Chapter 1:
Introduction & Literature Review

Production and quality of crops have to be improved to keep pace with the increasing food demand of the world population. From global agricultural land, about 40 % is used for maize, rice and wheat production (Skamnioti & Gurr, 2009; Oerke & Dehne, 2004). Amongst these three major crops, rice is the staple diet of one-half of world's population (Sharma et al., 2012). Notably 70 different types of diseases have been reported to affect the rice yield which have been demonstrated to be caused by different bacteria, fungi, viruses affecting the plant at early or middle stage of its growth phase by infecting different tissues of the plant like foliar, leaf sheath, grain, culm and roots (Saha et al., 2015). Of these known diseases, around 11 are caused by bacteria. Amongst the bacterial disease, Bacterial Leaf Blight (BB) is the most devastating and the oldest one (Verdier et al., 2012; Mew, 1987).

1.1. History of disease Bacterial Blight

Bacterial Blight of rice, caused by *Xanthomonas oryzae* pv. oryzae (Xoo), was first reported from southern region of Japan by farmers in 1884 (Devadath 1992; Mizukami & Wakimoto 1969). The disease became common throughout that region by 1910, but not in northern part of Japan and other Asian countries until 1960s and 1970s (Mizukami & Wakimoto 1969). By 1960s, other rice growing nations including India, Nepal and Sri Lanka reported the outbreak of BB of rice due to new 'high-yield' cultivars of rice TN1 and IR8 introduced during those times (Adhikari et al., 1994; Mew 1993; Mew et al., 1992). Apart from Asia, the disease occurs in Australia, Africa, Latin America, the Caribbean and the United States. Economically, the greatest impact is on Asian countries where several epidemics have been reported in the past few decades as shown by the shaded area in Fig 1.1 (Ou, 1985).

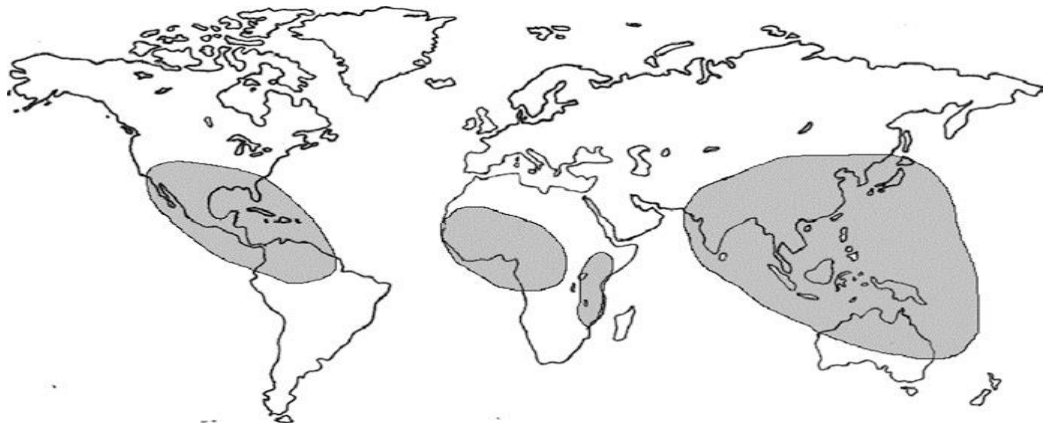

Fig. 1.1: Global geographical prevalence of BB.
Gray highlights indicate the regions affected by BB. (Adapted from Laha et al., 2017).

1.2. Economic impact of *Xanthomonas* genus

The bacteria of this genus has become an interesting research model owing to the prevalence of unique characteristics of different tissue specificity amongst the species of single genus, and to their ability to infect economically important plants.

Genus *Xanthomonas*, classified under *γ-Proteobacteria* comprises of 27 species. They infect approximately 124 monocotyledonous and 268 dicotyledonous plants including economically important plants like rice, citrus, banana, cabbage, tomato, pepper and bean. The pathogens of this group exhibit high level of host specificity. For example, *Xanthomonas campestris* comprises pathovars that infect different plants of families *Brassicaceae, Solanaceae*; whereas *Xanthomonas oryzae* infects rice and some of its wild relatives. Along with host specificity, these pathogens show tissue specificity as well. For example, both the species comprise pathovars that inhabit the vascular system (for example, *X. oryzae* pv. oryzae and *X. campestris* pv. campestris) or show specificity for the intercellular spaces of the parenchyma tissue (for example, *X. oryzae* pv. oryzicola and *X. campestris* pv. armoraciae) as depicted in Fig. 1.2 (Chan & Goodwin, 1999; Ryan et al., 2011).

In different countries, rice yield loss reported ranged between 20 -30% due to disease. In the case of severe infection, crop loss can go upto 80 % depending upon degree of susceptibility, the growth stage of plant, and the environmental conditions (Ou, 1985; Shin et al., 1992; Mew et al., 1993; Noh et al., 2007; Sundaram et al., 2009). In India, crop loss of rice varies from 6 -60% in different states again depending upon growth stage of plant, infection severity and cultivar types (Ou 1985; Singh et al., 1977). Study carried out by Reddy et al. (1979) on the epidemic that occurred in Palghat district of Kerala found a linear relationship between crop loss and disease severity which helped to develop a critical point model for predicting association of the rice yield losses with the BB disease. This disease is found in almost all the rice growing states and affect the yield of the crop (Mishra, 2013; Yashitola et al., 1997).

1.3. *Xanthomonas oryzae* pv. oryzae

Xanthomonas oryzae pv. oryzae (Xoo) is a vascular pathogen of rice where it causes Bacterial Blight. It is Gram-negative with long rods and round ends

(Fig.1.2a). The size of the bacterium varies from 0.7 µm to 2.0 µm in length and 0.4 µm to 0.7 µm in width. The cells are motile and have a polar, monotrichous flagellum. When grown in high glucose containing solid medium, the colonies seem convex, smooth, shiny, mucoid and yellow coloured, which is the typical trait of this genus, due to production of pigment called xanthomonadin as shown in Fig. 1.3b (Bradbury, 1984). It is aerobic and grows best at a temperature between 25 - 30 °C and pH 6.5-7.5.

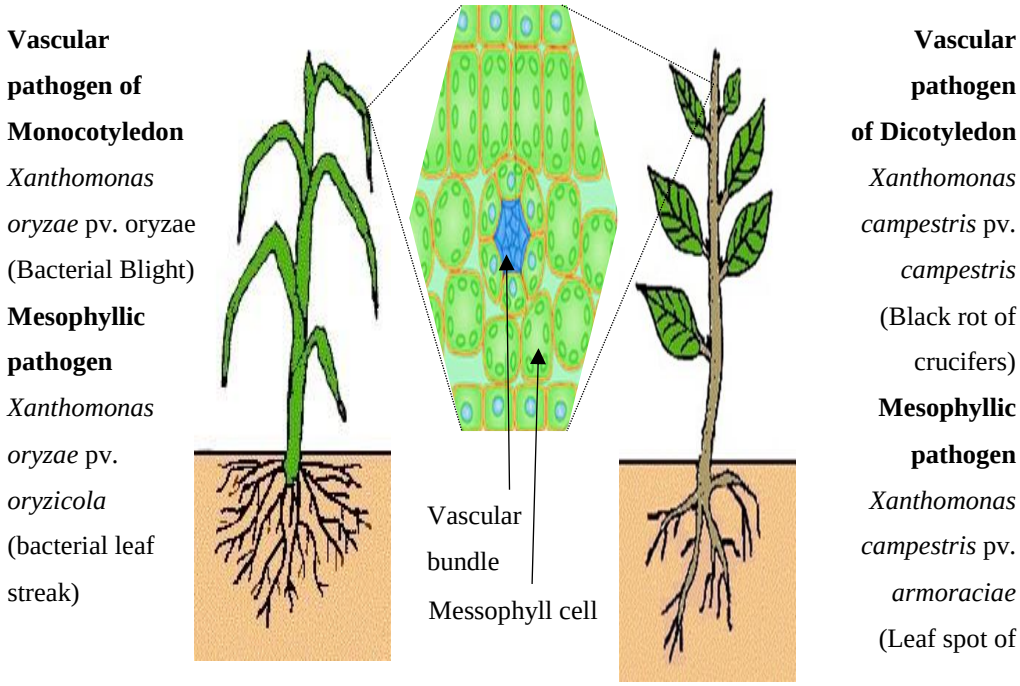

Fig. 1.2: Schematic diagram showing host and tissue specificity amongst Xanthomonads
The genus *Xanthomonas* infects broad range of plants both, monocots as well as dicots. They show host specificity and also tissue specificity. The enlarged portion shows the xylem vessels wherein Xoo inhabits and causes the disease.(Adapted and modified from Ryan et al., 2011).

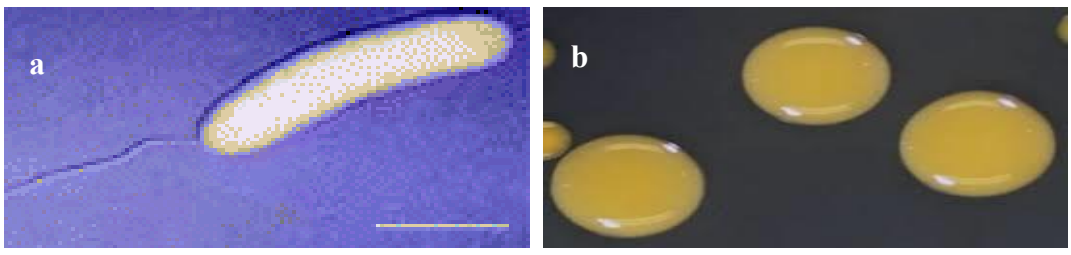

Fig. 1 3: Typical cells and colony morphology of Xoo
Scanning electron microscope image of single cell of Xoo (bar 1.0 µm); (b) Typical yellow shiny colonies of *X. oryzae* pv. oryzae when grown in glucose rich medium (PS agar medium). (Adapted from Nino-Liu et al., 2006).

1.4. Epidemiology and symptoms of disease caused by *X. oryzae* pv. oryzae

Xoo enters the host via hydathodes (water pores) and even through wounds and cracks. It spreads inside the plant by swimming, multiplies in spaces of underlying epithem and then it advances through xylem (Nini-Liu et al., 2006; Ou, 1985). Within few days, bacterial cells and extracellular polysaccharide (EPS) fill the xylem vessels and ooze out from hydathodes. The disease can affect rice plants at any plant growth stages (Mew & Vera Cruz, 1979; Reitsma & Schure, 1950). Two main disease symptoms caused by Xoo are depicted in Fig. 1.4 and can be categorized as follows:

1) Bacterial leaf blight (BB), and 2) Wilt or Kresek disease.

BB, is the most common disease symptom that develops at tillering stage and further peaks at flowering stage (Mew et al., 1993). Spots at the tips and margins of fully developed leaves are typical symptoms of bacterial blight. The spots expand along the veins, merge, and become chlorotic and then necrotic, forming opaque, yellow to grey coloured lesions that typically extend along the whole leaf tip (Fig.1.4a). The wilt, called Kresek disease is the most destructive form of this disease. It develops in seedling stage in plants less than 21 days old i.e. early tillering stage and as a result severely affect the crop yield (Mew & Vera Cruz, 1979). Infected plant leaves wilt and roll up and generally the whole plant dies or show stunted growth (Fig. 1.4b).

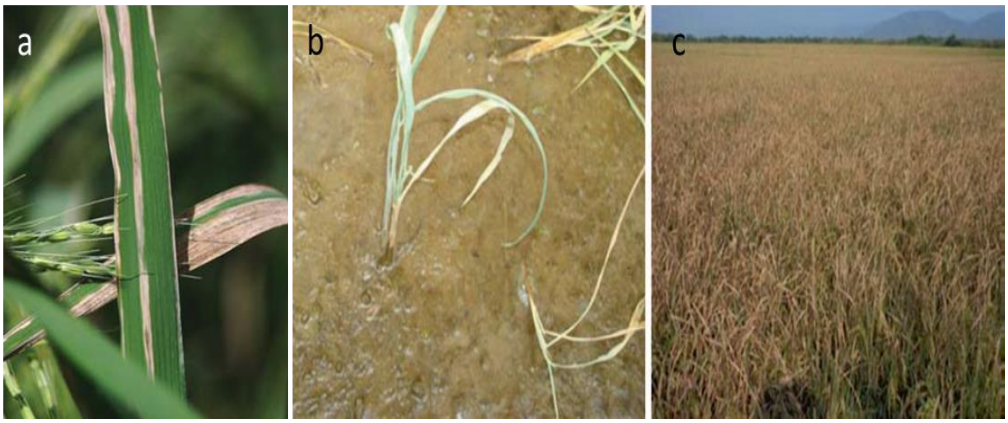

Fig. 1.4: Typical symptoms of the BB disease observed on the rice plant infected with Xoo

(a).continuous necrotic area along the leaf length formed due to blockage of xylem vessels of the adult plant, b) Infection showing wilting of the rice plant when Xoo infects the plant at an early stage, called Kresek disease, c) Severely infected rice field. (Adapted from Laha et al., 2017)

1.5. Virulence determinants of Xoo

Many virulence factors of Xoo are involved in successful invasion of pathogen in host plant (reviewed in Buttner & Bonas, 2010). They are xanthan gum, hydrolytic enzymes like xylanase, pectinases, cellulase, protease, etc. Type two secretion system (TIISS) secretes the cell wall degrading enzymes like xylanase (*xyn*), esterase (*lipA*), cellulase (*clsA*), cellobiosidase (*cbsA*), protease etc. (Rajeshwari et al., 2005; Sun et al., 2005; Furutani et al., 2004; Ray et al., 2000). Type III secretion system (TTSS) in many phytopathogens have been demonstrated to mediate secretion of various effectors which are injected directly in the host cells and cause virulence (Sugio et al., 2005; McCann & Guttman, 2008). These factors are believed to interact with the host cell components and thus subside a general resistance response of the host plant or otherwise enhance the colonization of bacteria in the plant (Alfano & Collmer, 1997; Willis et al., 1991). Mutants and complementation experiments of TTSS genes of *Xanthomonas oryzae* pv. oryzae and other species indicated the importance of TTSS in causing virulence on plants (Wang et al., 2007; Kamdar et al., 1993).

The extracellular polysaccharide i.e. Xanthan gum secreted by Xoo in copious amount after it enters and colonizes the xylem vessels of the host plant, is considered as important virulence factor. Mutation in *gum* genes, which encodes synthases for xanthan, lead to loss of EPS as well as virulence in *Xanthomonas oryzae* pv. oryzae (Dharmapuri & Sonti, 1999). Xoo has various adhesins, employed at different infection stages for successful entry and colonization in the host. Mutation in *Xanthomonas* adhesin-like protein A (*xadA*) or its paralog, *Xanthomonas* adhesin-like protein B (*xadB*) yielded avirulent strains and their role was demonstrated in initial attachment and entry in the leaf (Das et al., 2009). Mutation in *pilQ* gene that codes for Type IV pilus secretin has been demonstrated to play a role in spreading out within the leaf. *pilQ* mutant of virulent strain of Xoo was unable to cause infection in the host plant (Das et al., 2009). Xoo produces three types of quorum sensing factors called diffusible signal factor (DSF): DSF, BDSF and CDSF, wherein BDSF was found to be most abundant in *in vitro* studies. All three signalling factors regulate production of Xylanase and EPS in Xoo (He et al., 2010). Mutations in genes *rpfB, rpfF, rpfC* and *rpfG* cause reduction in the various virulence factors like EPS levels, xylanase activity, motility; thereby

reducing the virulence as demonstrated in Xoo strain KACC10331 (Jeong et al., 2008). Various virulence determinants of Xoo are depicted in Fig. 1.5. Hence, certain virulence factors whose mutation leads to significant loss of virulence of Xoo, could be potential targets to design strategies to control the pathogenesis of Xoo.

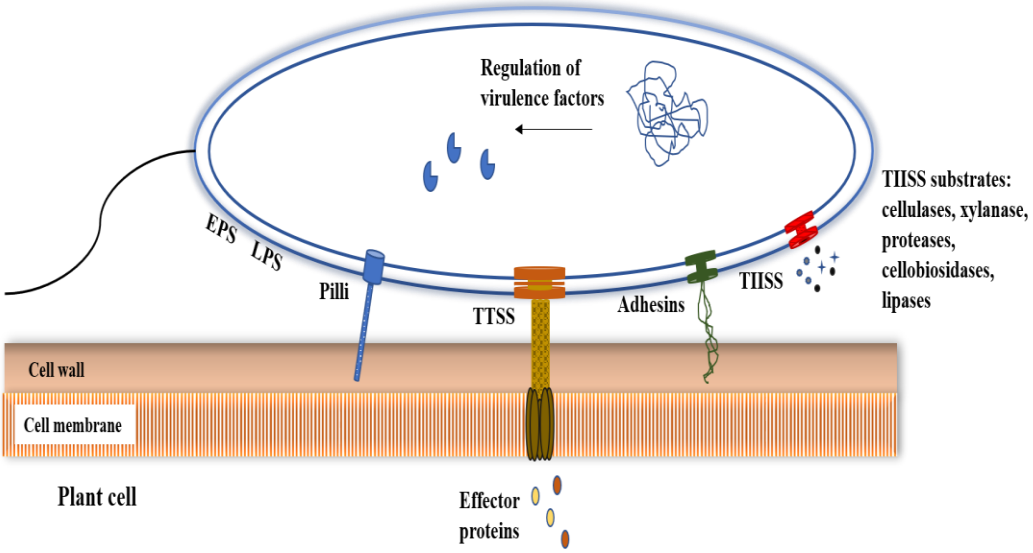

Fig. 1.5: Schematic diagram showing virulence determinants of Xoo and their interaction with host cell
(Adapted and modified from Buttner & Bonas 2010)

1.6. Control measures against Xoo

Many approaches like chemical, biological and introduction of host resistance genes have been implemented for rice disease management and its control during endemic and epidemic conditions as discussed below.

1.6.1. Conventional chemicals to control disease caused by Xoo

Various chemicals and pesticides have been used to control the spread of Xoo in fields. In China, Bismerthiazol is most commonly used to control BB disease (Xue, 2002; Ren et al., 1993). Disinfecting seeds with bleaching powder supplemented with 30 % chlorine before sowing was found to decrease the symptoms of BB disease (Chand et al., 1979). Chemicals like Bordeaux mixture, copper-soap mixture, copper-mercury fungicides also reduced the disease (Nino-Liu et al., 2006). Foliar spray of copper oxychloride and Streptomycin solution at

short intervals was recommended to control this disease (Sulaiman & Ahmed, 1965; Seki & Mizukami, 1956). Many chemicals like Tecloftalam were reported to be more effective when sprayed directly on the plant than application in soil. Bag et al. (2010) demonstrated effective control of BB using copper hydroxide containing 35 % metallic copper. Soil application of potash at 50 kg/ha in two splits at 40 and 50 DAS (days after sowing) effectively restricted the spread of BB and increased grain yield (Marimuthu, 1995). However, using these chemicals with high levels of copper are highly toxic to the ecosystem.

1.6.2. Biological control

Several potential antagonists from rice rhizosphere have been isolated and commercialized for rice disease management along with growth enhancement and seed emergence (Yang et al. 2008). Velusamy et al., (2006) screened 27 strains of plant-associated *P. fluorescens* which could produce 2,4-diacetylphloroglucinol (DAPG), a chemical which inhibited the growth of Xoo in lab conditions and suppressed rice BB up to 59 -64 % in net-house and in field experiments. Jeyalakshmi et al., (2010) found that *P. fluorescens* when used in the combination of seed treatment, soil application and foliar spray could significantly reduce BB disease symptoms and improved yield in comparison with the chemical control. Various bacteria like *Bacillus subtilis* and other *Bacillus* sp., *Pseudomonas fluorescens* and *Trichoderma* sp. were found antagonistic to Xoo pathovars and effectively decreased the disease intensity when used as biocontrol agent by spraying on plants or for seed treatment or pre-soaking followed by spraying (Hunjan et al., 2017; Gangwar & Sinha, 2010; Manmeet & Thind, 2002; Pant & Mukhopadhyay, 2012). A novel strain of *Lysobacter antibioticus* (strain 13-1) was found to suppress the disease up to 69.7 % in green house and upto 59.1 % in the field conditions (Ji et al., 2008). *Streptomyces toxytricini* VN08-A-12 was shown as biocontrol agent of Xoo (Van Hop et al., 2014). Similarly, *Bacillus oryzicola* sp. nov, an endophyte was demonstrated to protect rice plant against Xoo by induction of systemic resistance (Chung et al., 2015). Studies have shown that PGPR through various mechanisms like antagonism, competition for nutrients and ecological space, induction of host systemic resistance (host immune system), etc. could control the destructive diseases caused by various plant pathogens (Kumari &

Srivastava, 1999). Numerous *Bacillus* species are known to be antagonists that produce diverse secondary metabolites that are antimicrobial in nature and act against the growth of the phytopathogens (Stein, 2005).

1.6.3. Resistance genes in the host

There exists phenotypic and genotypic variation amongst the Xoo population, which in many studies have been reported on the basis of differential interactions with resistance genes of host plant (Adhikari et al., 1995; Noda et al., 1996; 1990; Leach et al., 1992). Till date, forty three resistance genes from rice (Xa genes), both recessive and dominant have been reported and are designated as Xa1 -Xa43 (Nino-Liu et al., 2006; Kim et al., 2015; Busungu et al., 2016; Kim & Reinke, 2019). Pathogen population is grouped in eleven pathotypes based on their virulence response for *Xa* genes *(Xa1, Xa3, Xa4, xa5, Xa7, xa8, Xa10, Xa11, xa13, Xa21)* present singly or pyramided (presence of more than one resistance genes in the host plant). The pathotype III amongst all the virulent types was found to be most widely spread in different states of India, whereas pathotype XI was the most divergent and was virulent to most of the resistance genes but not on rice cultivar pyramided with xa5, xa13 and Xa21 resistance genes (Mishra et al., 2013). Pathovars of Xoo have been found to possess highest number of IS elements as compared to other xanthomonads. They have very high number of TAL-effector genes which could be hot spots for recombination and rapid evolution (Salzberg et al., 2008).

The resistance of rice plant towards Xoo varies as per the genetic structure of the host. Hence, certain resistance genes confer resistance to the host at seedling stage (seedling resistance), while others render resistance at adult stage but are susceptible to pathogen at seedling stage (adult plant resistance) (Mew, 1987). Using resistance genes from various hosts has been proven as the most effective method in controlling the disease caused by Xoo. Studies have shown that genes *Xa4a, xa5, Xa10* confers resistance both at adult stage as well as seedling stage, while genes such as *Xa3, Xa4b, Xa6, xa8, xa9* and *Xa21* provide resistance only at adult stage of the host plant, which is a prominent difference to distinguish the two types of resistance (Khoshkdaman et al., 2014; Sundaram et al., 2008; 2009). Hence, gene pyramiding is a more effective method employed, wherein different

resistance genes are used in the same cultivar to make the host immune to pathogen at different growth stages. For this purpose more resistance genes are being discovered. Xa43 resistance gene was added to the list of reported genes and can be used in future in breeding (Kim & Reinke, 2019).

1.6.4. Limitations of the BB control methods

Although various approaches have been implemented for controlling the disease caused by Xoo, they have certain notable limitations. The chemicals which have been applied in the fields often target other non-pathogenic microbes and impact environment owing to their toxicity and non-degradable characteristics. Resistance against many commercially used antibiotics have also been reported (McManus et al., 2002). Streptomycin resistant pathovars of Xoo have been reported from Yunnan province of China (Xu et al., 2010). Similarly, the resistance genes used in the cultivars are being overcome by the pathogen giving rise to new pathogenic variants. Studies have demonstrated emergence of new races and significant shift in race distribution after the introduction of cultivars with resistant gene *Xa4* (Mew et al., 1992). New races of Xoo are being reported from Japan, Nepal, India, Philippines and Korea (Yugander et al., 2017; Lore et al., 2011; Cottyn & Mew, 2004; Jeung et al., 2006; Noda et al., 2001; Ochiai et al., 2000). Although many different methods have been applied for controlling and management of the BB disease and have been shown effective in the disease management, the pathogen eventually shows resistance against the deployed methods. Hence, there is a constant need to search for novel agents as well as different strategies for the effective control of this disease.

1.7. Alternative strategies to control BB disease

Investigations have been carried out in studying the effectiveness of many different methods and strategies in controlling the disease caused by Xoo. Diverse genes in plants encode functionally different defence related proteins to render resistance to the host plant. Such resistance genes exhibiting the potential to control BB disease can be implemented in transgenic approach. There have been reports on use of host pathogen species-non-specific broad-spectrum resistance (BSR) by DR

(defence-responsive /defence-related) genes for rice improvement as an efficient method to control diseases (Ke et al., 2017). An antibacterial peptide, Cecropin overexpressed in rice showed enhanced resistance against bacterial blight (Sharma et al., 2000). Similarly, antimicrobial peptide from onion was reported to give resistance against many pathogens along with BB (Patkar & Chattoo, 2006). However, the major problem with using transgenes is their instability and silencing (Repellin et al., 2001). Another approach reported was degradation of Xoo signalling molecule, DSF by bacteria like *Pseudomonas* sp. to decrease disease severity (Newman et al., 2008). Many synthetic as well as non-synthetic small molecule compounds, target the virulence factors of the pathogens such as adhesion to host, toxins or the specialized secretion systems of pathogen or interfere with the virulence by blocking the virulence factors at the regulation level (Rasko & Sperandio, 2010; Felise et al., 2008; Mahamoud et al., 2007; Lomovskaya et al., 2001).

These strategies can be integrated for achieving a higher level of crop protection and reducing the disease occurrence. Using a bacterial control treatment in combination with single resistance gene, whose expression was limited by growth stage of the host plant, showed enhanced level of plant protection against the pathogen (Gnanamanickam et al., 1999). Antibiotic Oxytetracycline and Streptomycin with copper oxychloride and plant products like neem oil and lemon grass oil showed increased efficacy of antibiotic in controlling and managing the disease caused by Xoo at field level (Singh et al., 2015; Khan et al., 2012; Tagami, 1962). Since such combinations involving antibiotic and chemical compounds for the control of Xoo have been found effective, newer combinations involving novel antibiotics and eco-friendly chemical compounds could have the potential for higher level of protection and sustainable rice yield. An account of antibiotic and other eco-friendly chemical approaches in Xoo control has been given in subsequent sections.

1.8. Antibiotics against Xoo

Use of antibiotic has been practiced for the control of BB since many years. Streptomycin is used as bactericide in controlling many phytopathogens along with Xoo since many decades (Xue et al., 1973). Certain reports have demonstrated the

antibiotics produced by various microorganisms exhibit inhibitory activity against Xoo. Antibiotics produced by fungus *Phomopsis longicolla* S1B4 have been shown by *in vitro* assay to be antibacterial against Xoo (Lim et al., 2010). Similarly, antibiotic of Iturin class produced by *Bacillus* species was shown to be effective and had biocontrol potentials against Xoo (Beric et al., 2012). Also, 1-Deoxy-N-acetylglucosamine from marine organism *Virgibacillus dokdonensis* MTCC 1A00493 was demonstrated to specifically supress Xoo (Huang et al., 2018). Bottromycin A2 and Dunaimycin D3S antibiotic from *Streptomyces bottropensis* was reported to be effective against Xoo (Park et al., 2011). Also, polyketides like Bacilysin and Difficidin were shown to be significantly effective in controlling disease symptoms on rice plant at green house level (Wu et al., 2015).

Polyketide class of antibiotics include structurally diverse compounds (Lim et al., 2016; Koskinen & Karisalmi, 2005). The typical characteristics of this class of antibiotics is that they are made of repetitive units of a monomer substrate (Caulier et al., 2019; Hertweck, 2009; Cane & Walsh,1999). The synthesis of Polyketides comprises of initiation, elongation and termination of the substrate units. Auxiliary domains can also be present on elongation modules which renders great diversity to this class of antibiotics (Hertweck, 2009). They are classified into three types, namely type I, type II and type III polyketides (Shen, 2003). type I and type II polyketides have been reported to be synthesized by certain *Bacillus* sp. (Caulier et al., 2019; Chakraborty et al., 2017b; Patel et al., 1995). However, presence of type III polyketide have been demonstrated to be present in bacteria only recently (Shimizu et al., 2017; Dibyendu, 2015; Hashimoto et al., 2014). The presence of type III polyketide genes in the whole genome sequence of *B. altitudinis* have been reported (Potshangbam et al., 2018; Kumaravel et al., 2018; Budiharjo et al., 2017). Certain strains of *B. altitudinis* have been demonstrated as biocontrol agents against Xoo (Potshangbam et al., 2018). It could be interesting to study polyketide group of antibiotics as potential control agents of phytopathogens, in this case Xoo.

1.9. Small molecule compounds and their application

Small molecules are non-peptide organic compounds generally < 1500 Da that can be semi-synthetic, synthetic compounds or natural product extracts (Ward

et al., 2002). Extracts from natural resources or large number of structurally diverse compounds can be used for studying the targeted biological effect by means of high-throughput screening (Selzer et al., 2000). Spectrophotometric absorbance, fluorescence, radioactivity, luminescence based screening assays can be designed and high-throughput read-outs can be easily carried out at microplate reader scale (Kariv & Chung, 2002; Blake, 2001). The physiological target can be perturbed in controlled manner at specific times and by using them at varying concentrations. This makes them powerful experimental tools for studying cell biological mechanisms using chemical genetics approach (Duncan et al., 2012; Ward et al., 2002; Schreiber, 1998; Mitchison, 1994). Several small molecule compounds have been studied and still are being explored for their potential in different applications. Small molecule compounds of plant origin have immense potential to control various diseases caused by bacteria, fungi, viruses and protozoans. Many different class of small molecule compounds have been studied as virulence attenuators, as efflux pump inhibitors, etc. as alternatives for new variants of pathogens which emerge owing to resistance towards deployed agents like antibiotics, resistance genes, etc. (Fan et al., 2017; Li et al., 2008; González-Lamothe et al., 2009).

Liu in 2004 classified the small molecule compounds from plant source broadly, into carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds, based on their biosynthetic origins and chemical structures. Silva (2016) and González-Lamothe et al.,(2009) in their review has compiled various compounds of plant origin that act on different virulence factors of different pathogens (Fig. 1.6). Phenolic acids that belong to phenolics group of small molecule compounds contain one or more hydroxy derivatives of benzene rings. Phenolics are widely distributed in plants and are used for defense related functions in many plant species (Boudet, 2007). Polymerization of polyphenols increases toxicity of the compounds to phytopathogens (Friedman, 1997) indicating potential of phenolics small molecule compounds to combat pathogenesis.

At present, only little about the efficacy of phytochemicals on *Xanthomonas* is known. Some reports have confirmed that alkaloids in some plants could strongly inhibit *X. axonopodis* pv. malvacearum, *X. axonopodis* pv. phaseoli and *Xanthomonas campestris*; however, the specific role of these alkaloids was not clear overall (Raghavendra et al., 2008, 2009; Venkatesh et al., 2015). The available

literature about such small molecules as virulence attenuators and as antibiotic potentiators, have been discussed in subsequent sections.

1.9.1. Small molecule compounds as virulence attenuators

A typical antibiotic aims at the bacterial cell viability by targeting many different means like inhibiting cell wall or destabilizing membrane, protein synthesis blockage or DNA replication inhibition. Antibiotics have been important in treating many microbial diseases, however emergence of resistance towards the traditional approaches has become a challenge (Levy & Marshall, 2004). Small molecule compounds act as agonists or antagonist to specific proteins and hence thereby target particular cell processes without killing the pathogen (Duncan et al., 2012; Schreiber, 1998). Additionally, this approach leads to increased range of targets for controlling pathogenesis by novel mechanisms of action of antimicrobial agents (Cegelski et al., 2008). In many pathogens, small molecule compounds reduce virulence of bacteria to the host by preventing the expression or blocking the activity of the virulence factors (Claworthy et al., 2007). These virulence factor blocking agents could be effective biocontrol agents (Duncan et al., 2012).

Many small molecules have been studied for their ability to attenuate various virulence factors of different pathogens (Ward et al., 2002) (Fig. 1.6). Bacterial quorum-sensing (QS) inhibition by quorum quenchers have been demonstrated as a strategy to control pathogenesis (Castillo-Juárez et al., 2017). Secretion systems of the pathogen, toxins and enzymes, biofilm formation, fimbriae, flagellum and two-component system could be alternative targets especially in bacteria showing resistance towards antibiotic (Silva et al., 2016; Gu et al., 2015; Gotoh et al., 2010; Costerton et al., 1995). For example, Catechin gallate has been reported to directly inhibit the lethal factor (LF) in *Bacillus anthracis* (Dell'Aica et al., 2004). Same compound has been shown to block the virulence factors regulated by QS in *P. aeruginosa* (Vandeputte et al., 2010).

Small molecule like phenolic compounds act as chemical barriers to invasion by phytopathogens and help in the plant defence mechanism (Lattanzio et al., 1994). Many small molecules have been reported as inhibitors of T3SS of many plant pathogenic bacteria like *Erwinia* sp. *and Psuedomonas* sp. (Myszka et al.,

2014; Khokhani et al., 2013; Truchado et al., 2012; Felise et al., 2008; Yang et al., 2008). Some studies have shown the effective reduction in pathogenesis in *Xanthomonas* sp. as well. For example, leaf extracts containing secondary small molecule metabolites have been studied in controlling the Xoo disease (Kagale et al., 2004; Grainge & Alvarez, 1987). In certain other reports investigators have used pure small molecule compounds and confirmed the ability of these small molecule compounds to reduce pathogenesis. In another report, Benzothiadiazole, a synthetic analog of salicylic acid has been shown to control disease caused by *X. axonopodis* pv. citri and other phytopathogens (Kouzai et al., 2018; Nayem et al., 2018). Notably, many phenolic compounds were screened against Xoo and have been studied for their ability to control the BB symptoms on rice plant (Fan et al., 2017). One such phenolic acid viz. p-coumaric acid has also been found effective against *X. campestris* pv. campestris in controlling disease in Chinese cabbage (Islam et al., 2018).

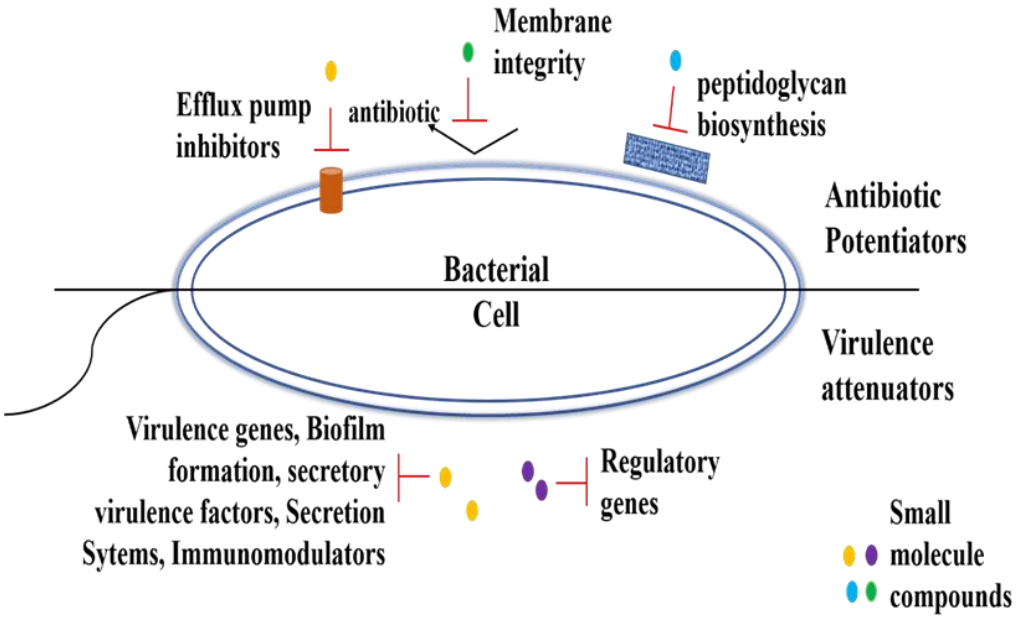

Fig. 1.6: Schematic representation of small molecule compounds as antibiotic potentiators and as virulence attenuators

Small molecule compounds acting on different targets of the bacterial cell as virulence attenuators or antibiotic potentiators. (Adapted and modified from Silva et al., 2016 and González-Lamothe et al., 2009)

1.9.2. Small molecule compounds that increase efficacy of antibiotics

There are many secondary metabolites, categorized into many different chemical classes, studied as inhibitors of virulence factors but very few are potent (Gibbons, 2004). Most of such compounds by themselves possess no antibacterial activity; however, when combined with antibacterial compounds the efficacy of the compound increases significantly. For example, 5'-methoxyhydnocarpin (5'-MHC), a metabolite of flavonolignan group, produced by *Berberis* species does not possess any antibacterial activity but potentiates the activity of berberine, a product of same plant, by blocking its efflux (Stermitz et al., 2000). Small molecule compounds, mainly natural products of plant origin act as antibiotic potentiators by various mechanisms like disrupting membrane integrity, by acting on bacterial cell wall or inhibiting efflux pumps of the bacterial system (González-Lamothe et al., 2009) (Fig. 1.6). Terpenoids being lipophilic in nature has been demonstrated to affect the cell membrane permeability and increase the susceptibility towards the antibiotic in *S. aureus* and *E. coli* (Brehm-Stecher & Johnson, 2003). Another compound farnesol inhibited C55 lipid carrier required in cell wall synthesis and increased susceptibility of MRSA towards β-lactam (Kuroda, 2007). Such small molecule compounds could potentially be used in combination with antimicrobials in a synergistic manner to extend their lifespan. The details of such compounds reported as efflux pump inhibitors are discussed in next section.

1.10. Efflux pumps and Efflux pump inhibitors

Efflux pumps are widely distributed in both prokaryotes as well as eukaryotes. The general information of various efflux pumps found in bacteria and their reported inhibitors called as efflux pump inhibitors (EPI) have been described below.

1.10.1. Efflux pumps (EPs)

Bacterial EPs are either primary active transporters which use energy from ATP hydrolysis for the efflux, or secondary active transporters which use proton motive force (PMF) as an energy source generated across the membrane due to H^+/Na^+ ions (Dwivedi et al., 2017; Putman et al., 2000). These active transporters

transport structurally diverse substances like sugars, amino acids, numerous intermediate metabolites of the various biochemical pathways and also the substances that could be toxic to the bacterium (Boudker & Verdon, 2010; Van Bambeke et al., 2003).

Based on the sequence homology, EPs are classified into 4 major families, namely (1) Major facilitator super family (MFS), (2) Small multidrug resistant transporter super family (SMR), (3) Multiple antimicrobial extrusion protein super family (MATE) and (4) Resistance-nodulation-cell division transporter super family (RND). Except for the EPs belonging to MATE which use Na^+/H^+ gradient, all the above mentioned superfamily members use H^+ gradient and possibly function via. multidrug or proton antiporter mechanism for the efflux of various substrates (Murakami & Yamaguchi, 2003; Paulsen et al., 1996). ATP-binding cassette (ABC) transporters which is an ATP dependent multidrug transporter have also been reported from bacterial system (Davidson & Maloney, 2007). Fig. 1.7 depicts the major families of EPs, their arrangement across the bacterial cells and functions as proton/drug antiporters (Tegos et al., 2011). MFS pump show narrow range specificity and is present in both Gram negative and Gram positive bacteria, while a broad spectrum RND, exclusively present in Gram negative bacteria; are the most common EPs present in bacterial system (Molnar et al., 2010; Ward et al., 2001). The efflux pumps which extrude structurally diverse compounds, including antibiotics, have not shown evolutionary changes corresponding to the stresses of the antibiotic era (Paulsen et al., 1996). It has been reported that the efflux pumps are responsible for the inherent resistance of many Gram negative bacteria to multiple antibiotics (Poole, 2000). In many pathogens, emergence of resistance has been attributed to increase in efflux pump activity and decrease in membrane permeability (Choudhury et al., 2016; Lister et al., 2009; Webber et al., 2006; Webber et al., 20003; Alekshun & Levy, 1997). The role of efflux pumps in clinically important bacteria have been well studied and their relevance in multidrug resistance have been documented (Poole, 2004; Van Bambeke et al., 2003). However, their role in general physiology of the bacteria has been only recently studied.

Du et al., (2018) and Blanco et al., (2016) reported that EPs not only extrude toxic compounds but also are involved in the virulence and adaptive responses.

With advances in sequencing technology, the whole genome sequencing projects have revealed that in plant and soil associated bacteria, very high number of such multidrug efflux pumps have been found (Paulsen, 2003). Since plants produce large number of diverse metabolites, these efflux pumps have been speculated to help pathogens invade the host plant. This assumption has quite been supported in reports wherein inhibitors of the efflux pumps have been shown to increase the efficacy of plant metabolites with antimicrobial activity, by about 2000 folds (Tegos et al., 2002). In phytopathogens, very limited number of efflux pumps have been characterized for their ability to confer antibiotic resistance. In *Erwinia amylovora*, NorM multidrug efflux pump have been reported to confer resistance to Norfloxacin, Berberine and many antibiotic compounds like the one produced by the epiphytes *Pantoea agglomerans* that colonize the plants (Burse et al., 2004). The same group have also worked on AcrAB multidrug efflux pump to show their involvement in virulence of the pathogen. In *X. oryzae* pv. oryzae, a putative RND type efflux pump has been shown to be involved in xanthomonadin pigment transport (Goel et al., 2002), while not much details about the efflux pumps of Xoo have been reported.

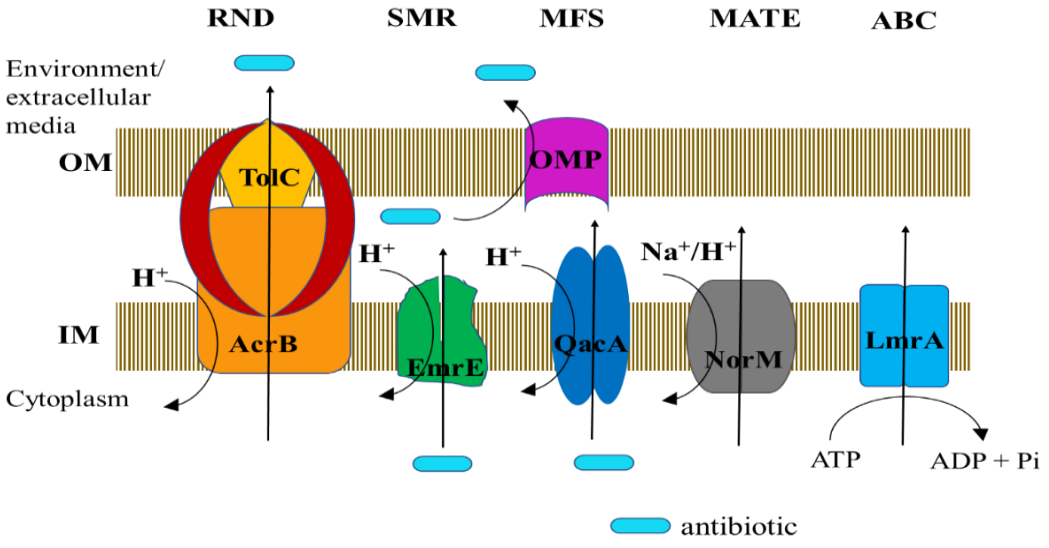

Fig. 1.7: Schematic diagram depicting Multidrug efflux pumps
(Adapted and modified from Tegos et al., 2011)

1.10.2. Efflux pump inhibitors (EPIs)

Many secondary metabolites produced by plants act as bactericidal or bacteriostatic compounds. Many such natural compounds of plant origin have been reported to possess potential to act as efflux pump inhibitors (EPIs) and increase the efficacy of antibiotics by acting as blocking agents of various efflux pumps. The antibiotics which otherwise are excreted out of the bacterial cells via. such efflux pumps, are unable to move out but are concentrated inside the bacterial cells and thus help in reaching the minimum bactericidal concentration (Marquez, 2005). There are many mechanisms proposed for inhibition of efflux pump by EPIs (Fig. 1.8). The mechanism of inhibition can be: transcription downregulation of efflux pump encoding genes, ion gradient disruption across the cell membrane, substrate binding inhibition by competitive or non-competitive manner, interference in assembly of efflux pump multiprotein complex or by changing outer membrane permeability of Gram negative bacteria. Studies have shown that EPIs increase the efficacy of antibiotics towards susceptible bacteria *in vitro*, reduce the minimum inhibition concentration (MIC) of antibiotics and improves on resistance development when used along with antibiotics (Mahmood, 2016; Kaatz, 2005)

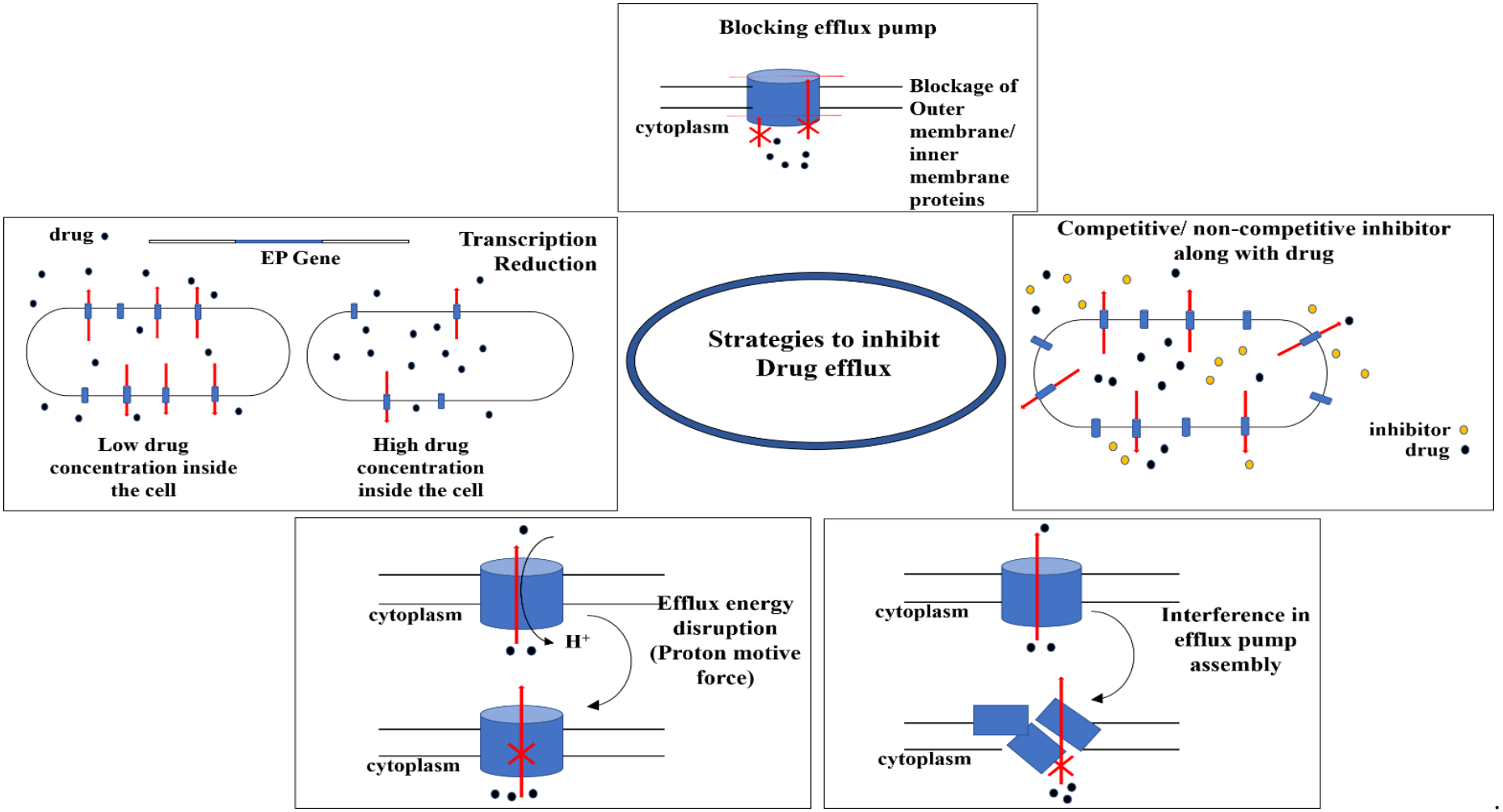

Fig. 1.8: Proposed mechanisms of efflux pump inhibition by EPIs
(Adapted and modified from Shriram et al., 2018)

Rationale and Objectives

Rationale

Xanthomonas oryzae pv. oryzae is a rice pathogen which infects the plant systemically by inhabiting the xylem vessels. Multiple means have been deployed for the control of this pathogen. Presently, the most effective commercially used method is introducing genetic resistance (Xa gene) from plant cultivars that confers resistance against the pathogen by plant breeding or transgenic approaches and notably even towards the multiple Xa genes used in gene pyramiding giving rise to new pathogenic variants. Hence, alternative strategies are needed to be explored that use newer approach or targets for controlling the pathogenesis.

Also, single method leads to easy and fast resistance development in pathogen therefore integrated approach which target pathogen by multiple mechanisms, could be more effective means in control of the pathogen. Eco-friendly methods like using natural products and biodegradable bactericidal agents will be more useful as they have minimal harmful effect on environment. Streptomycin and Polyketide antibiotics have been used against Xoo to control BB. However, instances of resistance development towards streptomycin have been reported from difference regions of Asia. Thus, there is a constant need for new antibiotics and novel antagonistic agents exhibiting strong inhibitory activity against the phytopathogen.

Many natural products like small molecule compounds are being studied for their potential to control pathogenesis of different bacteria. This approach is interesting in the fact that many of such compounds are studied as virulence attenuators, wherein they do not show bactericidal effect on the pathogen but rather disarm the pathogen by targeting different virulence determinants. Phenolic acids are one of the class of such small molecule compounds which have been studied as virulence attenuators in different plant and animal pathogens. However, not much studies have been reported for *Xanthomonas oryzae* pv. oryzae. Using such small molecules along with the bactericidal agents in integration approach could be an effective means of controlling pathogenesis of Xoo.

The approach of the present study was to screen bacterial antagonist to Xoo and small molecule compounds as virulence attenuators of Xoo, with an aim to use combination of the two approaches which combat pathogen by different

mechanisms.

Hence, the objectives designed were:

Objectives

I) Isolation and screening of antagonistic bacteria against *Xanthomonas oryzae* pv. oryzae and characterization of the mechanism of antibiosis.

II) Virulence attenuation studies using small molecule compounds with *Xanthomonas oryzae* pv. oryzae.

III) Studies on combined effect of inhibitory and virulence attenuating approaches on *Xanthomonas oryzae* pv. oryzae.