

ORIGINAL ARTICLE

Loline alkaloid production by fungal endophytes of *Fescue* species select for particular epiphytic bacterial microflora

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The leaves of fescue grasses are protected from herbivores by the production of loline alkaloids by the mutualist fungal endophytes *Neotyphodium* sp. or *Epichloë* sp. Most bacteria that reside on the leaf surface of such grasses can consume these defensive chemicals. Loline-consuming bacteria are rare on the leaves of other plant species. Several bacterial species including *Burkholderia ambifaria* recovered from tall fescue could use *N*-formyl loline as a sole carbon and nitrogen source in culture and achieved population sizes that were about eightfold higher when inoculated onto plants harboring loline-producing fungal endophytes than on plants lacking such endophytes or which were colonized by fungal variants incapable of loline production. In contrast, mutants of *B. ambifaria* and other bacterial species incapable of loline catabolism achieved similarly low population sizes on tall fescue colonized by loline-producing *Neotyphodium* sp. and on plants lacking this endophytic fungus. Lolines that are released onto the surface of plants benefiting from a fungal mutualism thus appear to be a major resource that can be exploited by epiphytic bacteria, thereby driving the establishment of a characteristic bacterial community on such plants.

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Introduction

A diversity of fungi, bacteria, and protozoa are found on the aerial surfaces of plants. The number of bacterial cells on leaves can exceed 10^7 cells per cm^2 (Lindow and Brandl, 2003; Vorholt, 2012). Such large and diverse microbial communities develop despite the fact that it is considered a relatively harsh habitat with highly variable environmental conditions typified by rapidly fluctuating conditions of water availability and incidence of ultraviolet (UV) irradiation. While all leaf colonists must endure such stresses, a key factor limiting bacterial population size on leaves is nutrient availability (Mercier and Lindow, 2000; Lindow and Brandl, 2003). While exogenous carbon sources such as pollen that are occasionally deposited on leaf surfaces can supply needed nutrients for microbial growth, they are not thought to be the dominant nutrient sources on plants (Tukey, 1970). Instead, a variety of compounds including carbohydrates,

amino acids, methanol and other organic compounds that passively leach onto the leaf surface from inside the plant are considered to be the major source of carbon and nitrogen for microbes on the phyllosphere. Although these sources are not abundant, usually less than $10 \mu\text{g g}^{-1}$ of plant tissue on most plants examined, they are sufficient to account for the large numbers of bacteria that develop on such plant species (Mercier and Lindow, 2000). Furthermore, features of plants that limit nutrient loss to the surface of the plant such as cuticular thickness, apparently dictate the size of microbial communities that develop on various plant species (Lindow and Brandl, 2003; Vorholt, 2012).

While epiphytic microbes are limited by the amount of nutrients that escape onto the surface of plants, certain microbes can avoid such limitations by exploiting the interior of plants for growth. While a variety of endophytic bacteria can be found inside plants, their abundance is often relatively low compared with the number found on the surface of the same plant perhaps because of limitations on entry into and movement within the plant and plant defenses against such invasion (Hallmann *et al.*, 1997; Kobayashi and Palumbo, 2000). In contrast, certain endophytic fungi extensively colonize the interior of certain plants (Porrás-Alfaro and Bayman, 2011). Prominent among this group are strains of

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Neotyphodium spp. and *Epichloë* spp. (asexual and sexual endophytes in the family Clavicipaceae) that colonize a variety of cool season (C-3) grasses such as *Lolium* and *Festuca* in the Poaceae subfamily Pooideae (White, 1987; Schardl and Moon, 2003). As obligate biotrophs, their entire life cycle is completed within the intercellular spaces of the grass and they are vertically transmitted due to their ability to colonize seeds.

The relationship between this group of fungi and their host grass is mutualistic, with the fungi protecting their host from herbivory through the production of several types of alkaloids, such as lolines (Bacon and White, 2003). Lolines are saturated 1-aminopyrrolizidine alkaloids having a variety of substitutions on the 1-amino group and an epoxide oxygen bridge between C₂ and C₇ (Schardl *et al.*, 2007). They are broadly toxic to many insect herbivores of grasses (Wilkinson *et al.*, 2000). However, unlike the hepatotoxic/carcinogenic pyrrolizidines produced by plants, loline alkaloids are non-toxic to mammalian herbivores (Schardl *et al.*, 2007). Of the several loline alkaloids produced by *Neotyphodium* strains, *N*-formylloline is typically found in the highest concentrations (Justus *et al.*, 1997). In some cases, total concentration of lolines can exceed 2% of the dry weight of the grass (Blankenship *et al.*, 2001; De-wen *et al.*, 2006).

Because lolines can be produced in such large amounts in infected grasses, we reasoned that they might constitute a major carbon source available to bacteria in the phyllosphere of infected grasses and hence select for a distinctive microflora. While there has been little evidence of plant selection for particular phyllosphere colonists, certain rhizosphere bacteria have been shown to benefit from novel plant-derived compounds. A subset of the rhizosphere bacteria that colonized flavonoid-secreting plants could catabolize these compounds, and those bacteria had a competitive advantage over non-utilizers when co-inoculated onto the roots of such species (Narasimhan *et al.*, 2003). In other examples, *Agrobacterium tumefaciens* alters the metabolism of plants to benefit itself by genetically transforming plant tissues to initiate galls that produce one or more opines that only it can catabolize (Kim, 2000). Likewise, rhizopines produced by nodules in leguminous plants infected with certain rhizobia can also be consumed by these bacteria (Murphy *et al.*, 1995; Gordon *et al.*, 1996; Gardener and Bruijn, 1998). Given that the microbial community on leaf surfaces is generally much less abundant than that on roots, apparently because it is more nutrient limited (Lindow and Brandl, 2003; Hartmann *et al.*, 2009), additional sources of nutrients such as lolines might strongly select for epiphytic bacterial colonists capable of consuming them. In this study, we explored whether a multi-trophic level interaction could occur between *Neotyphodium* spp. and tall fescue (*Festuca arundinacea*) and that of the closely related *Epichloë*

festucae that infects meadow fescue (*Festuca pratensis*), whereby enough lolines would become available to bacterial colonists of the plants to provide them a sufficiently large and unique resource to select for a higher and distinct bacterial population on the leaves. Not only do we find that loline-consuming bacteria are found mainly only on *Neotyphodium* spp.-infected grasses but also find that these strains attain a much higher population size on such plants, which is dependent on their ability to consume loline. This indicates that loline is a dominant carbon resource on grasses infected with this fungal endophyte and that a large trophic selection for loline consumption results.

Materials and methods

Alkaloid extraction

Tall fescue (Kentucky-31) seed (500 g) was ground to a fine powder with a Thomas-Wiley mill fitted with a #20 screen. Ground seeds were wetted with 1 N NaOH and incubated for 1 h in 1000 ml of chloroform. The chloroform–seed mixture was then sonicated for 20 min and then filtered through cheesecloth to remove the seed debris. The remaining chloroform solution was then acid hydrolyzed for 1 h at 80 °C, the pH adjusted to 11.0, and material extracted into fresh chloroform. The chloroform layer was dried with anhydrous sodium sulfate and filtered through a #2 Whatman filter. Crude loline salts were precipitated from the liquid under a stream of dry HCl gas, and air dried overnight. Crude loline salts were analyzed via gas chromatography (GC) and mass spectrometry (GC–MS), revealing a mixture of *N*-formylloline (*m/z* 183.3), loline (*m/z* 154) and trace amounts of *N*-acetylloline (*m/z* 197.3). Loline species were then separated by thin layer chromatography and further characterized with GC–MS to compare peaks to reference data (Takeda *et al.*, 1991; Justus *et al.*, 1997).

Isolation of epiphytic bacterial strains

Loline-catabolizing bacteria were recovered from *Neotyphodium*-infected tall fescue (Kentucky-31) collected at the Sunnyvale Municipal Golf Course, Sunnyvale, CA, USA. The grass had received minimal horticultural intervention since planting. Epiphytic bacterial communities were described on (1) leaves of mature tall fescue plants from a commercial planting near Hillsboro, OR, USA, (2) leaves of coyote bush (*Baccharis pilularis*) and black sage (*Salvia mellifera*) collected near Point Reyes National Seashore, CA, USA in an area where there were no grass species growing within 200 m, and (3) leaves of Japanese maple (*Acer palmatum*) and English ivy (*Hedera helix*) and tall fescue grown in mixed horticultural plantings collected from the campus of the University of California Berkeley (Berkeley, CA, USA). Bacteria were obtained by

sonicating 3–4 leaves (~10 g) in 20 ml washing buffer (0.1 M potassium phosphate, pH 7.0 containing 0.1% bactopectone). Appropriate dilutions of leaf washings were plated on 10% tryptic soy agar (TSA) containing benomyl (50 µg ml⁻¹) to inhibit fungal growth.

Loline-catabolizing bacteria were obtained by enrichment culture in M9 minimal medium (Sambrook *et al.*, 1989) lacking added carbon sources but containing 200 µg ml⁻¹ crude loline 2HCl salts (loline minimal medium). All bacterial colonies recovered on 10% TSA plates were removed by scraping, washed in distilled water and appropriate dilutions were inoculated into loline minimal medium and incubated at 28 °C with shaking for 7 days. A 1-ml aliquot was then removed and inoculated into 100 ml of fresh loline minimal medium. After three successive transfers, a 1-ml aliquot was diluted and plated onto 10% TSA. Morphologically distinct colonies were transferred to 5 ml of loline minimal media and incubated at 28 °C for 72 h. Bacterial strains showing abundant growth in loline minimal medium were selected for further analysis and identified by 16S rRNA sequencing.

Molecular methods

Bacterial strains were inoculated into 10% tryptic soy broth for 24 h and total genomic DNA was extracted using a DNeasy Plant kit (Qiagen, Valencia, CA, USA). The 16S rDNA was amplified by PCR using universal primers 27F (5'-AGAGTTG ATCMTGGCTCAG-3') and 1525R (5'-AAGGAG GTGWTCCARCC-3'). Reactions were performed in 50 µl volumes containing 5 µl PCR buffer (Promega, Madison, WI, USA), 1.25 mM MgCl₂, 15 pmol of each primer, 200 µM of each dNTP, 25 µg BSA, 1 µl template DNA and 2.5 U Taq DNA polymerase (Promega). Reactions were performed in a thermal cycler (Peltier Thermocycler PTL-200, MJ Research, Watertown, MA, USA) with an initial denaturation step at 95 °C for 3 min followed by 27 cycles at 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min, with final extension at 72 °C for 10 min. Amplified PCR products were purified using a Qiagen PCR purification kit. Further characterization of strain 7R was conducted with specific 16S rRNA primers for the *Burkholderia cepacia* complex: PC-SSF and PCSSR (specific for *B. cepacia* genomovars I and III and *B. stabilis*), BC-GII and BC-R (specific for *B. multivorans*) and BC-V and BCR (specific for *B. vietnamiensis*), and BCRGC1 and BCRGC2 (specific for *B. ambifaria*), with PCR performed as previously described (Coenye *et al.*, 2001). Amplified PCR products were cloned using a TOPO TA cloning kit (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) by standard methods (Sambrook *et al.*, 1989) and sequenced at the UC Berkeley Sequencing Facility using primers for the cloning vector (M13F and M13R). The BLAST search tool (BLASTN) of the

National Center for Biotechnology Information was used to identify the resulting sequences. Sequences adjacent to sites of Tn insertions are provided in Supplementary Figure S3.

Phyllosphere community analysis

Total genomic DNA isolated from a mixture of all bacteria initially recovered on 10% TSA from leaf washings of field-grown plants as well as DNA from each step in enrichment cultures in loline minimal medium (described above) was collected and purified using a Qiagen DNeasy Blood and Tissue Kit. Bacterial 16S rRNA genes were amplified by PCR as above but the forward primer contained the 5' label 6-FAM, a 6-carboxyfluorescein (Sigma-Genosys, The Woodlands, TX, USA). Approximately 0.5 µg of each PCR product was digested overnight with *HhaI* (New England Biolabs, Beverly, MA, USA). The digested DNA was then precipitated, and resuspended in 20 µl dionized formamide containing the size standard 500-ROX (Applied Biosystems, Foster City, CA, USA). Samples were then denatured at 95 °C for 5 min, and fragment lengths were determined using an ABI Prism 3100 automated capillary sequencer (Applied Biosystems).

Loline catabolism

A variety of phyllosphere bacteria and common laboratory strains (Supplementary Table 1) were inoculated individually into 20 ml *N*-formylloline minimal medium and shaken at 28 °C. A 300 µl aliquot of each strain was removed every 24 h for up to 168 h, filter sterilized, and stored at -20 °C until analysis. An additional 100 µl collected at each time point was diluted and plated onto 10% TSA to quantify bacterial population sizes. The supernatants of 100 µl aliquots were adjusted to pH 11 with 1 N NaOH and vortexed for 30 s with 1 ml of chloroform to extract the remaining alkaloids. The samples were then centrifuged at 8000 *g* for 1 min and the chloroform phase removed. After a second extraction, both chloroform extracts were combined and spiked with 500 µg of the internal standard phenylmorpholine (Sigma-Aldrich, St Louis, MO, USA) and concentrated in a rotary evaporator (SVC 100 speedvac, Savant Instruments, Holbrook, NY, USA) until dry. The dried samples were resuspended in 10 µl 50:50:1 chloroform/methanol/ammonium chloride solution and subjected to GC analysis. Alkaloid concentrations were determined by GC by the method previously described (Yates *et al.*, 1990). *N*-Acetylloline was not easily separated by GC and was not quantified. GC was performed with a Hewlett-Packard 5890A (Sunnyvale, CA, USA) equipped with a flame ionization detector.

Isolation of loline catabolic mutants

The pUTmini-Tn5TP (trimethoprim resistance) transposon was introduced into *B. ambifaria* 7R by

conjugal mating with the donor strain *E. coli* S17-1 λ pir. The recipient was grown at 28 °C in King's B broth (KB) (King *et al.*, 1954) while the donor strain was incubated in Luria Broth (LB) overnight at 37 °C before bi-parental mating. Matings were conducted with a 1:2 mixture of donor and recipient on LB overnight at 28 °C. Insertion mutants were selected on KB containing 10 mg ml⁻¹ trimethoprim and 5 mg ml⁻¹ tetracycline and inoculated into loline minimal medium containing 300 μ g ml⁻¹ cycloserine. After a 5-day incubation at 28 °C with shaking, a 1-ml aliquot was removed and cells were washed two times in 10 mM KPO₄ buffer and then plated on 10% TSA. Plates were incubated for 3 days at 28 °C, and colonies were resuspended in buffer and again inoculated into fresh loline M9 supplemented with cycloserine. This process was repeated four times. Strains recovered after the final transfer step were further investigated. Mutants verified to be incapable of catabolizing loline as above were further characterized by arbitrary PCR to locate the DNA regions flanking the transposon. The left-specific primers (Int L (5'-CTGACTCTTATACAC AAGTGC GGC-3') and ExtL (5'-TGTTTGATGTTATG GAGCAGCAAC-3')) and right-specific primers (IntR (5'-NNNAAATCTGGTGCCGCTTGCCAG-3') and ExtR (5'-TTGTTTCAATCCGTCGGCAACACG-3')) were designed from the pUTmini-Tn5TP sequence. Arbitrary primers ARB6 (5'-GGCCACGCGTCTGACTAGT ACNNNNNNNNNNACGCC-3') and ARB2 (5'-GGC CACGCGTCTGACTAGTAC-3') were run in 25 μ l reactions using the PCR conditions previously described (O'Toole and Kolter, 1998). The resulting PCR products were sequenced at the UC Berkeley sequencing facility and the open reading frame affected by the transposon were determined using ORF Finder (NCBI).

Plant inoculations

Cells of wild-type loline-catabolizing *B. ambifaria* strain 7R, its isogenic mutant incapable of loline catabolism, LCMS1, and *Pseudomonas fluorescens* A506 (Wilson and Lindow, 1993) were grown on KB for 2 days at 28 °C, removed from plates by scraping, and resuspended in 10 ml of 10 mM KPO₄ buffer, pH 7.0 and adjusted to a final concentration of 10⁶ colony-forming units (c.f.u.) per ml. Meadow fescue (*Festuca pratensis*) infected with either a loline-producing (lol+) *Epichloë festucae* endophyte or a variant strain (lol-) incapable of producing lolines (Wilkinson *et al.*, 2000) were used in some studies. In addition, tall fescue (*Festuca arundinacea*) infected with *Neotyphodium* spp. (lol+) or free of infection (lol-) were also used. Plants free of infection were prepared by soaking tall fescue seed (Kentucky-31) in 50 μ g ml⁻¹ benomyl for 12 h and then washing in sterile water three times. Next, the seeds were soaked in 6 μ g ml⁻¹ propiconazole for 12 h and washed three times in sterile water. The seeds were planted in a greenhouse and allowed to grow for 6 weeks. The leaf sheaths were then

observed microscopically as in other studies (Bush *et al.*, 1982) to verify the lack of hyphae of *Neotyphodium*. Bacterial strains were spray-inoculated to wetness onto eight plants for each treatment with a hand-held sprayer. After inoculation, all plants were placed in a chamber maintained at ~100% relative humidity at 25 °C for 48 h with a 14-h light period each day. To determine population sizes of epiphytic bacteria, three randomly chosen leaves were cut from plants in each pot and immersed together in 20 ml of washing buffer. The test tubes were then sonicated for 10 min in an ultrasonic bath and vortexed for 10–20 s. Appropriate serial dilutions of each sample were plated onto 10% TSA containing kanamycin and natamycin (for *B. ambifaria* LCS), trimethoprim and natamycin (mutant LCMS1) or rifampicin and natamycin (*P. fluorescens* A506) using a spiral plater (Model D; Spiral Systems, Inc. Cincinnati, OH, USA). Population sizes were estimated from plate counts after a 3-day incubation at 28 °C. The experiments were repeated three times.

Analysis of lolines in leaf washings

Both lol+ and lol- grasses were subjected to 5 h of 100% humidity in a mist chamber to wet leaves. Wet leaves were then carefully covered with a plastic bag, excised near their base while capturing any water droplets that were shed. Ten to twelve excised leaves were added to another clean plastic bag with the cut ends protruding from the bag. A volume of 10 ml of sterile distilled water was then added to the bag and gently passed over the surface of each leaf (avoiding the cut end), and the leaves then carefully removed to limit water loss. Additional leaves were added to the same fluid and washed in the same manner until all had been washed. The wash fluid was then combined with the initial water droplets, filter sterilized through a 0.2- μ m filter, adjusted to pH 11 with NaOH, and lolines partitioned into chloroform as described above. The washed leaves were dried to the touch with a paper towel and weighed. The presence of lolines was determined with thin layer chromatography as previously described (Blankenship *et al.*, 2001), except that dragendorff reagent (Sigma-Aldrich) was used to visualize the spots. Samples containing lolines were further analyzed with GC to measure the total concentration of the alkaloids (Yates *et al.*, 1990), which were normalized per gram of leaf weight.

Results

Isolation of loline-catabolizing bacterial strains

Loline-catabolizing bacteria were readily isolated from the tall fescue phyllosphere. Enrichment culturing of bacterial epiphytes washed from the leaves of *Neotyphodium* spp.-infected tall fescue collected from a field site was performed in a

minimal medium containing purified lolines extracted from tall fescue seeds infected with *Neotyphodium* spp. as the sole carbon source. Four morphologically distinct strains capable of consuming this alkaloid were consistently recovered in repeated experiments (Supplementary Figure S4). These strains grew nearly as rapidly on minimal media containing lolines as a sole carbon source as with other compounds such as sucrose. No growth of any of 11 other laboratory or other plant-associated bacterial strains tested was observed in loline-containing minimal medium under these same conditions (Supplementary Table S1). Sequencing of the 16S rRNA gene from the loline-catabolizing strains identified them as *B. ambifaria*, *Serratia proteamaculans*, *Pseudomonas fluorescens* and *Stenotrophomonas maltophilia*. As *B. ambifaria* exhibited the highest growth rate and achieved the highest population size when grown on lolines and was likely to be the most genetically tractable, it was chosen for further analysis. As several substituted forms of loline can occur in infected plants (Scharld *et al.*, 2007), we examined which forms were present in our extracts by both thin layer chromatography (TLC) and GC-MS analysis (Supplementary Figures S1 and S2). *N*-Formylloline was preferentially consumed by *B. ambifaria*, as indicated by GC analysis (Yates *et al.*, 1990). After growth for 3 days in a medium containing the mix of loline derivatives, nearly all *N*-formylloline was consumed by *B. ambifaria* (Figure 1, Supplementary Figures S5), while little catabolism of loline was evident (Supplementary Figure S6). No such depletion of any loline species was seen in cultures by other bacterial taxa such as *Pseudomonas fluorescens* strain A506, even after extended incubation (Supplementary Figure S7). *B. ambifaria* grew rapidly in a minimal medium containing NFL, and its concentration decreased concomitantly with the increase in cell numbers in such cultures (Figure 1). By 72 h, *B. ambifaria* had consumed most of the NFL

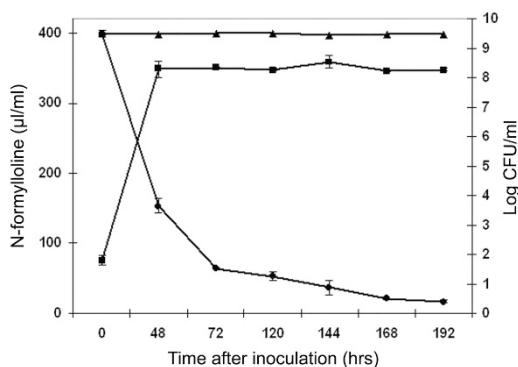


Figure 1 Growth of *B. ambifaria* 7R with *N*-formylloline as a sole carbon source in a minimal medium (squares) and depletion of this resource with time as quantified by GC, in the presence of cells (circles) or in cell-free minimal medium (triangles). The vertical lines represent the s.e. of the determination of the mean.

from the media. Stoichiometric analysis of cell growth revealed that each microgram of NFL yielded about 5.3×10^5 cells of *B. ambifaria* when available as the sole carbon source in this minimal medium.

Lolines as a nutrient source on leaves

The presence of lolines on grass leaf surfaces was assessed by gas chromatographic analysis of leaf washings. No lolines were detected in leaf washings from *Neotyphodium*-free tall fescue or from leaves of meadow fescue infected with a loline-deficient variant of *E. festucae*. In contrast, an average of about 0.8 µg per gram of total lolines were recovered in surface washes of *Neotyphodium*-infected tall fescue, and *N*-formylloline was the most abundant form recovered, as in seed extracts. Thus, at least some of the loline produced by *Neotyphodium* endophytes reaches the surface of grass and can be recovered in brief washes of the leaves.

To assess the contribution of lolines to the growth of bacteria on leaves, the population sizes of loline-utilizing and non-utilizing strains were compared on isogenic grasses differing in loline content. The maximum population sizes attained by loline-catabolizing bacterial strains such as *B. ambifaria* were much greater on tall fescue colonized by loline-producing *Neotyphodium* endophytes than on uncolonized plants. While the population size that *B. ambifaria* attained on non-infected tall fescue was about 3×10^6 cells per gram, it was about 10-fold higher on grasses infected with loline-producing *Neotyphodium* spp. (Figure 2). Importantly, the population size of *P. fluorescens* A506, an epiphytic strain unable to catabolize lolines was also only about 3×10^6 cells per gram on both loline-free tall fescue and on *Neotyphodium*-infected plants (Figure 2). Thus, infection by loline-producing *Neotyphodium* spp. greatly increased the carrying capacity of tall fescue for this loline-catabolizing

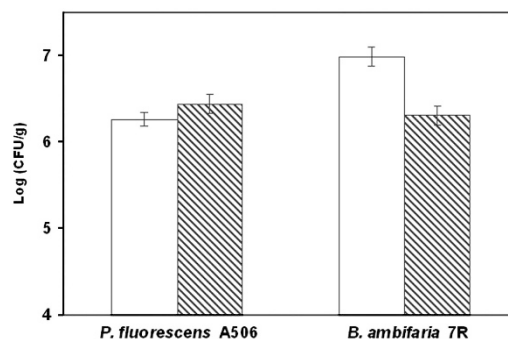


Figure 2 Population size of *Pseudomonas fluorescens* strain A506 and *B. ambifaria* strain 7R attained after 48 h incubation on moist leaves of tall fescue infected with loline-producing *Neotyphodium* spp. (white bars), or plants free of infection (striped bars). The vertical lines represent the s.e. of log-transformed population size.

bacterium, but not for strains that could not consume lolines.

To distinguish between loline production and the simple presence of fungal endophyte infection of grasses as contributing to the growth of epiphytic bacteria, we compared the population sizes attained by *B. ambifaria* and *P. fluorescens* strain A506 on meadow fescue that was infected with strains of *E. festucae* that differed in their ability to produce lolines. While strain A506 achieved very similar population sizes on plants infected with the loline-producing fungal endophyte as on plants infected by the non-producing variant, *B. ambifaria* achieved a population size that was about ninefold higher on plants infected with the loline-producing endophyte (Figure 3). Thus, fungal endophyte infection *per se* does not lead to enhanced growth of the loline-consuming *B. ambifaria* strain, and thus loline itself serves as the selective agent.

To more directly assess the role of loline consumption by *B. ambifaria* in its enhanced colonization of plants harboring lolines, we generate mutants of this strain that were unable to catabolize the alkaloid. To select for loline catabolism mutants, cycloserine was added to minimal medium containing NFL to kill cells that could consume lolines and thus were actively growing. Loline catabolic mutants would not be able to grow, and therefore would survive exposure to cycloserine. The loline catabolic mutants were then rescued by growth on TSA without antibiotics. Several independent mutation events were found to disrupt loline catabolism in this strain. Most mutants such as LCMS1 exhibited very little growth on minimal medium with NFL as the sole carbon source and did not deplete NFL from culture media (Supplementary Figure S8), while they grew normally on rich culture media or in minimal medium with glucose as a carbon source (Supplementary Figure S9). Mutant

LCMS1 was further investigated, as it exhibited the most pronounced deficiency in growth with lolines as a sole carbon source, only reducing NFL concentrations from $350 \mu\text{g ml}^{-1}$ to $329 \mu\text{g ml}^{-1}$ in 120 h, while the wild-type strain reduced NFL to only $21 \mu\text{g ml}^{-1}$. The gene that was disrupted in mutant LCMS1, determined by sequence analysis of the chromosomal region adjacent to the transposon insertion site by arbitrarily primed PCR, was homologous to Bam_1330 in another *B. ambifaria* strain, encoding a XRE family transcriptional regulator.

The population size of the loline catabolic mutant LCM1 that was attained on both loline-containing tall fescue infected with *Neotyphodium* spp. and on loline-free plants lacking this fungal endophyte was only about 10^6 cells per gram. In contrast, the wild-type strain reached a population size of over 10^7 cells per gram on plants infected with *Neotyphodium* spp., attained a population size that was about eightfold higher than that on plants not infected with this endophyte (Figure 4). Thus, loline catabolism is required by *B. ambifaria* to exploit this resource for epiphytic growth.

Phyllosphere community analysis

To determine the extent to which the presence of lolines on plants in nature selected for bacterial communities capable of catabolizing this resource, we examined the culturable epiphytes on a variety of plants differing in the presence of lolines in two different ways. In one approach, we compared the epiphytic bacterial community composition using T-RFLP analysis of amplified 16S rRNA genes of cells of bacteria cultured on non-selective media and after three rounds of enrichment for loline catabolism in loline minimal media as described earlier. A variety of bacterial colony types were observed when cells washed from leaves of

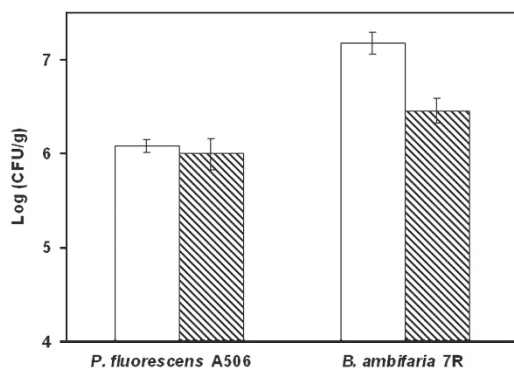


Figure 3 Population size of *Pseudomonas fluorescens* strain A506 and *Burkholderia ambifaria* strain 7R attained after 48 h incubation on moist leaves of meadow fescue infected with a loline-producing *Epichloe festucae* strain (white bars), or with a near isogenic loline-deficient variant of this fungal endophyte (striped bars). The vertical lines represent the s.e. of log-transformed population size.

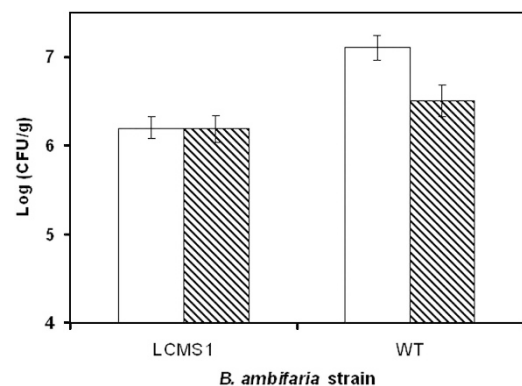


Figure 4 Population size of wild-type *B. ambifaria* strain 7R or of the loline catabolic mutant LCMS1 attained after 48 h incubation on moist leaves of tall fescue infected with loline-producing *Neotyphodium* spp. (white bars), or plants free of infection (striped bars). The vertical lines represent the s.e. of log-transformed population size.

Neotyphodium-infected tall fescue were cultured on 10% TSA (Supplementary Figure S4). A modest number of putative taxa could be resolved when these cultures were exemplified by T-RFLP analysis (Figure 5a). Importantly, after three rounds of enrichment for bacteria that could consume lolines, nearly half of the apparent taxa resolvable by T-RFLP remained (Figure 5b), suggesting that a relatively large proportion of the more numerically dominant bacteria on the leaves of tall fescue could catabolize lolines. In contrast, a somewhat higher diversity of bacteria as evident by larger number of terminal restriction fragments were recovered from cells cultured from leaves of Japanese maple (Figure 6a) and English Ivy (Figure 6c) growing nearby the tall fescue, yet very few of these putative taxa remained after enrichment for loline-utilizing bacteria and those that were recovered after growth with loline as a sole carbon source appeared to be relatively rare taxa on these plants before enrichment (Figures 6b and d, respectively). Thus, loline-consuming taxa appear to be common on *Neotyphodum*-infected tall fescue but quite uncommon on adjacent plants that do not harbor lolines. Further evidence for the development of a loline-supported epiphytic flora was provided by the finding of a significantly larger epiphytic bacterial population size on *Neotyphodum*-infected tall fescue than that on endophyte-free plants grown in a replicated field trial. The total epiphytic bacterial population size was over twofold higher on loline-containing fescue than on the loline-free plants (6.8×10^5 cells per gram vs 3.3×10^5 cells per gram, respectively).

In a second approach to associating the frequency of loline catabolism among epiphytic bacteria with loline availability on host plants, we isolated colonies of bacteria having morphologically

distinguishable features that could be cultured from a variety of plants on a non-selective medium and subsequently assessed their ability to consume lolines in a minimal medium. An estimate was also made of the numerical dominance of each colony morphotype so that the proportion of the total culturable phyllosphere microflora with ability to catabolize lolines could be estimated. While less than 6% of the taxa producing morphologically distinct colonies recovered from *Neotyphodium*-infected tall fescue could consume lolines, those taxa that could consume lolines were numerically quite dominant on all tall fescue plants examined. From these two parameters, we estimated that over 70% of the culturable bacteria on tall fescue could consume lolines. In contrast, less than 10% of culturable epiphytes from English ivy and Japanese maple consumed lolines. The predominance of loline-consuming epiphytes on tall fescue but their apparent rarity on plants that do not contain lolines that were estimated in this manner closely match the results obtained in the more indirect method based on T-RFLP analysis described above.

It was unclear whether the small proportion of loline-consuming bacteria recovered from plants not containing lolines reflected a low, but measurable population of loline consumers that might be expected on most non-loline-harboring plant species or whether it reflected a local accumulation of immigrant strains due to their close proximity to

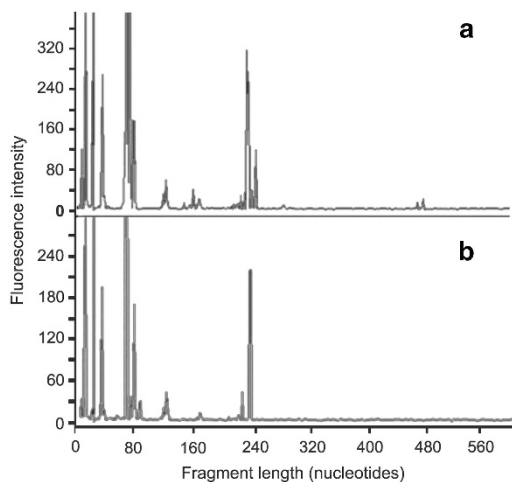


Figure 5 T-RFLP profile of amplified rRNA genes recovered from the culturable bacterial community on tall fescue leaves before (a) and after (b) enrichment of mixed cultures with lolines as the sole carbon source in a minimal medium.

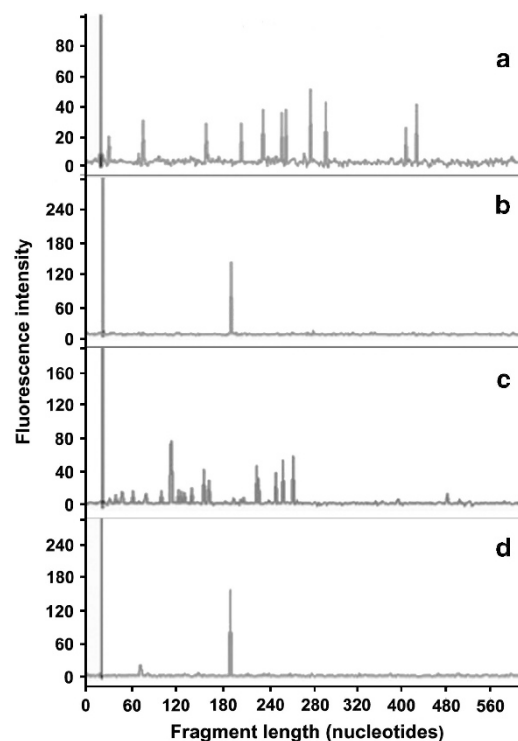


Figure 6 T-RFLP profile of amplified rRNA genes recovered from the culturable bacterial community on Japanese maple (a and b) and English ivy (c and d) both before (a and c) and after (b and d) enrichment of mixed cultures with lolines as the sole carbon source in a minimal medium.

tall fescue on which strong selection of loline consumers had occurred. To test this issue, we assessed the epiphytic bacterial populations on two common California chaparral plant species that grow in areas devoid of grasses. No loline-catabolizing bacteria were found in random samples of 50 or more bacteria recovered from leaves of coyote bush (*Baccharis pilularis*) or black sage (*Salvia mellifera*) that were collected in an area in which no grass species was present within at least 200 m. The lack of loline-consuming bacteria on these loline-free plants grown away from local sources of loline-consuming bacteria suggest that loline consumption is indeed an uncommon trait in epiphytic bacteria, but one that is strongly selected for on grasses infected with loline-producing fungal endophytes.

Discussion

The results of this study reveal that loline-producing fungal endophytes mediate a very strong, multi-trophic level change in the bacterial microflora of the plants that they infect. The epiphytic bacterial community analyses suggest that *Neotyphodium* sp. confers strong selection for loline-consuming bacteria through its production of such compounds, as such bacteria were very rare on all other plants studied. The apparent dominance of loline-catabolizing strains in the phyllosphere community of loline-containing plants indicates that they are being strongly enriched for on the leaf surface. As nutrient availability on leaf surfaces limits bacterial population sizes (Mercier and Lindow, 2000), the ability of some strains to use this additional nutrient source gives them an advantage. The selective advantage is apparently quite strong, as loline-catabolizing strains achieved as much as a 10-fold higher population size on loline-containing plants than on isogenic plants lacking lolines (Figures 2–4). While somewhat higher bacterial populations were also seen on some powdery mildew-infected plants (Suda *et al.*, 2009), it seemed likely that such growth occurred on the extensive aerial hyphae present on plant surfaces, and not due to any particular fungal exudate.

Lolines may egress onto the leaf in several ways from the apoplast from where they are produced. As they are in high concentration in the apoplast and phloem (Justus *et al.*, 1997), wounding by herbivory or damage to the plant could cause efflux onto the leaf. The passive leakage of various organic compounds is also known to increase with leaf age, presumably as the cuticle becomes more permeable (Tukey, 1970). As lolines are found in higher concentrations in mature leaves (De-wen *et al.*, 2006), the potential for leaching would be maximal in such plants. NFL and *N*-acetyl lolines have also been detected in the guttation fluid of tall fescue leaves (Koulman *et al.*, 2007), where it could then be

deposited onto the leaf blade. While we measured much higher concentrations of loline species in extracts of entire leaves than of leaf washings, it is clear that a subset of the lolines produced by fungal endophytes reaches the leaf surface. Our estimate of the abundance of lolines on leaves is likely also underestimated given the difficulties in recovering this relatively insoluble material from leaves. Some of the loline released onto leaves may already have been consumed by indigenous bacteria as well. The former conjecture is supported by the observation that as many as 10^7 more cells of *B. ambifaria* grew on each gram of loline-containing leaf tissue on than on loline-free plants (Figures 2–4); such excess growth was as much as 10-fold more than expected given the relatively small amount of lolines ($\sim 1 \mu\text{g g}^{-1}$) recovered in leaf washings unless our estimates of the resource demand for cell production ($< 10^6$ cells per μg of loline) is underestimated. It thus seems likely that bacteria can access considerable amounts of lolines that are inaccessible to the recovery methods used here.

The difference in growth of LCMS1 and wild-type strain on loline-containing leaves seen here is clearly due to the inability of the mutant to properly catabolize the alkaloids (Supplementary Figure S8). While the Bamb_1339 XRE family transcriptional regulator that was disrupted is apparently involved in loline catabolism, its precise role is unclear, as most of the XRE family regulators are hypothetical proteins, and thus no prediction can be made of general functions for most members of this protein family (Gerstmeir *et al.*, 2004; Galperin, 2006). XRE (xenobiotic-responsive elements) are found in many bacteria and are often responsible for the processing of foreign materials. It is most likely that the XRE regulator controls one or more genes enabling *B. ambifaria* to degrade heterocyclic compounds such as *N*-formylloline.

The assembly of the epiphytic bacterial community on loline-containing tall fescue appears to reflect a process of niche partitioning. That is, the high proportion of bacterial cells that can consume lolines on such plants compared to other plants that do not suggest that loline catabolism is very strongly selected for, and that lolines represent a dominant resource on such plants. The rarity of bacterial strains capable of loline catabolism, even on plants collected nearby *Neotyphodium*-infected tall fescue, support a model whereby loline catabolism is uncommon in the metacommunity of bacteria yet is very strongly selected only on those plants harboring fungal endophytes that produce lolines. Lolines would thus represent both a very unique and abundant resource that could be consumed only by specialist colonists. The presence of large numbers of methylotrophs on some plants that emit large amounts of methanol is another example of a major resource that supports distinct community members (Delmotte *et al.*, 2009; Vorholt, 2012). Unlike lolines, however, many plants apparently

release methanol (Sy *et al.*, 2005) and one might expect that methylotrophs might be relatively common in the metacommunity of bacteria. The relative importance of lolines as a resource on *Neotyphodium*-infected tall fescue can be estimated from the relative population sizes attained by *B. ambifaria*, which can consume lolines, and its isogenic mutant that cannot, as well as that of *P. fluorescens* A506 which also cannot consume lolines. Strain A506 is a very successful epiphyte that has a wide resource utilization profile, capable of consuming a wide variety of sugars, amino acids and other compounds that might be expected to be leached onto plant surfaces (Wilson and Lindow, 1994). Despite this, both its population size and that of the loline-deficient mutant of *B. ambifaria*, was about 8 to 10-fold lower than that of the wild-type *B. ambifaria* strain on loline-containing tall fescue (Figures 2–4). This suggests that lolines constituted up to 90% of the nutrient resources on leaves. The fact that a variety of other bacteria that are incapable of consuming lolines are also found on loline-containing tall fescue, although in much lower abundance, suggests that they share the many other common carbon compounds that are found in lesser amounts. A more complete assessment of the community composition of bacteria on adjacent loline-containing and loline-free plants using culture-independent methods should prove useful in better defining the magnitude of apparent loline-driven selection of strains that can catabolize this compound.

While we focused our examination of the effects of loline production on leaf surface bacterial communities, it seems likely that the rhizosphere bacterial community might also be influenced by loline production. *Neotyphodium* infection of root tissues and loline accumulation has been noted in the roots of tall fescue (Schardl and Moon, 2003), thus making it likely that lolines are released into the rhizosphere. If true, then a very distinctive bacterial microflora might also be expected on the roots of *Neotyphodium*-infected grasses.

Loline consumption might also prove to be a highly useful selective trait to be exploited in establishing particular loline-catabolizing bacteria on leaf surfaces for useful purposes. For example, hydrophobic air pollutants such as phenol were shown to accumulate in the cuticle of plants where they could be more readily consumed by indigenous epiphytic bacteria (Sandhu *et al.*, 2007). It might be possible to establish particular bacterial strains that catabolize both loline and xenobiotics on loline-containing plants; the higher population size of such strains that might be sustained due to loline enrichment might be expected to accelerate the process of bioremediation. Such a method might be both more effective and more acceptable than strategies such as the production of opines in transgenic plants to enhance opine-catabolizing bacterial strains (Wilson *et al.*, 1995). As fescues

are hardy grasses that are easily propagated, and *Neotyphodium* infection is a positively selected trait in such plants due to its secondary benefits to the host for insect resistance and drought tolerance (Wilkinson *et al.*, 2000), such a naturally occurring mutualism might be readily exploited to enhance a variety of processes.

Conflict of Interest

The authors declare no conflict of interest.

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