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A. Leonardo Iniguez
University of Wisconsin-Madison

Yuemei Dong
University of Wisconsin-Madison

Heather D. Carter
Ohio State University

Brian M. M. Ahmer
Ohio State University

Julie M. Stone
University of Nebraska-Lincoln, jstone2@unl.edu

See next page for additional authors

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Authors

A. Leonardo Iniguez, Yuemei Dong, Heather D. Carter, Brian M. M. Ahmer, Julie M. Stone, and Eric W. Triplett

Regulation of Enteric Endophytic Bacterial Colonization by Plant Defenses

A. Leonardo Iniguez,[1] Yuemei Dong,[1] Heather D. Carter,[2] Brian M. M. Ahmer,[2] Julie M. Stone,[3] and Eric W. Triplett[4]

[1]University of Wisconsin-Madison, Department of Agronomy, 1575 Linden Drive, Madison 53706 U.S.A.; [2]The Ohio State University, Department of Microbiology, 484 West 12th Ave. Columbus 43210 U.S.A.; [3]University of Nebraska-Lincoln, Department of Biochemistry, 1901 Vine St., Lincoln 68588-0664 U.S.A.; [4]University of Florida, Department of Microbiology and Cell Science, P.O. Box 110700, Gainesville 32611-0700 U.S.A.

Bacterial endophytes reside within the interior of plants without causing disease or forming symbiotic structures. Some endophytes, such as *Klebsiella pneumoniae* 342 (Kp342), enhance plant growth and nutrition. Others, such as *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), are human pathogens that contaminate raw produce. Several lines of evidence are presented here to support the hypothesis that plant defense response pathways regulate colonization by endophytic bacteria. An ethylene-insensitive mutant of *Medicago truncatula* is hypercolonized by Kp342 compared to the parent genotype. Addition of ethylene, a signal molecule for induced systemic resistance in plants, decreased endophytic colonization in *Medicago* spp. This ethylene-mediated inhibition of endophytic colonization was reversed by addition of the ethylene action inhibitor, 1-methylcyclopropene. Colonization of *Medicago* spp. by *S. typhimurium* also was affected by exogenous ethylene. Mutants lacking flagella or a component of the type III secretion system of *Salmonella* pathogenicity island 1 (TTSS-SPI1) colonize the interior of *Medicago* spp. in higher numbers than the wild type. *Arabidopsis* defense response-related genotypes indicated that only salicylic acid (SA)-independent defense responses contribute to restricting colonization by Kp342. In contrast, colonization by *S. typhimurium* is affected by both SA-dependent and -independent responses. *S. typhimurium* mutants further delineated these responses, suggesting that both flagella and TTSS-SPI1 effectors can be recognized. Flagella act primarily through SA-independent responses (compromising SA accumulation still affected colonization in the absence of flagella). Removal of a TTSS-SPI1 effector resulted in hypercolonization regardless of whether the genotype was affected in either SA-dependent or SA-independent responses. Consistent with these results, *S. typhimurium* activates the promoter of PR1, a SA-dependent pathogenesis-related gene, while *S. typhimurium* mutants lacking the TTSS-SPI1 failed to activate this promoter. These observations suggest approaches to reduce contamination of raw produce by human enteric pathogens and to increase the number of growth-promoting bacteria in plants.

Additional keywords: food safety, nitrogen fixation.

Corresponding author: Eric W. Triplett; Telephone: 352-392-5430; Fax: 352-392-5922; E-mail: ewt@ufl.edu

Current address of Y. Dong: Johns Hopkins University, Department of Molecular Microbiology & Immunology, 615 Wolfe St., Baltimore, MD 21205 U.S.A.

Bacteria interact with plants in four ways, as pathogens, symbionts, epiphytes, or endophytes. Of these four types of bacteria–plant interactions, endophytic interactions are the least studied and least understood. Endophytes are defined here as bacteria that enter the interior of plants without causing disease symptoms or eliciting the formation of symbiotic structures. Endophytic bacteria are of agronomic interest because they can enhance plant growth and improve the nutrition of plants through nitrogen fixation (Boddey et al. 2003; Sevilla et al. 2001). They are also of medical interest because some bacterial endophytes are human pathogens that cannot effectively be removed by surface sterilization (Beuchat et al. 2001; Proctor et al. 2001; Taormina et al. 1999; Weissinger and Beuchat 2000; Weissinger et al. 2001).

In previous work, different species or strains of enteric bacteria were found to differ greatly in their ability to colonize the interior of *Medicago sativa* (alfalfa) roots (Dong et al. 2003a). However, the mechanism of this strain specificity is not known. A strain isolated from maize, *Klebsiella pneumoniae* 342 (Kp342), colonizes the interior of several host plants in higher numbers than any other strain tested (up to 10^7 cells/g of fresh weight) (Dong et al. 2003a and b). This strain, originally isolated from a nitrogen-efficient maize line (Chelius and Triplett 2000), fixes N_2 and increases maize yield in the field (Riggs et al. 2001). Kp342 also expresses nitrogenase in planta (Chelius and Triplett 2000) and occupies the interior of plants in much higher numbers than *Klebsiella* spp. that were not of plant origin (Dong et al. 2003a). Fewer than 10 cells of Kp342 are sufficient in the inoculum to fully colonize the plant (Dong et al. 2003a). Similarly various *Salmonella* strains differed in their ability to colonize alfalfa roots (Dong et al. 2003a). To understand this strain specificity, an in-depth analysis of the basic biology of endophytic colonization has begun to elucidate the mechanisms that allow specific bacterial strains to be successful endophytes. Here, four lines of evidence are presented in support of the hypothesis that plant defense response pathways regulate endophytic colonization.

RESULTS

Four lines of evidence suggest that plant defenses regulate the number of endophytic bacteria within plant tissues: i) ethylene, a signal molecule for induced systemic resistance in plants, decreases endophytic colonization; ii) presence of bacterial extracellular components decreases endophytic colonization; iii) increased endophytic colonization in host genotypes with diminished plant defense responses; and iv) activation of

a promoter that controls an SA-dependent pathogenesis-related gene upon endophyte inoculation

Ethylene, a signal molecule for induced systemic resistance in plants, decreases endophytic colonization.

Ethylene has been studied extensively as a secondary messenger in the induction of a salicylic acid (SA)-independent plant defense pathway referred to as induced systemic resistance (ISR) (Knoester et al. 1998; Pieterse et al. 1998; Ton et al. 2001, 2002). Kp342 hypercolonized an ethylene-insensitive (*sickle*) mutant of *M. truncatula* (Fig. 1). This mutant also is hypernodulated following inoculation with the nitrogen-fixing symbiont *Sinorhizobium meliloti* (Penmetsa and Cook 1997). Consistent with this result, addition of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), to the growth media significantly reduced endophytic colonization in wild-type *M. sativa* by Kp342 and *Salmonella enterica* serovar Typhimurium strain 14028 (*S. typhimurium*) by three and four orders of magnitude, respectively (Fig. 2). The number of Kp342 cells within *M. truncatula* roots does not change significantly with ACC treatment until 4 days after inoculation. This evidence suggests that ACC does not inhibit invasion of Kp342 cells into the plant but triggers a response that can significantly lower the number of Kp342 cells 4 days after ACC treatment (Fig. 3). To test the effects of ethylene on *M. truncatula* before, during, and after inoculation, a time-course experiment was conducted (Fig. 4). In this experiment, gaseous ethylene (C_2H_4) was used rather than ACC because addition of C_2H_4 was required each day for up to 6 days during the time course. No difference in endophytic colonization was observed in plants exposed to C_2H_4 or ACC for the same time period (Fig. 4). This time course experiment showed that ethylene must be applied to the plants prior to or at the time of inoculation for maximal inhibition of endophytic colonization (Fig. 4). These results further corroborate those of Figure 3. That is, the effects of ethylene on endophytic colonization become significant 96 h after ethylene exposure. To determine whether ethylene affects endophytic colonization in monocots, wheat seedlings were exposed to varying amounts of ACC and inoculated with Kp342 and 14028 (Fig. 5). ACC caused a decline in
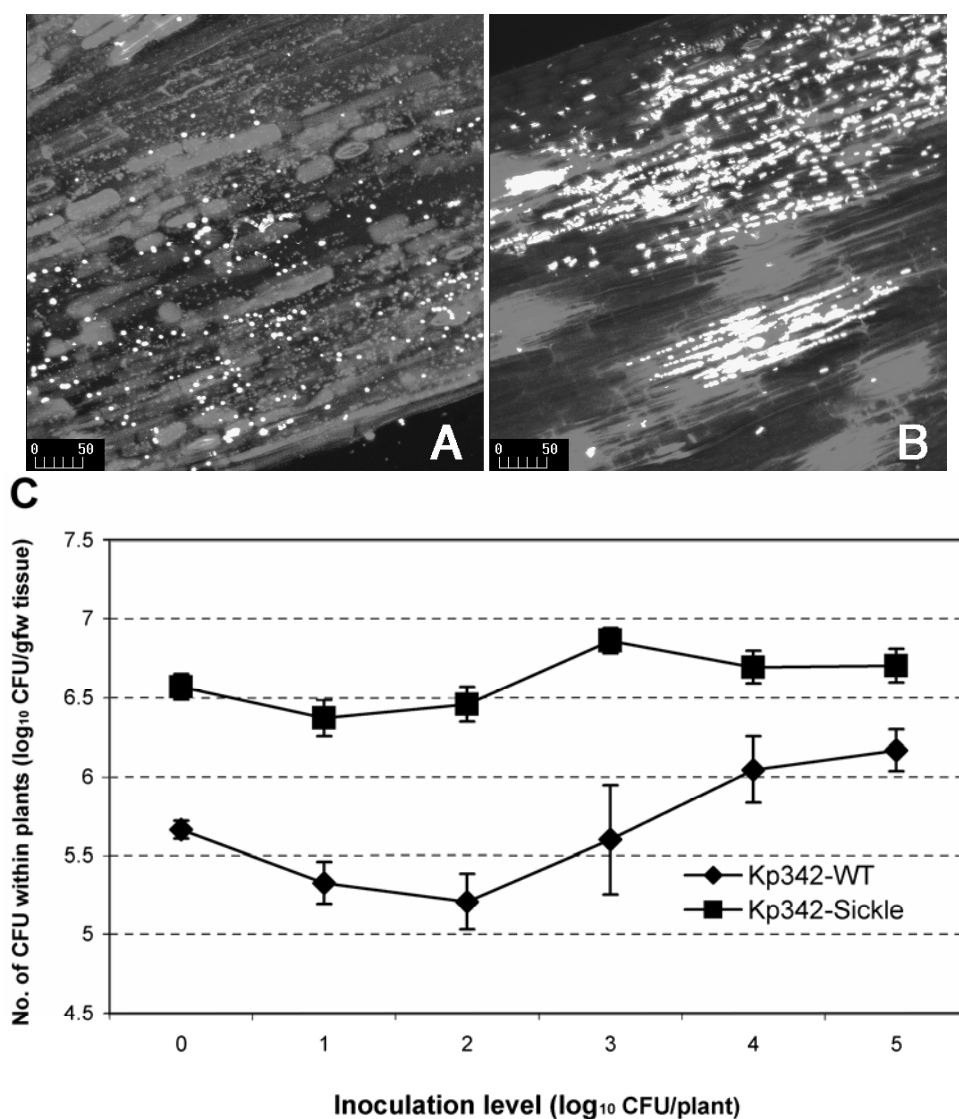

Fig. 1. Scanning laser confocal microscopy at ×20 magnification of longitudinal sections of *Medicago truncatula* **A,** wild-type and **B,** *sickle* mutant hypocotyls showing colonization by green fluorescent protein-labeled *Klebsiella pneumoniae* 342 (Kp342). Sections were visualized 9 days after inoculation. The inoculum level was 10^4 CFU/plant. Bars, 50 μm. **C,** Numbers of bacterial CFU recovered from interior of *M. truncatula* Gaerten cv. A17 wild-type and *sickle* mutant plant tissues 7 days after inoculation. Two-day-old seedlings were inoculated with Kp342 at different inoculum levels. Data points represent the means and the bars represent the standard errors about the mean resulting from four replicates with each replicate consisting of four plants.

the number of Kp342 and 14028 cells within wheat roots of 1.85 and 1.2 orders of magnitude, respectively (Fig. 5).

To confirm that the effects observed with ACC were specific to ethylene production, a specific inhibitor of ethylene-mediated signaling, 1-methylcyclopropene (1-MCP) (Porat et al. 1999; Serek et al. 1995), reversed the reduction in endophytic colonization of alfalfa observed with ACC (Fig. 2). Also, treatment of plants with 1-MCP resulted in significantly higher endophytic colonization regardless of the presence or absence of exogenous ACC in *M. truncatula* (Fig. 2).

These results suggest that endogenously produced ethylene limits the extent of endophytic colonization in *M. truncatula* but not in *M. sativa*.

Presence of bacterial extracellular components decreases endophytic colonization.

Bacterial extracellular components, such as flagella, are known to induce plant defenses (Felix et al. 1999; Gomez-Gomez and Boller 2000). A *Salmonella* 14028 mutant lacking both flagellin genes, *fliC* and *fljB*, fails to produce flagella in
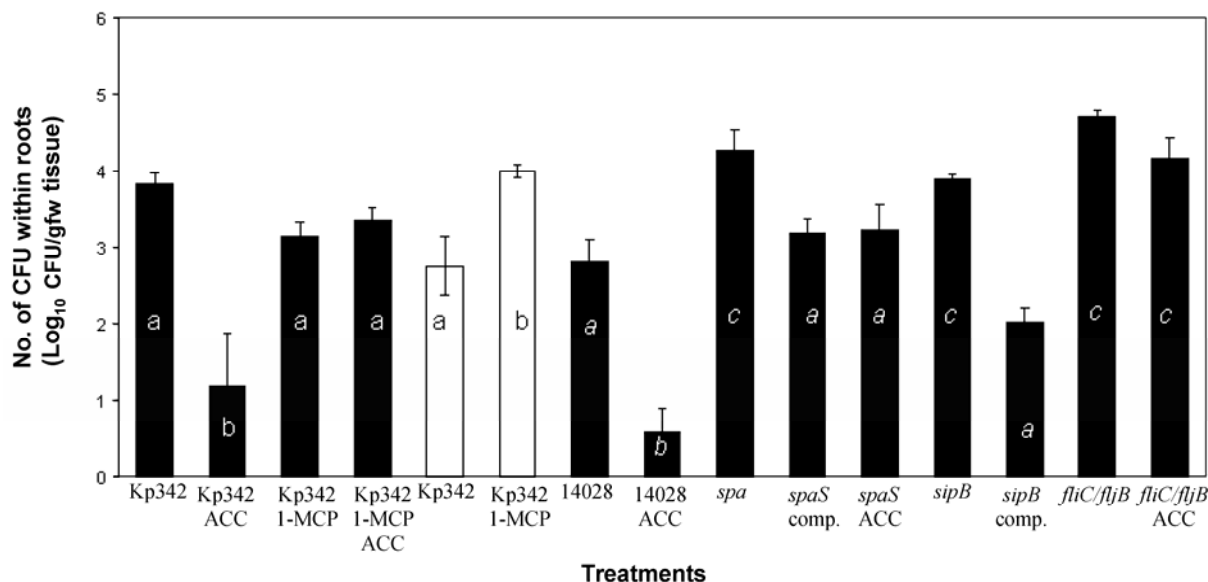

Fig. 2. Number of CFU recovered from the interior of *Medicago sativa* (closed columns) or *M. truncatula* (open columns) roots and hypocotyls were determined 5 and 7 days, respectively, post inoculation. Seedlings of *M. truncatula* were inoculated with 10^2 CFU of *Klebsiella pneumoniae* 342 (Kp342) in the presence and absence of 1 ppm of the ethylene action inhibitor, 1-methylcyclopropene (1-MCP). Seedlings of *M. sativa* were inoculated with Kp342, *Salmonella enterica* serovar Typhimurium strain 14028 (14028), the *spaS* mutant of 14028, the *spaS* mutant complemented with the *spaS* gene, the *sipB* mutant, the *sipB* mutant complemented with the *sipB* gene, and the double flagellin mutant with insertions in *fliC* and *fljB*. Treatments included an untreated control, application of the ethylene precursor, 5 μM 1-aminocyclopropane-1-carboxylic acid (ACC), or treatment with the ethylene action inhibitor, 1 ppm 1-MCP. The bars represent the standard errors of the mean resulting from four replicates, each replicate consisting of four plants.
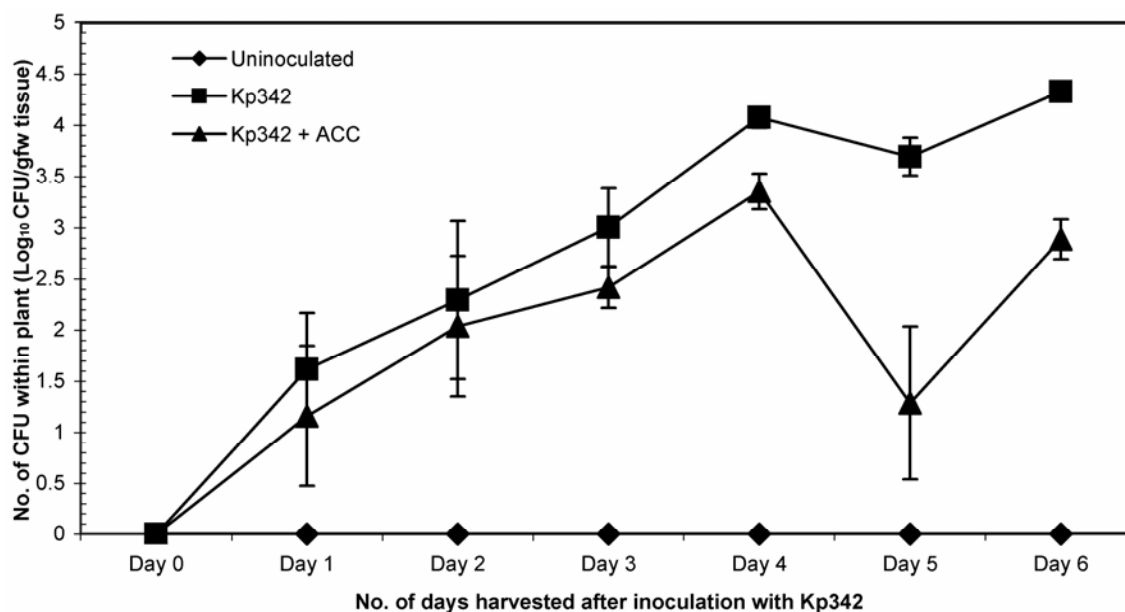

Fig. 3. Effect of 1-aminocyclopropane-1-carboxylic acid (ACC) on endophytic colonization over time. The number of CFU recovered from the interior of *Medicago truncatula* roots and hypocotyls was determined each day for 6 days after inoculation with 10^2 cells of *Klebsiella pneumoniae* 342 (Kp342) per plant. Plants were treated with and without ACC (5 μM) at the time of inoculation. The columns represent the mean CFU recovered from the plants, and the bars represent the standard errors of the means resulting from four replicate treatments; gfw = gram (fresh weight). ACC treatments are statistically different from the controls on days 4, 5, and 6 at the 5% level of confidence.

culture. This mutant showed significantly higher endophytic colonization, consistent with the notion that *Salmonella* flagellar components are specifically recognized and induce plant defenses. Another extracellular component of enteric bacteria, the type III secretion system encoded by *Salmonella* pathogenicity island 1 (TTSS-SPI1), also affects endophytic colonization. The TTSS-SPI1 is a virulence factor that promotes invasion of mammalian cells and elicits fluid secretion and inflammation in animal models (Zhang et al. 2003). The *sipB* and *spaS* genes are encoded within SPI1. The *spaS* gene encodes a structural component of the type III secretion appara-

tus, whereas the *sipB* gene encodes a protein with dual functions. SipB is required for translocation of other effectors and has effector properties of its own (Collazo and Galan 1997). Furthermore, secretion of SipB is independent of bacteria–host cell contact and, therefore, is not necessarily concomitant with translocation to host cells (Collazo and Galan 1997). Mutations in *spaS* and *sipB* resulted in much higher levels of colonization in alfalfa roots (Fig. 2). When these mutants were complemented with a wild-type copy of the gene, the reduced colonization phenotype was restored (Figs. 2). Similar results were obtained with the *sipB* mutant on wheat seedlings (Fig. 6).
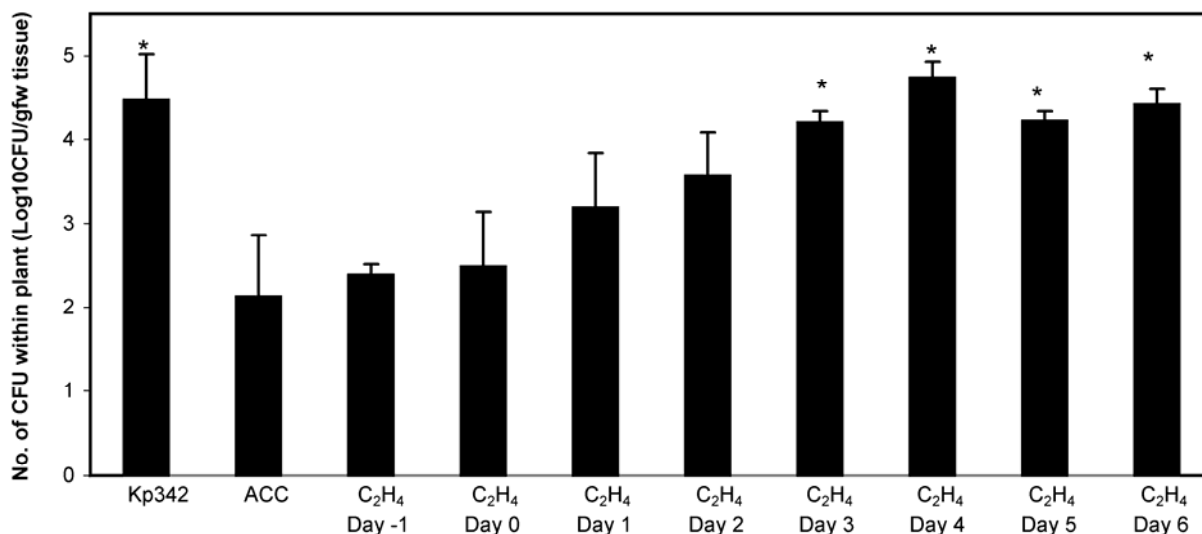

Fig. 4. Endophytic colonization of *Medicago truncatula* roots and hypocotyls treated with C_2H_4 on successive days. *Medicago truncatula* seedlings were inoculated with 10^2 cells per plant of *Klebsiella pneumoniae* 342 (Kp342). 1-Aminocyclopropane-1-carboxylic acid (ACC) (5 μM) was used as a control on day 0 to show that the effects of ACC and C_2H_4 are similar. C_2H_4 (5 μM) was applied to different sets of plants beginning 1 day prior to inoculation (day 1) and continuing each day up to 6 days after inoculation. The columns represent the mean CFU recovered from the plants 7 days post inoculation. The bars represent the standard errors of the means resulting from four replicate treatments; gfw = gram (fresh weight). Asterisks represent differences that are statistically significant from plants treated with C_2H_4 at day 0 at the 5% level of confidence.
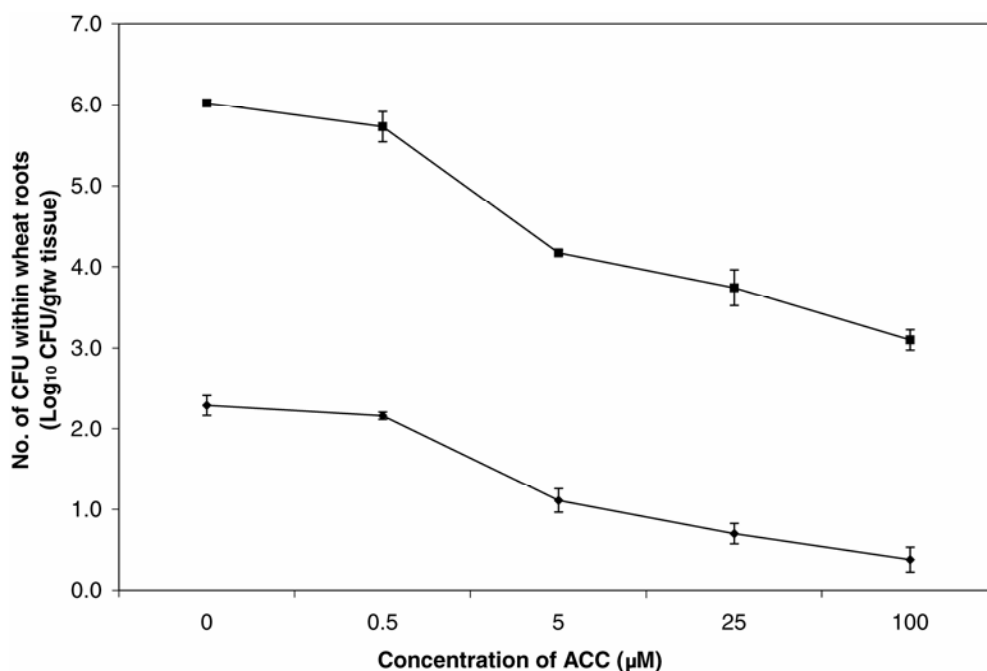

Fig. 5. Endophytic colonization of *Triticum aestivum* roots at in the presence of increasing concentrations of 1-aminocyclopropane-1-carboxylic acid (ACC). Number of CFU recovered from the interior of the roots and hypocotyls of wheat seedlings. Roots of 1-day-old seedlings were inoculated with 10^4 cells of *Salmonella enterica* serovar Typhimurium strain 14028 (diamonds) and 10^2 cells of *Klebsiella pneumoniae* 342 (squares). Plants were harvested 5 days after inoculation. The data points represent the means and the bars represent the standard errors of the means resulting from four replicate treatments.

With the removal of these extracellular components, ethylene-mediated inhibition of endophytic colonization, although still significant, was greatly reduced compared with the wild-type strain (Fig. 2). ACC decreases endophytic colonization by over two orders of magnitude for the wild-type strain (Fig. 2), whereas the ACC-induced decrease is only 0.5 to 1.1 orders of magnitude when the seedlings were inoculated with the *spaS* or double flagellin mutants, respectively (Fig. 2). The *Salmonella sipB* and double flagellin mutations also caused an increase of 2.5 and 2.4 orders of magnitude, respectively, in the number of *Salmonella* cells within wheat roots compared with wild-type *Salmonella* 14028. Complementation of the *sipB* mutant completely reversed the increase observed from the *sipB* mutation (Fig. 2).

Increased endophytic colonization in host genotypes with diminished plant defense responses.

The importance of plant defenses on endophytic colonization were examined using *Arabidopsis* lines impaired in plant defense. Strain 14028, the *sipB* and double flagellin mutants of 14028, and Kp342 were inoculated individually onto the roots of *Arabidopsis* wild-type Col-0, a *nahG* transgenic plant, and an *npr1* mutant (Fig. 7). The *nahG* transgenic plant produces a bacterial salicylate hydroxylase (Friedrich et al. 1995) that prevents the accumulation of SA in plants. The NPR1 protein regulates the DNA binding ability of transcription factors involved in plant defense (Despres et al. 2003; Mou et al. 2003), and the *Arabidopsis npr1* mutant is disrupted in both SA-mediated and SA-independent defense responses (Ton et al. 2002).
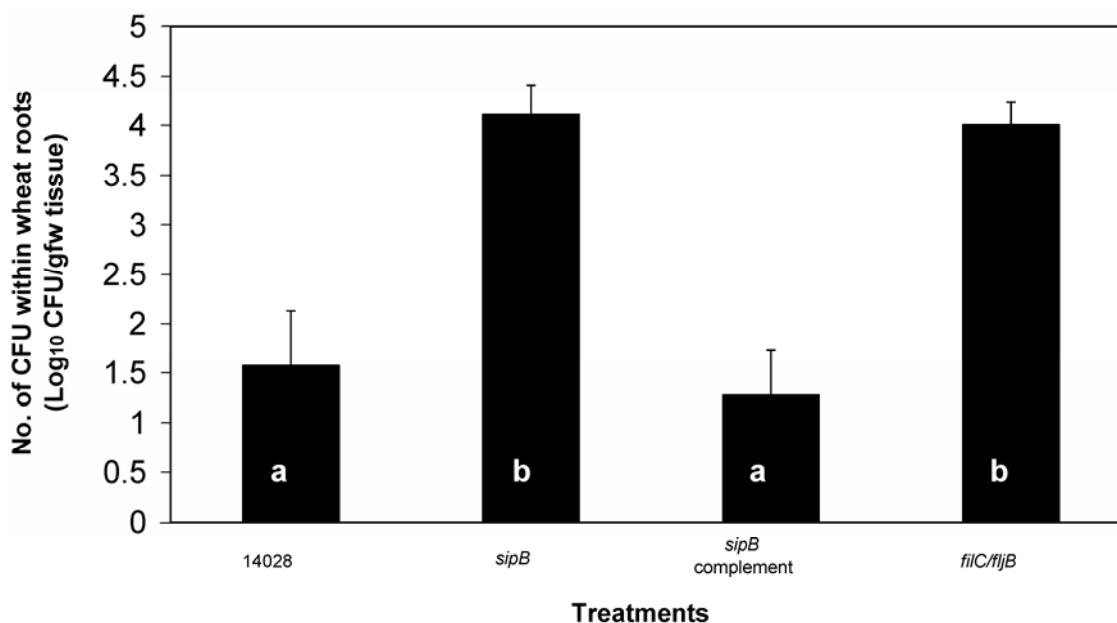

Fig. 6. Number of CFU recovered from the interior of wheat roots and hypocotyls. Roots of 1-day-old seedlings were inoculated with 10^4 cells of *Salmonella enterica* serovar Typhimurium strain 14028 (14028), the *sipB* mutant of 14028, the *sipB* mutant complemented with the *sipB* gene, and the double flagellin mutant (*fliC/fljB*) of 14028. Columns represent the means of each treatment and the bars represent the standard errors of the means resulting from four replicate treatments; gfw = gram (fresh weight).
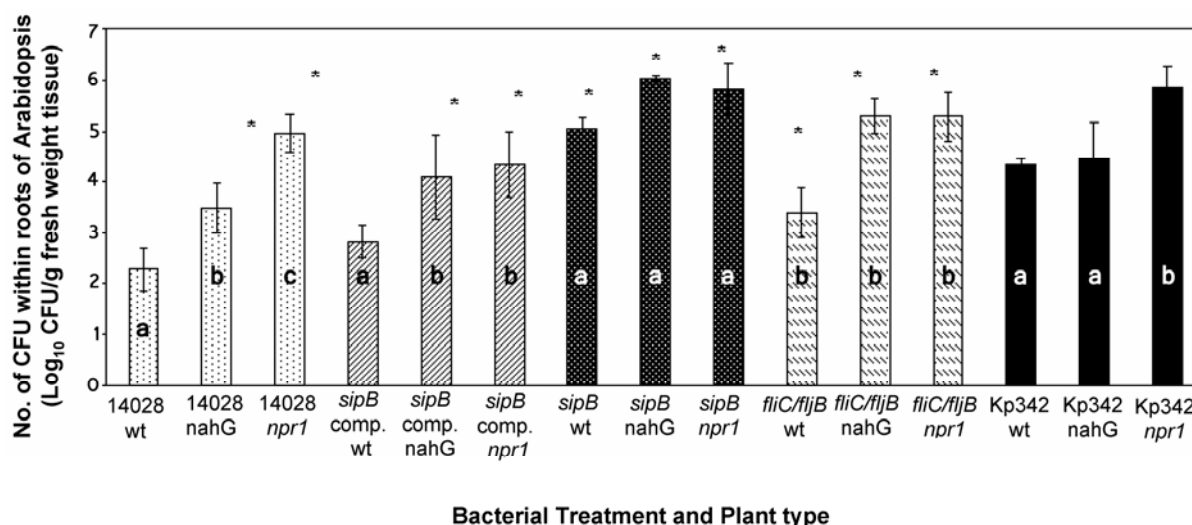

Fig. 7. Root endophytic colonization of three *Arabidopsis thaliana* genotypes inoculated with *Salmonella enterica* serovar Typhimurium strain 14028 (14028), the flagella mutant of 14028, the *sipB* mutant of 14028, the complemented *sipB* mutant, and *Klebsiella pneumoniae* 342 (Kp342). Number of CFU recovered from the interior of roots of *A. thaliana* cv. wild type, *nahG*, and *npr1*. The columns represent the means of each treatment. Each treatment consists of four replicates and each replicate consists of four plants. The bars represent the standard errors about the mean; gfw = gram (fresh weight). The letters in each column represent statistical differences with respect to the wild-type plant. The asterisks represent statistical differences with respect to the wild-type plant inoculated with 14028.

Colonization by Kp342 was not significantly different on wild-type *Arabidopsis* spp. compared with the *nahG* transgenic plants, suggesting that accumulation of SA is not important for restricting colonization by Kp342. However, colonization of the *npr1* mutant by Kp342 was 1.5 orders of magnitude greater than in wild-type *Arabidopsis* spp. These data suggest that SA-independent defense responses (defective in the *npr1* mutant) may contribute to reduced colonization by Kp342.

The interior colonization of *Arabidopsis* roots by 14028 was 1.2 to 2.7 orders of magnitude greater in the *nahG* transgenic and *npr1* mutant, respectively (Fig. 7), compared with wild-type plants, suggesting that both SA-dependent and -independent pathways are involved in restricting colonization. The roles of flagellin and TTSS-SPI1 in colonization were examined by mutational analysis. Both the *Salmonella* double flagellin mutant (*fliC/fljB*) and the TTSS-SPI1 (*sipB*) mutants colonized the roots of wild-type *Arabidopsis* spp. in significantly greater numbers than the wild-type strain 14028 (Fig. 7), supporting roles for both of these extracellular components in plant recognition.

Colonization by the flagella mutant was 1.9 orders of magnitude greater in the *nahG* transgenic and *npr1* mutant than in wild-type plants (Fig. 7). For the *nahG* transgenic plants, these results are consistent with colonization behavior observed for 14028. However, no difference was observed in endophytic colonization of the *npr1* mutant by 14028 or the flagella mutant, but the wild-type host was colonized significantly more by the flagella mutant compared with 14028. Equal colonization of the *nahG* transgenic and the *npr1* mutant by the *Salmonella* flagella mutant imply that endophyte recognition and the subsequent defenses induced by flagella are largely SA-independent. That is, a plant defective in SA accumulation still allows more colonization by a flagella-defective endophyte, whereas a mutant defective in both SA-dependent and -independent responses fails to exhibit super-enhanced colonization (as was observed for wild-type bacteria).
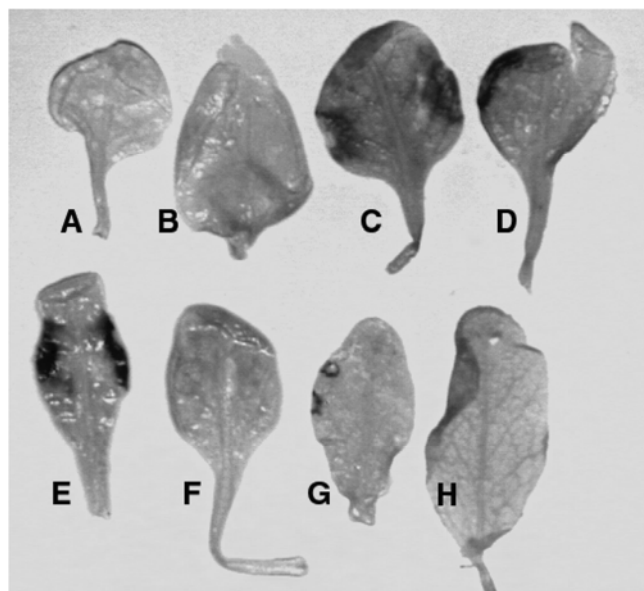

Fig. 8. Histochemical assays of **A–G,** *Arabidopsis thaliana PR1::GUS* and of **H,** *A. thaliana* wild type. The treatments were **A,** uninoculated, **B,** inoculation with H_2O, **C,** sprayed with 5 mM salicylic acid, **D,** leaves infiltrated with 10^7 CFU of *Pseudomonas syringae* DC3000, **E,** root inoculation with *Salmonella enterica* serovar Typhimurium strain 14028 (14028), **F,** root inoculation with the *sipB* mutant of 14028, **G,** and root inoculation with the *sipB* mutant complemented with the *sipB* gene. **H,** Uninoculated wild-type *A. thaliana*.

In contrast, data obtained with the TTSS-SPI1-defective *sipB* mutant suggest that the lack of TTSS-SPI1 effectors permits the avoidance of SA-dependent and -independent responses. Whereas colonization of wild-type plants was enhanced by the *sipB* TTSS-SPI1 mutation, colonization by *sipB* was not significantly different in *nahG* transgenic and *npr1* mutants. Therefore, although colonization by a bacterium defective in TTSS-SPI1 was significantly enhanced in wild-type plants, it was unaffected by compromising both SA-dependent and -independent defense pathways in the host plant. These data suggest that *sipB*-regulated TTSS-SPI1 effectors act downstream of SA and *npr1* in this system. As predicted, the increased colonization observed with the *sipB* mutant was reversed when the mutant was complemented with the wild-type gene.

These data also support the notion that the TTSS-SPI1 of 14028 induces both the SA-mediated and -independent responses, in agreement with 14028 induction of the SA-mediated PR1 promoter.

Activation of a promoter that controls an SA-dependent pathogenesis-related gene upon endophyte inoculation.

In support of the elicitation of plant defenses during endophytic colonization, the expression of the extensively studied plant defense response gene *PR1* (Beilmann et al. 1992) was tested by inoculation of *Arabidopsis thaliana PR1::GUS* with our enteric endophytes. As expected, the positive controls, application of SA or inoculation with the plant pathogen *Pseudomonas syringae* DC3000 PV288, strongly induced a *PR1::GUS* fusion in planta. The GUS activity of the *P. syringae* DC3000 PV288 and SA controls were 545 and 139 pmol 4-MU/mg of protein/min, respectively. Inoculation of roots with St14028 also induced *PR1::GUS* expression in distal leaves, displaying a GUS activity of 42 pmol 4-MU/mg of protein/min, (Fig. 8). In contrast, inoculation of roots with the *sipB* mutant and Kp342 showed no GUS induction with either the qualitative in vivo or the quantitative in vitro assays. Complementation of the *sipB* mutation restored GUS expression and activity (19 pmol 4-MU/mg of protein/min) (Fig. 8). Negative controls, where plants were sprayed with H_2O, leaf infiltration with phosphate-buffered saline (PBS), or root inoculation with PBS failed to induce *PR1::GUS* expression. Because the *PR1* promoter is induced by the SA signaling pathway (Stone et al. 2000), these data suggest that the TTSS-SPI1 induces SA-mediated defense signaling. Unlike 14028, inoculation with Kp342 did not result in *PR1::GUS* expression, suggesting that this endophyte does not induce SA-dependent defense responses (data not shown), consistent with *Klebsiella* spp. lacking flagella and TTSS-SPI1.

DISCUSSION

S. enterica serovar Typhimurium strain 14028, a human pathogen that does not cause disease in plants, induced both SA-dependent and -independent plant defenses. Reducing the virulence of the strain by removing TTSS-SPI1 increased endophytic colonization. The effector molecules secreted by this TTSS-SPI1 seem to be deleterious to the bacterium due to their ability to elicit both SA-mediated and -independent plant defenses. Another extracellular component, flagella, which have very little effect on virulence in animals, also induces SA-independent defense responses.

Another aspect of the strain specificity discovered here is that Kp342 is either resistant to SA-dependent responses or fails to induce them. This is based on two lines of evidence. First, nearly equal endophytic colonization of the wild-type and *nahG*-transformed plants by Kp342 was observed. Second, inoculation with Kp342 did not induce the expression of an SA-dependent gene, *PR1*. Thus, some strains may be insensi-

tive to SA-mediated responses (such as Kp342) whereas other strains (such as 14028) are sensitive to these responses. This may be a significant component of the mechanism for the strain specificity of endophytic colonization. Based on the results above, both extracellular components studied here elicit SA-independent defense responses. However, only the SA-dependent defense responses elicited by TTSS-SPI1 are effective in reducing colonization.
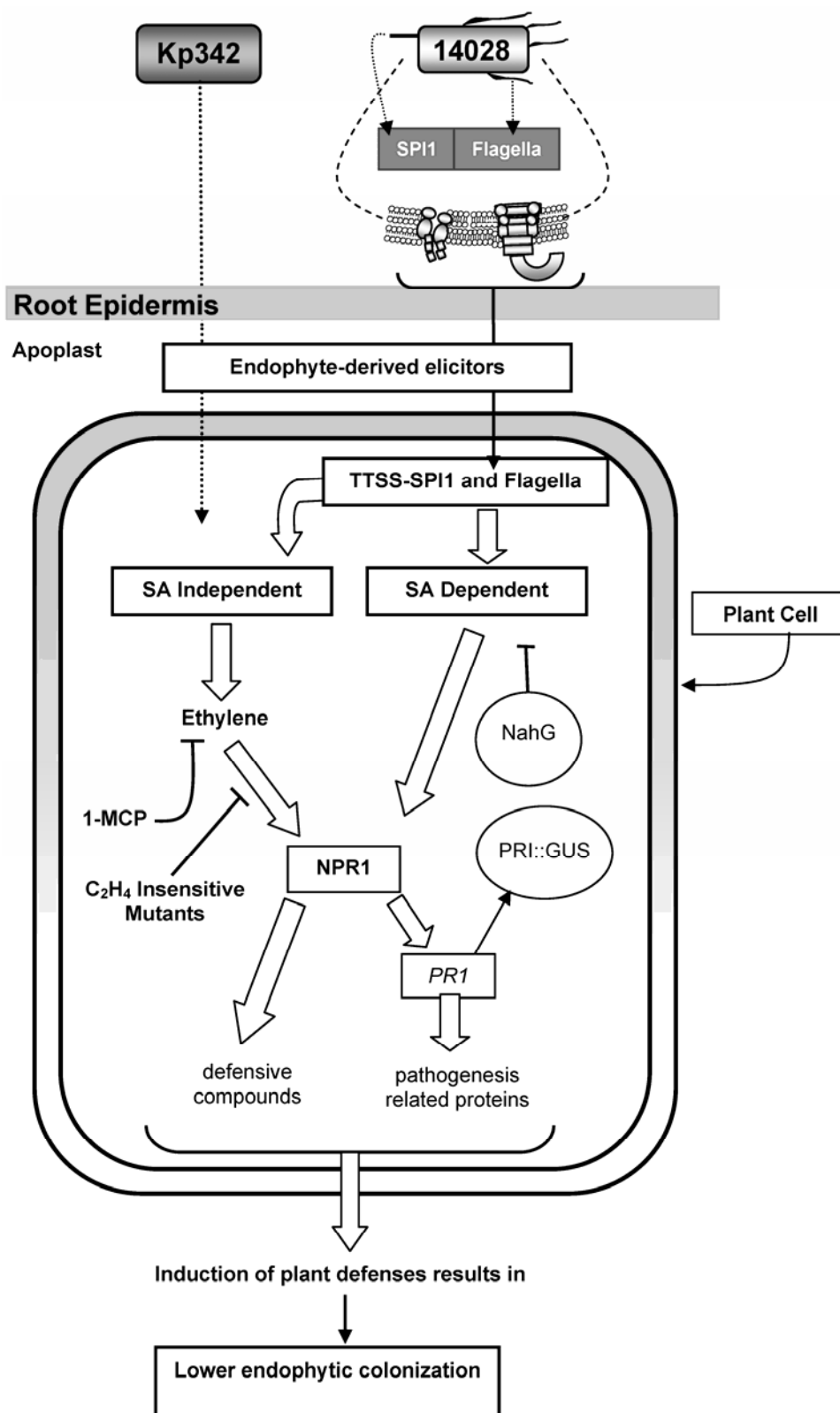

Fig. 9. Model for the regulation of the endophytic colonization of plants by enteric bacteria. Abbreviations: Kp342 = *Klebsiella pneumoniae* 342, 14028 = *Salmonella enterica* serovar Typhimurium strain 14028, SPI1 = *Salmonella* pathogenicity island 1, TTSS = type III secretion system, SA = salicylic acid, NahG = a bacterial salicylate hydroxylase (Friedrich et al. 1995), 1-MCP = 1-methylcyclopropene, C_2H_4 = ethylene, NPR1 = protein that regulates transcription factors involved in plant defense (Despres et al. 2003; Mou et al. 2003), and *PR1* = pathogenesis-related gene 1.

This work has two significant implications that likely are broadly applicable to monocots and dicots given the similar results obtained with *A. thaliana*, *M. sativa*, *M. truncatula*, and *Triticum aestivum*. With regard to food safety and the presence of *Salmonella* spp. and other bacterial pathogens residing within plant tissue, this work suggests a means to eliminate contamination of raw produce. According to the model of Latimer and associates (2001) and based on the number of 14028 cells observed here in alfalfa tissue, human consumption of 10 g of 14028-contaminated, surface-sterilized alfalfa is sufficient to virtually ensure salmonellosis. This estimate assumes that the plant defenses activated by 14028 do not influence the virulence of this pathogen in humans. After induction of SA-independent defense responses with ethylene treatment, the amount of surface-sterilized fresh tissue that must be consumed by a person to cause disease increases to 30 kg. Clearly, the induction of ethylene-mediated defense responses eliminates the possibility of illness from the consumption of contaminated tissue. However, in practice, because ethylene alters plant morphology, a means to induce these responses other than with ethylene treatment would be required in a commercial setting. A solution to this problem may be the overexpression of *npr1*, which is known to confer broad disease resistance in plants (Cao et al. 1998; Chern et al. 2001). Overexpression of this gene in vegetables is likely to reduce the number of enteric bacteria within plants to a level that is harmless to those who consume the raw produce.

Conversely, it may be desirable to increase the number of beneficial endophytes that enhance plant growth or nutrition. Here we show that colonization by a nitrogen-fixing endophyte is limited by SA-independent plant defense responses. Colonization of the *Arabidopsis* plant defense mutant (*npr1*) with Kp342 was significantly greater compared with the wild-type, whereas transgenic depletion of SA had no effect. Therefore, a Kp342 mutant resistant to SA-independent plant defense responses should be able to colonize plants in very high numbers.

In keeping with these observations, Kp342 lacks flagella, as do all *Klebsiella* spp., and TTSSs have not been identified in Kp342 (Dong et al. 2001). During the evolution of the Kp342–host plant association, Kp342 may have lost extracellular components that induce SA-mediated plant defense. Alternatively, the *Klebsiella* sp. may have lost these components prior to any association with plants, resulting in it being a common inhabitant of plants.

For a bacterial endophyte to colonize plants in high numbers, it must be able to avoid plant defenses by failing to produce those extracellular components that elicit plant defense. Bacteria that elude plant defenses can be thought of as "stealth bacteria." The data presented here suggest that endophytic colonization may be modulated to reduce levels of harmful bacteria or enhance levels of beneficial bacteria by altering the genotypes of endophytic bacteria and their host plants.

These data suggest a model that, for a bacterial endophyte to colonize plants in high numbers, it must be able to avoid plant defenses by failing to produce those extracellular components that elicit plant defense (Fig. 9). Future experiments may show that an overexpression of plant defenses still may be able to recognize endophytic bacteria that resist wild-type levels of plant defense expression.

MATERIALS AND METHODS

Bacterial strains and inoculum preparation.

The bacterial strains used in this work are listed in Table 1. BA3104 was constructed by sequential P22 transduction into 14028 using a P22HT*int* lysate grown on the SL3201 *fliC*::Tn10 *fljB*::MudJ strain kindly provided by Dr. Allison O'Brien (Schmitt et al. 2001). pHC112 was constructed by amplifying the *spaS* gene of 14028 (nucleotides 28 to 1,327 of GenBank accession number AE008832), using *Taq* DNA polymerase. The *spaS* fragment was cloned into pCR-2.1-TOPO (Invitrogen, Carlsbad, CA, U.S.A.), removed using *Eco*RI, and cloned into the *Eco*RI site of pWSK29 (Wang and Kushner 1991). pHC113 was constructed in the same way except that the *sipB* gene was amplified (nucleotides 18,133 to 20,138 of GenBank accession number AE008831). Bacterial strains were cultured and inoculum prepared as described previously (Dong et al. 2003a and b). In some cases, very low inoculum doses were used. To be confident that we were applying such low doses, each data point consists of a minimum of four plants

Table 1. Bacterial strains used in this work[a]

Strains	Abbreviations	Comments	Reference
Klebsiella pneumoniae 342 (Kp342)	Kp342	Maize endophyte Kp342	Chelius and Triplett 2000
Salmonella enterica serovar Typhimurium ATCC 14028	14028	Type strain provided by ATCC	ATCC
BA1502 (14028 *spaS1502*::MudJ)	*spaS*	TTSS SPI1 structural mutant	Ahmer et al. 1999
BA1502/pHC112	*spaS* complement	*spaS* mutant complemented with the *spaS* gene	This work
BA1577 (14028 *sipB1577*::MudJ)	*sipB*	TTSS SPI1 structural mutant	Ahmer et al. 1999
BA1577/pHC113	*sipB* complement	*sipB* mutant complemented with the *sipB* gene	This work
BA3104 (14028 *fliC*::Tn10 *fljB*::MudJ	*fliC/fljB*	Lacks two flagellin biosynthetic genes	This work
Pseudomonas syringae DC3000*avrRpt2*	NA	Contains *avrRpt2* on plasmid PV288[b]	Kunkel et al. 1993

[a] ATCC = American Type Culture Collection; TTSS SPI1 = type III secretion system of *Salmonella* pathogenicity island 1; NA = not available.
[b] Provided by Andrew Bent University of Wisconsin–Madison.

Table 2. Plants used in this work

Plant line	Comment	Reference
Medicago sativa cv. CUS101	Common line for alfalfa sprout production	…
M. truncatula Gaerten cv. A17	Provided by Doug Cook, University of California, Davis	…
M. truncatula mutant sickle (*skl*)	Provided by Doug Cook, University of California, Davis	Penmetsa and Cook 1997
M. truncatula Jester	Provided by Barry Rolfe, Australian National University	…
M. truncatula Jermalong	Provided by Edwin Bingham, University of Wisconsin-Madison	…
Arabidopsis thaliana cv. Col-0	Arabidopsis Biological Resource Center	…
A. thaliana cv. Col-0 *PR1*::*GUS*	Provided by Julie Stone University of Nebraska-Lincoln	Cao et al. 1994
A. thaliana cv. Col-0 *nahG*	Provided by Julie Stone University of Nebraska-Lincoln	Reuber et al. 1998
A. thaliana cv. Col-0 *npr1-4*	Provided by Julie Stone University of Nebraska-Lincoln	Cao et al. 1994
Triticum aestivum cv. Trenton	Hard red spring wheat line developed at North Dakota State University in 1995	…

per replicate and four replicates per treatment in those experiments where the inoculum dose size was varied.

Scanning confocal laser microscopy.

The methodology used here for scanning confocal laser microscopy (SCLM) was previously described (Dong et al. 2003a and b). Using this methodology, hypocotyls of *M. truncatula* mutant sickle (*skl*) and *M. truncatula* Jermalong were observed under SCLM with ×20 magnification through z sections ranging from 0.5 to 2 µm in thickness.

Seed surface sterilization, germination, inoculation, plant culture, and harvest.

The plants used in this work are listed in Table 2. The manipulation of plants, from seed surface sterilization to plant harvest, were carried out by methods developed previously (Dong et al. 2003a and b).

Determination of microbial population within surface-sterilized plant tissue.

With the exception of the *M. truncatula* sickle experiment, where the whole plant tissue was used to determine microbial populations, only the root and hypocotyl were examined for bacterial colonization. The procedures used for surface sterilization, determination of endophytic microbial populations, and statistical analysis were done as described previously (Dong et al. 2003a and b).

Assurance of endophytic colonization results.

To ensure that the endophytic colonization numbers presented reflect only the number of cells within the interior of plant tissue, previously developed methods were followed (Dong et al. 2003a and b). Furthermore, day 0 of the time course experiment (Fig. 3) served as a control to ensure that the endophytes did not enter the plants through wounds caused during harvesting or through the root surface as a result of the surface sterilization procedure. Day 0 data show that no Kp342 cells were recovered from the interior of alfalfa seedlings within 1 h after inoculation. This suggests that the methods used here to estimate microbial population within plants do not contribute to endophytic invasion of the apoplast.

Induction of ethylene response in seedlings.

To induce ethylene responses, seedlings were cultured in growth medium as described previously (Dong et al. 2003a) supplemented with 5 µM ACC. ACC was dissolved in water and filter sterilized prior to its addition to autoclaved plant growth media. In most experiments, seedlings were exposed to media containing ACC for 12 h prior to inoculation.

In the ethylene time course experiments, gaseous ethylene was added to the plants cultured in closed tubes to a final concentration of 5 µM. The stopper on these tubes was removed each day, flushed with fresh air, stopped, and re-treated with sufficient ethylene to bring to a final concentration of 5 µM.

Preparation and use of 1-MCP.

The gaseous ethylene action inhibitor, 1-MCP, was prepared and stored as described by Hall and associates (2000). 1-MCP was generated from EthylBloc, which was provided by A. B. Bleecker (University of Wisconsin, Madison, WI, U.S.A.). The concentration of 1-MCP in EthylBloc is 0.14%. A stock of 1-MCP of 100 ppm was created in a serum bottle 121.5 ml in volume. This was accomplished by adding 19.44 mg of EthylBloc and 0.5 ml of hot H_2O to the serum bottle and setting it to rest for 15 min. The stock was used to dispense 0.3 ml of headspace gas to 30-ml stopped test tubes where the plants were cultured, resulting in a final concentration of 1 ppm per tube.

The plant cultures were placed under conditions described previously (Dong et al. 2003a and b), with the exception of a rubber stopper used to conceal 1-MCP. The stoppers were removed daily, flushed with air, stopped again, and, finally, freshly prepared 1-MCP was added to the desired final concentration.

GUS histochemical staining and GUS fluorogenic assay.

Roots of transgenic *A. thaliana* Col-0 harboring a pathogenesis-related 1 (PR1) gene promoter fused to the bacterial *uidA* (β-glucuronidase) reporter gene (PR1::GUS) were inoculated with 10^7 CFU of *S. enterica* 14028, the 14028 *sipB* mutant, and the complemented *sipB* mutant. Exogenous application of SA (5 mM) and infiltration of leaves with 10^7 CFU of an avirulent strain of *P. syringae* DC3000 carrying the *avrRpt2* on plasmid PV288 were used as positive controls (Kunkel et al. 1993; Ton et al. 2002). The histochemical assay was performed as described by Sundaresan and associates (1995) with slight modifications (Sundaresan et al. 1995). Plants were immersed in staining buffer (50 mM sodium phosphate, pH 7, 10 mM EDTA, 0.1% Triton X-100, chloramphenicol at 100 µg/ml, 5 mM potassium ferricyanide and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Glc) at 0.5 mg/ml. Plants then were vacuum infiltrated, incubated overnight at 37°C, and destained with 70% ethanol.

To conduct the quantitative GUS fluorogenic assay, whole plants were flash-frozen in liquid N_2 and crushed. The fluorogenic assay and protein extraction were done as described by Jefferson and associates (1987). Protein concentration of the samples was determined using a BCA protein assay kit (Pierce, Rockford, IL, U.S.A.). GUS activity was measured as pmoles of 4-methyleunbelliferyl produced per milligram of protein per minute, abbreviated as pmol 4-MU/mg protein/min.

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