 2012; Vlamakis et al., 2013).

1.5.4 Sporulation

Sporulation is an energy demanding complex developmental process that lasts for several hours from the onset to the release of mature spore. Once committed, it is an irreversible process and the cell divides asymmetrically into a larger mother cell and a smaller daughter cell that is called the 'forespore' or 'prespore'. After completion of division, the forespore is internalized, followed by lysis of the mother cell and release of the mature spore. Sporulation in *B. subtilis* is primarily controlled at the transcriptional level by four sporulation-specific sigma factors, σF, σE, σG, and σK which are post-translationally activated at specific times in specific compartments. This pattern of regulation produces distinct lines of gene expression in the mother cell and forespore that drive sporulation through its morphological stages.

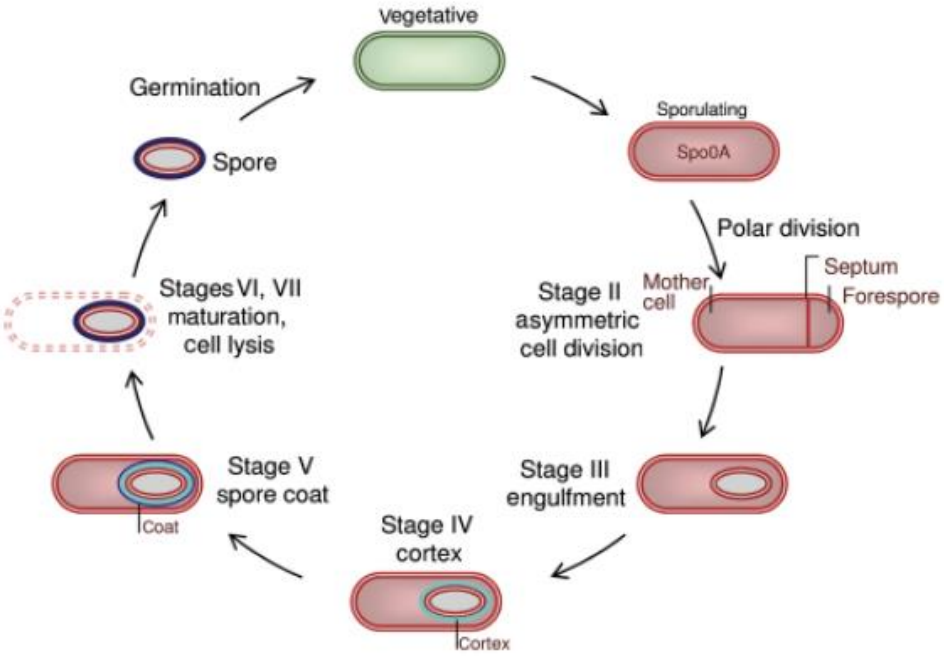

Fig. 1.13: The sporulation cycle of spore formation (Gonzalez-Pastor, 2011).

The key stages of the cycle are shown. Sporulation is divided into seven stages based on the morphological landmarks. However, there are no distinct boundaries between the stages and there exists an overlap in terms of when a stage begins and ends. At stage 0, chromosomes are replicated, but no obvious morphological landmarks of sporulation are yet present. Stage I is defined by chromosome condensation and the anchoring of the origins of replication to the extreme poles of the cell. In stage II, the polar septum is elaborated, followed by engulfment of the forespore in stage III. Stage IV and V represent cortex and coat assembly respectively. Stage VI refers to 'spore maturation'; a particularly obvious morphological feature elaborated at this stage is the tightly condensed, toroidal structure of the forespore chromosome. In stage VII, the mother cell lyses, releasing the mature, largely dormant spore into the environment. A high threshold level of Spo0A is required to trigger sporulation in response to nutrient deprivation and oxygen limitation (Piggot and Hilbert 2004; Higgins and Dworkin 2011; Tan and Ramamurthy 2014).

1.6 Rationale

Bacillus is best known for its biocontrol potential to inhibit variety of plant pathogens and spore forming capacity. Although spore formation is the most salient feature of *Bacillus* for survival under adverse conditions, spore dormancy may shut off many genes and metabolic functions which otherwise benefit plant growth. Sporulation is the last resort for survival and normal physiological regime also works in the direction of avoiding/delaying the sporulation if it is possible. Considering its rhizospheric abundance and versatile potential as biofertilizer, *Bacillus* strains secreting oxalic acid in sufficient amounts will be very effective in mineralizing inorganic phosphate. Mitigation or delay of sporulation while maintaining a longer vegetative phase of *Bacillus* spp may be advantageous for their PGP performance in the rhizosphere. This study is an effort to genetically modify *Bacillus subtilis* DK1042 for mitigation of sporulation and oxalic acid mediated phosphate solubilization.

1.7 Objectives

The objectives of the present study were defined as follow:

1. Developing oxalic acid secretion ability in *Bacillus* sp. by incorporation of oxaloacetate acetyl hydrolase (*oah*) and oxalate transporter (*FpOAR*) genes.
    a. Incorporation of *vgb* and *gfp* genes into *Bacillus* sp.
    b. Incorporation of *oah* and *FpOAR* genes into *Bacillus* sp. using pSW4 shuttle vector.
    c. Developing genomic integrant of *Bacillus* sp. containing *oah, FpOAR, vgb* and *gfp* genes.
2. Biochemical characterization of the MPS and MKS ability of *Bacillus* sp. secreting oxalic acid.
    a. Characterization of MPS ability of *Bacillus* sp.
    b. Characterization of MKS ability of *Bacillus* sp.
3. Evaluation of effect of VHb on biofilm formation and sporulation in *B. subtilis*