

Chapter 2

Bacillus mojavensis*: Its Endophytic Nature, the Surfactins, and Their Role in the Plant Response to Infection by *Fusarium verticillioides

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2.1 Introduction

Preharvest fungicides are used to control or reduce pathogenic fungi, their inoculum, and infection in hosts that also should reduce the formation of undesirable contaminants such as mycotoxins. However, the emphasis in greater sustainability and an increase in public concern for hazards associated with synthetic chemical pesticides and transgenic plants have produced a resurgence of interest in the use of introduced microorganisms for biological control of plant pathogens. Biological control is another one of the several measures used as pre- and postharvest control of several pathogens including the species of *Fusarium*. Most of these microorganisms are inconsistent in their performance in biological control resulting in reduced commercial development and widespread use. The major reason for this lack of performance is inadequate colonization of the target site, variation in expression of control at that site, and the need for numerous applications. Most of the biocontrol organisms are either soil or surface dwellers and have very little affinity to plants as specific colonizers as evidenced by ineffective controls of disease following repeated applications (see Thomashow and Weller 1990 and Hallmann et al. 1997 for review).

There is a unique group of bacteria that form endophytic associations with plants, and several of these have been reported to be successful in preventing disease development. The uses of bacterial endophytes in agriculture for general and specific biological control applications are current, widespread (Chanway 1996; Hallmann et al. 1997; Kobayashi and Palumbo 2000; Reinhold-Hurek and Hurek 1998; Sturz et al. 2000), and might afford protection to host plants similar to those provided by fungal endophytes.

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Bacterial endophytes can be distinguished from nonbacterial endophytes by their unique behavior with plant hosts. Large numbers of bacterial species are endophytic and are regarded as symbiotic with plants as biotrophic and several of these form mutualistic endophytic associations (Hallmann et al. 1997; Chanway 1996, 1998; Bacon and Hinton 2006; Kobayashi and Palumbo 2000; Bacon and Hinton 2006). These bacterial endophytes actively colonize above- (foliage) and below-ground (root) host tissues and establish long-term associations, actually lifelong natural associations, without doing substantive harm to the host. These associations are to be distinguished from transient bacteria, usually dormant or latent infections, which form associations as happenstances that will not survive long. Endophytic bacteria are further distinguished by controlling the growth of several fungi, bacteria, and nematodes (Bacon and Hinton 2006; Hallmann et al. 1997). Endophytic bacteria include both obligate and facultative species. These bacteria offer several advantages for control of pathogenic fungi, and they are expected to control both endophytic fungi, such as *Fusarium verticillioides*, and epibiotic to endophytic species of other *Fusarium*.

Bacillus mojavenis Roberts, Nakamura, and Cohan was distinguished from the *Bacillus subtilis* complex on the basis of whole-cell fatty acid composition, divergence in DNA sequence, and resistance to genetic transformation (Roberts et al. 1994). The type of this bacterium was an isolate from the Mojave Desert, California, which served as the specific epithet for this and the other desert isolates as *B. mojavenis* Roberts, Nakamura, and Cohan. This group of species in addition to the desert origins has additional physiological traits that serve as characteristics of the species. All strains of this species are antagonistic to fungi and are all endophytic. These traits suggest that this species has outstanding traits as an endophytic biocontrol agent.

The genus *Bacillus* has several traits in common and one that is emerging is the ability to produce specific lipopeptide biosurfactants such as surfactins A, B, and C, pumilacidin, esperin, lichenysin, fengycin, iturin, the bacillomycin, mycosubtilin, surfactants 86, arthrofactin, and the fungicines. We recently discovered that this species also produced a mixture of closely related cyclic lipopeptide isoforms of the biosurfactant, surfactin A (Snook et al. 2009; Fig. 2.1). Among the many anticipated uses of surfactin, this biosurfactant is inhibitory to fungi, some bacteria, mycoplasmas, and viruses (Bonmatin et al. 2003; Desai and Banat 1997; Georgiou et al. 1992; Peypoux et al. 1999; Seydlova and Svobodova 2008).

In addition to *B. mojavenis*, the surfactins are produced by *B. subtilis*, *B. licheniformis*, *B. pumilus*, and *B. amyloliquefaciens*. Only two other genera are reported as producer of lipopeptide biosurfactants and these include *Serratia marcescens* that produces serrawettin W2 and species of *Pseudomonas* that produces the putisolvins and arthrofactin (Desai and Banat 1997; Matsuyama et al. 1992).

A major thrust is on bacterial endophytes that offer biocontrol potential for fungal endophytes such as *Fusarium* species. This genus consists of several species that are endophytic (Kuldau and Yates 2000), and one species in particular is *F. verticillioides* (synonym *Fusarium moniliforme*), Holomorph: *Gibberella*

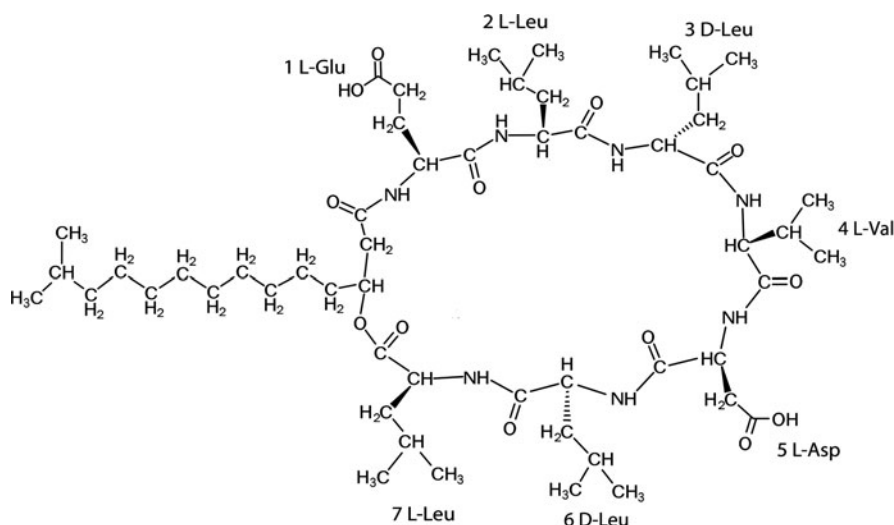


Fig. 2.1 Structure of surfactin A, Leu⁷-surfactin with the amino acid sequence of Glu, Leu, Leu, Val, Asp, Leu, Leu. Surfactin B has an amino acid sequence Glu, Leu, Leu, Val, Asp, Leu, and note for this isomer Val at position 7. Surfactin C has an amino acid sequence of Glu, Leu, Leu, Val, Asp, and note for this isomer Ile at position 7

moniliformis Wineland, a pathogen of maize and other crops. In maize, it produces the fumonisin mycotoxins that are toxic to horses, swine, poultry, and are correlated with human toxicity (Bullerman 1996; Marasas et al. 1988, 2004; Riley et al. 1993; Ross et al. 1992; Voss et al. 2001).

Since the fumonisins are common occurrence on maize, most probably due to the endophytic nature of *F. verticillioides*, it is imperative to find a control for both the endophytic infection and the saprophytic state. The endophytic state serves both as a source of mycotoxin production and as an infection source. The endophytic state also makes it difficult to control by fungicides registered for use on food crops. *B. mojavensis* has the potential to control infections by *F. verticillioides* and for reducing the content of fumonisin in maize (Bacon et al. 2001). However, the practical uses of bacterial endophytes under field conditions are problematic. The basis for this we feel is due to the unknown mechanism or mechanisms responsible for biocontrol by endophytic bacteria. Such mechanisms obviously are complicated by genetics of biocontrol and target organisms, interacting biotic and abiotic factors, and most importantly competing organisms. Further, there might be several mechanisms operating within a given environment, each operating for specific species of biocontrol organism. Intuitively, competitive exclusion is used to describe competition between two organisms for the same niche. What is described as competitive exclusion is envisioned as replacing one organism with another is too simplistic. The method of obtaining dominance or excluding another from a niche is operationally a complex scenario of which very little is known. In this regard, nutrition is often used as the explanation, although most theories encompass

an antagonistic response such as the production of an antibiotic. In the instance of *B. mojavensis* versus *F. verticillioides*, antibiotic production by the biocontrol organisms is suggested since the production of antagonistical substances is observed on Petri plates.

Surfactins are produced by the majority of strains of *B. mojavensis* tested (Snook et al. 2009). Further, since all *B. mojavensis* strains examined are endophytic, we believe this species can offer valuable biocontrol information from which we can draw conclusions and extend to other bacterial endophytes. Moreover, *B. mojavensis* along with other *Bacillus* species produce surfactins, which implies that there is a natural role in the ecology of a species for surfactin and others in this class of biosurfactants (Ron and Rosenberg 2001). We review the chemistry of surfactin, surfactin production in this natural endophytic species, as well as the biocontrol potential for surfactins. We also review the biocontrol activities and host–bacterial relations along with in planta growth properties. The target of our control is primarily *F. verticillioides* and other endophytic species of fungi, although other pathogens might equally fit the model and be controlled as well.

2.2 Surfactin Chemistry and Structural Features

The surfactins consist of a group of several surface-active agents produced by microorganisms and conveniently grouped as biosurfactants because they are surfactants of microbial origin, and they are readily biodegradable. Thus, they are environmentally friendly. Surfactin is one of the small molecular weight lipopeptide biosurfactants that range from 1,003 to 1,127 Da (Cooper et al. 1989). Surfactin was first isolated from *B. subtilis* by Arima et al. (1968), and the name assigned to it is due to its surfactant activity (Arima et al. 1968). The complete structure was determined as a macrolide lipopeptide, which was confirmed by Kakinuma et al. (1969). Since this initial discovery, surfactin serve as the major class of this cyclic lipopeptides.

The surfactins consist of a polar end of seven α -amino acids and an acyl chain of hydrophobic β -hydroxy fatty acids. The amino acids are arranged in the chiral sequence LLDLLDL with hydrophobic amino acids located at carbons 2, 3, 4, 6, and 7, while hydrophilic amino acids are located at carbons 1 and 5 as glutamyl and aspartyl, respectively (Fig. 2.1). Surfactin is rated one of the most biologically active biosurfactant that has been discovered (Kakinuma et al. 1969).

Surfactin is soluble in polar and nonpolar solvents due to the hydrophilic and hydrophobic moieties, which explains most of its many attributes. Either moiety has the chemical ability to partition interfaces between different surface polarities. This class is a highly active compound with high ratings for detergency, foaming, dispersing, and emulsifying qualities. While surfactins belong to the major group of biosurfactants, they are microbial in origin and have several advantages over chemical derivatives or surfactants and these include lower toxicity, higher biodegradability, and ability to remain biologically active over a wide range of pH values

and temperatures (Cameotra and Makkar 1998). Again this is due to the amino acid composition, and specifically aspartic acid and glycine are the major amino acids that make surfactin amenable to microbial degradation and chemical decompositions.

According to Seydlova and Svobodova 2008, surfactin is the most powerful biosurfactants. It lowers the surface tension of water from 72 to 27 mN m⁻¹ at a concentration as low as 10 µM. Further, surfactin is amphoteric, possessing both a positive and negative charge. This low molecular weight biosurfactant is characterized due to this weight as having the basic property of lowering surface, interfacial tensions efficiently (Cooper 1980) and other physiochemical properties (Bonmatin et al. 2003). Additional structural features of surfactin and other small molecular weight biosurfactants such as binding to heavy metals, detachment and attachment to and from surfaces, increasing surface, biofilm, and emulsifier production have been reviewed (Ron and Rosenberg 2001).

2.2.1 *In Vitro Production and Heterogeneity of Surfactins*

Surfactin was originally discovery from a nutrient broth culture of *B. subtilis* (Arima et al. 1968). Current procedures for most producing organisms are based on strains of *B. subtilis* cultured on a variety of synthetic and natural media (Das and Mukherjee 2007) and mixed fermentation procedures and with a variety of incubation temperatures (Makkar and Cameotra 1998). The initial media used to screen strain were the synthetic minimal mineral salt medium (Cooper et al. 1981) or on a semisynthetic medium (Landy et al. 1948). The yield of surfactin by *B. mojavensis* on either of these is very low, and in most instances, there is no surfactin produced at all by some strains, including submerged or stationary cultures. In our experience, the synthetic media reported above do not induce the synthesis of surfactin in *B. mojavensis*. However, the nutrient broth and nutrient broth amended with agar are excellent media and more surfactin is produced on the nutrient agar than broth (Snook et al. 2009). Nutrient agar is by far the best in terms of yield, although another medium that works well is a modified nutrient solution made from cottonseed formulation, Pharmamedia (Traders Protein, Southern Cotton Oil Company), amended with nutrient broth (Snook et al. 2009). On these media, most *B. mojavensis* strains tested produced a variety of isomers (Table 2.1). Some strains produce high levels of the desirable C-14 and C-15 alkyl chain lengths (unpublished data).

The biosynthesis of surfactins is nonribosomal (Desai and Banat 1997; von Dohren 1995) as is the synthesis of most secondary metabolites of bacteria. Regulation of this process is very complex because genes encoding nonribosomal peptide synthetases are organized into operons (Marahiel et al. 1997). The synthesis and regulation involve modular nonribosomal peptide synthetases that are transcriptionally induced under nutritionally limiting or stressful conditions (Desai and Banat 1997; Davis et al. 1999). Thus, the poorer the medium usually the better the product, which is the case of *B. mojavensis* as attempts at increasing the yield of

Table 2.1 Percentage of isolates producing specific surfactin isoforms by *B. mojavensis* in culture

| Acyl chain length | % Producing ^a surfactins | % Producing none |
|-------------------|-------------------------------------|------------------|
| C11 | 28 | 72 |
| C12 | 28 | 72 |
| C13 | 58 | 42 |
| C14 | 51 | 49 |
| C15 | 58 | 42 |
| C16 | 12 | 88 |
| C17 | 15 | 85 |

^aA total of 33 strains from all the major deserts were cultured on the nutrient broth amended with Pharmamedia, a cotton seed product, and tested for specific isoforms of surfactin after 30 h of incubation

surfactins by increasing or modifying carbohydrate and nitrogen levels result in luxuriant growth but no surfactin. Under each domain of a module, the seven amino acids of surfactin are bound to carriers, activated and chemically united to produce the characteristic head. Additional domains such as epimerizations within the module modify or catalyze specific amino acids to produce either D- or L-amino acids.

It was also reported that stressful conditions also induce sporulation by *Bacillus* species and indeed whose sporulation specific locus have been identified and shown to transcribe the synthesis of surfactin due to promoting transcription of the surfactin synthetase operon but only when glucose is in excess (Marahiel et al. 1993). In general, surfactin is induced by nitrogen starvation (Davis et al. 1999), although Marahiel et al. (1993) indicate that it is repressed by glutamate. There are variations in specifics of surfactin fermentation requirements, which are strain specific. For example, in one strain of *B. mojavensis* (synonym *B. licheniformis* JF-2), maximum biosurfactant synthesis occurs during the log phase, but when the cells enter stationary phase, which is controlled by a promoter gene, it stops the synthesis of surfactin and that which is produced is absorbed by the producing cells (Lin 1996). This indicates that the series of genes are controlled by extracellular environmental factor during dense cell growth and its associated biochemical pathways suggesting a “quorum sensing mechanism.”

Resorption of surfactin might be an explanation for data published earlier (Bacon and Hinton 2002), indicating that on nutrient agar all strains were antagonistic to *F. verticillioides*, but 42% were negative for surfactin C15, the most biologically active form (Table 2.1). Perhaps these stains reabsorbed the surfactins, but remained toxic when the fungal and bacterial cells interact during the in vitro assay for toxicity on nutrient agar surfactin. This form of inhibition was referred to as surface contact inhibition in that early work (Bacon and Hinton 2002). Yet to be explained is the ability of strains of any species to tolerate massive production of surfactin without self-destruction.

In summary, surfactin biosynthesis is regulated by genes relating to nutrient limiting conditions, which apparently are under transcriptional control. Surfactin biosynthesis and sporulation are coregulated under nutrient poor condition. Thus,

measures to increase its production under laboratory conditions should include a limit of nitrogen sources and concentration as well as amounts of carbon available to cell, usually under log phase. Strategy to improve strain for product formation might be achieved by altering specific cultivation conditions and by genetic manipulation (Abu-Ruwaida et al. 1991; Das and Mukherjee 2007; Davis et al. 1999).

2.2.2 Surfactin Biocontrol Features

The acyl chain length varies from C13 to C16 fatty acids that are linear, *iso*-, or *anteiso*-branched. The terminal amino acid is linked to the carboxyl group of the fatty acid and the carboxyl terminus is linked to the hydroxy or amino group of the acid resulting in a lactone cyclic peptide configuration with a hydrophobic tail. There are three structural analogues of surfactin, A (Fig. 2.1), B, and C, in which each differs in the sequence of amino acid, particularly in position 7. The essential difference is that Leu located at position 7 of surfactin A is replaced by valine and isoleucine in surfactin B and C, respectively (Baumgart et al. 1991).

Substitution of specific amino acids with other L- or D-amino acids exerts a change on the entire lipopeptide structure that modifies both the chemistry and biological activity of the molecule (Kikuchi and Hasumi 2002). For example, lichenysin is another cyclic heptapeptide with similarity to surfactin, except Glu in surfactin as opposed to the presence of Gln in lichenysin which confers a remarkable decrease in biological activity compared to surfactin (Konz et al. 1999). However, in lichenysin the makeup and length of the fatty acid tail also affect antimicrobial activity (Seydlova and Svobodova 2008; Yakimov et al. 1996), which was not measured in the Konz et al. (1999) work, but speculated here as being important for activity of surfactin to which it is structurally similar. Further, it was noted that surfactant activity also varied with the branching type in an increasing order from *anteiso* to *iso* to *normal*, which imparts the highest biocontrol activity relative to fungal toxicity (Maget-Dena and Ptak 1995; Yakimov et al. 1996). Thus, *normal* alkyl chains are more active than *iso* and *anteiso* being last active, but as observed in Table 2.1, several isomers coexist in the same extract that might be more active due to synergism. Although, of several strains tested, 42% did not produce any surfactins, they were all antagonistic to *Fusarium verticillium* (Bacon and Hinton 2002), suggesting the role of additional inhibitors, resorption of surfactin as described above, or failure of an inhibitory substance to diffuse into the test medium (and see below). The pattern of β -hydroxy fatty acids synthesized depends on the branched amino acids in the medium such as valine and isoleucine (Besson et al. 1992).

The physical characteristics of surfactins increase the benefits as biocontrol agents. Most lipopeptides are physically active over a variety of conditions and it is expected that surfactins will follow this generalization. Surfactin is active at below room temperature, and up to 80 or 100°C, and over a range of pH values from 3 to 11 (Cameotra and Makkar 1998; Joshi et al. 2008; Makkar and Cameotra

1998). *B. mojavensis* is an osmophile that tolerates seawater and salt concentrations up to 50 g l^{-1} and capable of anaerobic growth at 45°C (Javaheri et al. 1985; Janneman et al. 1983). However, we do not know if surfactin and other biocontrol modulators are produced or active at this concentration of salt. Further, *B. mojavensis* JF-2 is capable of anaerobic growth, provided DNA or deoxyribonucleoside are supplied to the medium (Folmsbee et al. 2004). The ability to tolerate high salt concentrations, extreme pH, and other factors describes the versatility of the bacterium to most environments.

The major uses of biosurfactants such as surfactin are for bioremediation of oil recovery and spills as well as emulsifiers, detergents, and cleaners, with some small uses in the cosmetic industry (Rahman et al. 2006). Worldwide well over 17 million tons of biosurfactants are produced and used for these industrial purposes (Whalley 1995). Surfactins specifically have a large range of applications for use in medicine (Rodrigues et al. 2006).

Very little of this was used for agriculture and almost none for biocontrol uses as advanced here. The anticipated uses in agriculture are expected to include biocontrol of phytopathogenic fungi, mycoplasmas, viral, and as an antitumor agent. Surfactin is also inhibitory to some extracellular enzymes, and evidence indicates that it has biocontrol activity over mosquito larva (Das and Mukherjee 2006; Geetha et al. 2010). To our knowledge, the only reported use of a biosurfactant as a biocontrol is against three zoosporic plant pathogens: *Pythium aphanidermatum*, *Phytophthora capsici*, and *Plasmopara lactuceae-radicis* (Stanghellini and Miller 1997). The uses of endophytic bacteria as biocontrol agents while widespread are not specifically designed for use in the in situ endophytic production of a biosurfactant as a biocontrol for specific disease of plants. Thus, the field is wide open for uses of specific biosurfactants for the control of specific diseases.

2.3 Plant–Bacterial Endophytic Relationships

Bacterial endophytes as intended here are those that are strictly intercellular (Bacon and Hinton 2002; Chanway 1998; McCully 2001), although the bacterium may colonize either below, above, or both plant axes. Our definition continues to exclude those species that are only transient dwellers of the intercellular habit which can and do within their life cycle become intracellular, oftentimes invading xylem tissues which usually become problematic and cause diseases in plants. Further, xylem endophytes, while not causing a disease, are viewed as a problem since endophytes that live within it are considered not ideal endophytes for biocontrol uses since such infected vessels are weakened and become nonfunctional and therefore detrimental to the plant (McCully 2001). *B. mojavensis* is associated with plants as biotrophic symbionts, but it is not known if this association is obligate or facultative, since all strains are readily isolated from soils (as spores) and their natural colonization from desert habitats such as plants have not been

documented. It is logical to assume that this species naturally inhabits desert plants as endophytes. However, we do know that under experimental conditions, the preferred niches colonized are roots and seedling stems immediately upon germination and the association remains throughout the life of the plant.

The method by which infection occurs is extremely important not only from a biotechnological viewpoint but also from understanding the basic biology of this bacterium. The infection process of *B. mojavensis* occurs from the topical application to seed, where we assume the infection takes place directly from cuts and abrasion of roots during the germination process. Upon germination the roots and mesocotyl are already infected and this infection is perpetuated throughout the plant axis during the entire season (Bacon and Hinton 2002). There are no direct studies on how plants are initially infected and most studies suggest that infection is accomplished through breaks, tares, or wounds of the roots of seedling during either germination or shortly thereafter (Patriquin and Dobereiner 1978). Foliage and stem infection can take place through the pores and stomatas. As we are dealing with nonpathogenic bacteria, there is very little evidence that they possess the arsenal of hydrolytic enzymes that are used to gain entry into roots and leaves characteristic of plant pathogens. We see no evidence for the activity of hydrolytic enzymes characteristic of pathogenic bacteria during or after infection of plants by *B. mojavensis*.

B. mojavensis and other bacterial endophytes benefit from inhabiting the plant's interior because it is a protected niche in which there is a constant source of nutrition. However, the concentrations of such nutrients have been reported as being sparse, suggesting that intercellular bacteria might live under oligotrophic conditions. This, however, is not correct as intercellular spaces are actually high in organic and inorganic nutrients (see review of Bacon and Hinton (2006)) necessary to support the growth of a complex intraspecific mixture of endophytes (Bacon and Hinton 2006; Canny 1995; Dong et al. 1994; Kursanov and Brovchenko 1970). This is due to the recent consideration that the apoplast, the intercellular spaces, and symplast are structurally the same. Nutrients within the apoplast are similar to and in similar concentrations as those in the symplast. Nutrient concentrations in the apoplast and symplast are interactive with the phloem, dispelling the earlier notion that the apoplast is relatively free of nutrients. Indeed, current research indicates that nutrient transport within plant tissues is considered to occur through an apoplastic route via the cell wall continuum and via the symplastic route via the plasmodesmata (Canny 1995).

Bacterial endophytes in general and *B. mojavensis* in particular are not oligotrophs, but rather copiotrophs. Thus, the intercellular space in maize is rich in substances necessary to support the oligotrophic growth of *B. mojavensis* supporting the observation of large numbers of bacterial cells observed in the several intercellular spaces (Fig. 2.2). Additional information on bacterial-induced morphological and chemical changes within cellular types of the endophytic niche has been described in an earlier review (Bacon and Hinton 2006). These morphological changes are presumably harmless as diseases are not observed, not even under stresses such as drought.

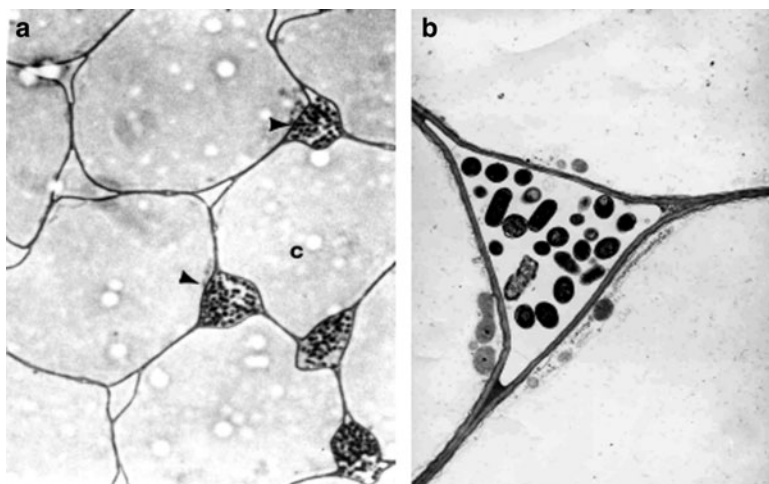


Fig. 2.2 *Bacillus mojavensis* in the intercellular spaces of maize: (a) Light microscopy showing bacterial cells located within the intercellular spaces of the cortex, c, of maize root (arrow), 40 \times . (b) Transmission electron micrograph of maize root showing bacteria between the intercellular spaces formed by three adjacent cells, 7,250 \times (Hinton and Bacon 1995)

2.4 Plant Growth Effects

Plant growth responses to bacteria are well established for several crop plants and we presented evidence of the effect of *B. mojavensis* on maize and bean growth indicating growth enhancements on both a monocot and a dicot (Figs. 2.3 and 2.4). The factors responsible for growth enhancements are unknown, but might reflect the exogenous production of phytohormones such as ethylene, auxins, and cytokinins. We have not examined the *in vitro* production of plant growth hormones by *B. mojavensis*, and it has been demonstrated that a plant-associated bacteria (Kuklinsky-Sobral et al. 2004) but not necessarily endophyte as defined in this review can produce hormones. However, it is not uncommon for bacteria to produce phytohormones, which have been demonstrated for several species (Arshad and Frankenberger 1991). Growth enhancements are also observed on *B. mojavensis*-infected maize plants grown in the presence of pathogenic isolates of *F. verticillioides* (Fig. 2.3).

In addition to the production of phytohormones plant growth, other factors of abiotic nature should also be considered. Other plant growth effects attributed to endophytic bacteria are enhanced mineral uptake such as the solubilizing of bound soil iron and phosphorus and by providing the plant with siderophores and nitrogen (Sturz et al. 2000; Surette et al. 2003). The siderophore producing ability of *B. mojavensis* has been demonstrated, but its interaction at the maize root level has not been determined (unpublished). Additional effects are those that interact with soil bacterial population, prior crop history and the buildup of soil bacteria and

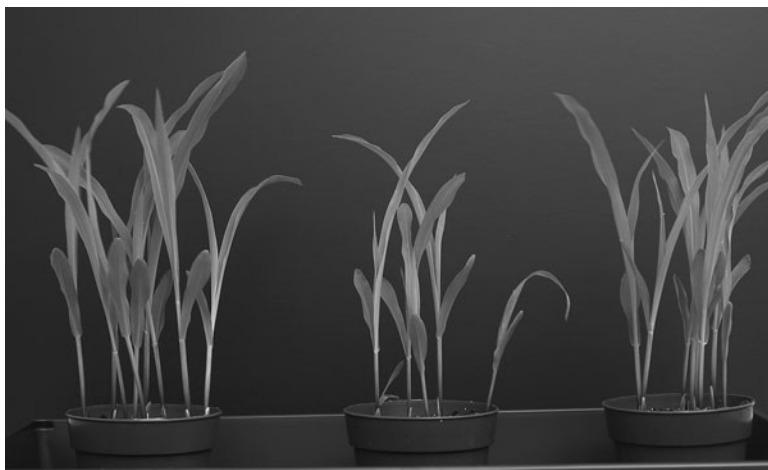


Fig. 2.3 Effects of *Bacillus mojavenensis* endophytic infection on plant growth: *Left*, growth of maize shoot inoculated with bacterium; *Middle*, inoculated with *Fusarium verticillioides*; *Right*, inoculated with the fungus and bacterium (Bacon and Hinton 2002)

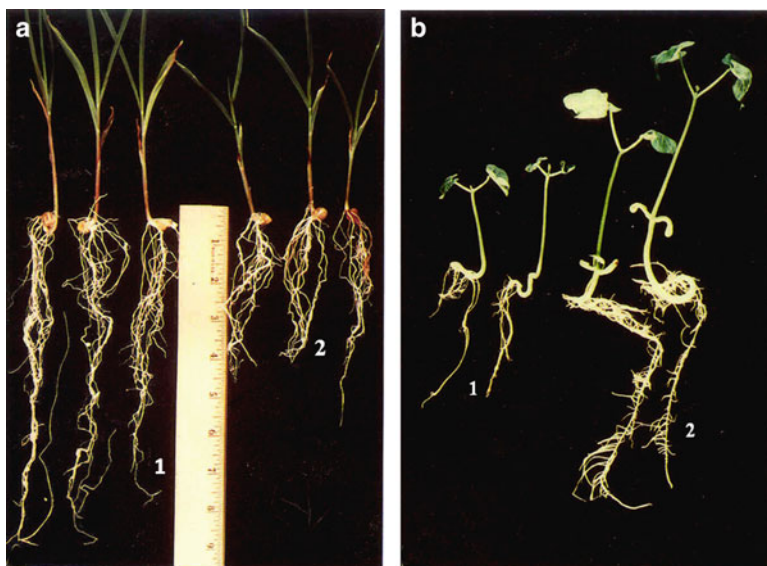


Fig. 2.4 (a) Effects of bacterium on maize root growth, (1) and without the bacterium (2). (b) Effects of bacterium on 2-week-old growth of beans inoculated without (1), and with bacterium (2); (Bacon and Hinton 2002)

the resulting benefits derived from allelopathy as indicated in the above section on transformation. In addition to soil nitrogen, i.e., a root effect, recent evidence has indicated the endophytic fixation of nitrogen in rice by a leaf endophyte isolated from wild rice by a species of *Herbaspirillum* (Elbeltagy et al. 2001), and similar

analyses by other foliage endophytes such as in sorghum (James et al. 1997). In rice it has been estimated that as much as $200 \text{ kg N ha}^{-1} \text{ year}^{-1}$ can be produced by the use of endophytes in plants (Ladha and Reddy 1995). Thus, nitrogen fixation can be transferred to endophytic bacteria, which can be accomplished through the use of *B. mojavensis* and if necessary by genetic alteration to accomplish the task.

The discussion above describes direct effects on plant growth by the bacterium. There are other effects that relate to plant health, which bacterial endophytes such as *B. mojavensis* might relieve. It is anticipated that with the control of pathogenic organisms, plant growth can be maximized with basic cultural condition. The resulting growth is due to lack of parasitisms and diseases, as well as decreased susceptibility to abiotic stresses such as drought and frost tolerance or resistance.

2.5 Product Transformations

Maize and other cereals produce a group of phytoanticipins the benzoxazinones and their decomposition products (benzoxazolinones and their methyl derivatives) as part of a constitutive defense system capable of deterring insects and pathogenic fungi (Niemeyer 1988). In addition to serving as defensive metabolites, they possess allelopathic qualities (Singh et al. 2003). Due to this very wide spectrum of biological activity, they are commonly referred to as a group of allelochemicals and offer a great deal of promise for natural controls of pests. The main benzoxazinones produced by maize are 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one and its dimethoxy derivative 2,4-dihydroxy-6,7-dimethoxy-1,4-benzoxazin-3(4*H*)-one with the former occurring in greater concentrations (Cambier et al. 2000; Whitney and Mortimore 1959). Since these benzoxazinones are highly toxic and reactive, they are also toxic to the plant but are stored as the nontoxic conjugate, a glycoside. The mechanism is put into operation from injury from insects or pathogenic response and are degraded to their unstable and highly toxic agluconic forms, which spontaneously decompose to the corresponding nonplant toxic benzoxazolinones, such as MBOA (6-methoxy-2-benzoxazolinone) and BOA (benzoxazolin-2-one) (Niemeyer 1988). These toxic decomposition products are very stable and remain in debris and maize stubble. Over time apparently maize pathogens have developed resistance to these benzoxazolinones, which is offered as the major reason why maize fields are characterized by fungi that are predominantly maize pathogens with resistance to MBOA and BOA (Glenn 2001; Glenn and Bacon 1998) as observed in *F. verticillioides* (Glenn et al. 2001). Thus, MBOA and BOA are found in plant debris and soil as stable degradation products of DIMBOA and DIBOA, which are considered useful allelochemicals for other uses (Fomsgaard et al. 2004) but are of no consequence for biocontrol of maize pathogens.

It was recently discovered that *B. mojavensis* has the ability to tolerate the benzoxazolinones and in fact transforms BOA and MBOA into 2-amino-3*H*-phenoxazin-3-one (APO), which is stable and very toxic transformation product to

F. verticillioides and other maize pathogens (Bacon et al. 2007). This substance is now a much better fungicide than the original BOA since *F. verticillioides* is not resistance to it. The results indicate that the bacterium metabolizes a plant metabolite, preventing the usual series of transformations of this same metabolite by *F. verticillioides* and other maize fungal pathogens to nontoxic products, resulting in the accumulation of APO. Thus, an enhanced biocontrol is suggested by this in vitro study. Additional evidence of biotransformation by *B. mojavensis* and other novel enzymatic activities have yet to be reported presumably due to the relative recent attempts at exploiting this species.

2.6 Fungal–Bacterium Interaction

The successful use of a biocontrol bacterium depends on its value under field condition to control the target species, usually a pathogenic fungus that may be equally competitive within specific environments. *F. verticillioides* produces a number of secondary metabolites some of which are considered as antibiotics providing this fungus with a competitive edge. Fusaric acid (5-butylicpicolinic acid) was first implicated in the pathogenesis of tomato wilt cause by *F. oxysporum* f.sp. *lycopersici*. Fusaric acid is now known to be a common metabolite of the genus *Fusarium* (Bacon et al. 1996) and other biological activities are assigned to it. It is a mild mycotoxin (Porter et al. 1995; Voss et al. 1999) and is pharmacologically active (Porter et al. 1995; Ballio 1981; Bungo et al. 1999).

In addition to mammalian toxicity, fusaric acid is toxic to vegetative growth of plants in general (Corden and Diamond 1959; D’Alton and Etherton 1984; Drysdale 1984; Marrè et al. 1993) as well as to a variety of physiological processes of plants (Luz et al. 1990; Marrè et al. 1993; Mace and Solit 1966; MacHardy and Beckman 1981; Sanwal and Waygood 1961). Thus, plants infected with *Fusarium* species, endophytic and otherwise, should also show degrees of growth restrictions, provided fusaric acid is produced in planta.

Fusaric acid also has antibiotic effects on both Gram-negative and Gram-positive bacteria including *Bacillus*, *Paenibacillus*, and *Pseudomonas* species (Arias 1985; Landa et al. 2002; Luz et al. 1990; Notz et al. 2002; Schnider-Keel et al. 2000). We have determined that high levels of fusaric acid exhibited toxicity to some strains of *B. mojavensis* (Table 2.2) (Bacon et al. 2006). This indicates that the endophytic states of *Fusarium* species might have a competitive edge over *B. mojavensis* and any other biocontrol bacterium sensitive to fusaric acid. Fusaric acid sensitivity is not established since the data use what might be considered high concentrations of fusaric acid. These levels are, however, low enough and do not show a response to maize and are within the concentrations used by others to demonstrate. We have developed mutants of *B. mojavensis* that are tolerant to fusaric acid at these concentrations and higher. These mutants might prove superior under field conditions provided all the other qualities such as endophytism and surfactin production are maintained. Fusaric acid is a common contaminate in

Table 2.2 Inhibitory activity of fusaric acid ($100 \mu\text{g ml}^{-1}$) on the specific rate of growth of *Bacillus mojavensis* strains and their antagonism to *F. verticillioides* (Bacon et al. 2006)

| Strains | Origin | % Fusaric acid inhibition ^a (100 g ml^{-1}) |
|---------------------------|---------------|--|
| RRC 101 | Corn, Italy | 59a |
| RRC 112 | Mutant of 101 | 81b |
| NRRL B-14698 ^T | Mojave | 92b |
| NRRL B-14699 | Mojave | 21c |
| NRRL B-14700 | Mojave | 76a |
| NRRL B-14703 | Gobi | 91a |
| NRRL B-14708 | Gobi | 0d |
| NRRL B-14706 | Gobi | 26c |
| NRRL B-14710 | Gobi | 87b |
| NRRL B-14714 | Sahara | 64a |
| NRRL B-14716 | Sahara | 77a |
| NRRL B-14817 | Sahara | 62a |
| NRRL B-14824 | Sahara | 69a |

^aPercentage of fusaric acid inhibition was determined within a 48-h incubation period at 30°C . Values represent the means of six replicate cultures. Means within a column followed by a different letter were significantly different at a *P* value of 0.05 according to Fisher's protected least significant difference test

feedstuffs made from maize (Smith and Sousadias 1993), indicating its natural occurrence. Nevertheless, fusaric acid and possibly other *Fusarium* specific metabolites must be considered a major problem with the use of this and other bacteria. The manner by which fusaric acid tolerance is expressed, i.e., production of an extracellular degrading enzyme or it is impermeable to membrane-regulated transport, is not known but must be determined before such mutant will have a valid place to control the growth of *Fusarium* species under field conditions.

2.7 Conclusion

Endophytic bacteria as defined in this review describe *B. mojavensis* as a bacterium that dwells within the intercellular spaces of plants, is highly compatible with plants, it does not impose negative reactions to the plant, and its interactive metabolism with the host influences growth, maturations, and tolerance to diseases. *B. mojavensis* offers additional qualities of being competitive with some fungi, particularly those that are also endophytic by the production of isomers of surfactins, a biosurfactant that is described as being the most potent known of the lipopeptide group. The surfactins are very active, and are readily degradable, thus increasing its desirability for use in biological controls. The target group of fungi such as *F. verticillioides* and the other *Fusarium* species are also endophytic and competent in production of similar antibiotics some of which are toxic to bacteria. However, there are strains naturally resistant and laboratory generated mutants that should obviate this problem. These should compete well under field conditions with

F. verticillioides resulting in mycotoxin reduction because under controlled conditions the fumonisin mycotoxins were considerably reduced. Several plasmids have been identified useful for transformation of *B. mojavensis* with several fluorescent proteins to serve as ecological marker for field studies (Olubajo and Bacon 2008). This species of bacteria also produces a battery of extracellular specific enzymes capable of the transformation of maize phytoanticipins into more stable and fungitoxic compounds. Its role in related environmental processes such as phytoremediation is noteworthy, which while not a direct plant growth response is an important process that should facilitate plant growth. Current technology can be used to modify *B. mojavensis* for application in increasing the nutritional value of agronomic plants that are lacking in specific animal and human growth requirements. The process, surrogately transformation of plants, offers an alternative to genomic transformation of plants, which is controversial and objectionable to some.

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