Indole-Diterpene Biosynthetic Capability of *Epichloë* Endophytes as Predicted by *ltm* Gene Analysis[∇]

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Bioprotective alkaloids produced by *Epichloë* and closely related asexual *Neotyphodium* fungal endophytes protect their grass hosts from insect and mammalian herbivory. One class of these compounds, known for antimammalian toxicity, is the indole-diterpenes. The *LTM* locus of *Neotyphodium lolii* (Lp19) and *Epichloë festuce* (Fl1), required for the biosynthesis of the indole-diterpene lolitrem, consists of 10 *ltm* genes. We have used PCR and Southern analysis to screen a broad taxonomic range of 44 endophyte isolates to determine why indole-diterpenes are present in so few endophyte-grass associations in comparison to that of the other bioprotective alkaloids, which are more widespread among the endophtyes. All 10 *ltm* genes were present in only three epichloë endophytes. A predominance of the asexual *Neotyphodium* spp. examined contained 8 of the 10 *ltm* genes, with only one *N. lolii* containing the entire *LTM* locus and the ability to produce lolitrems. Liquid chromatography-tandem mass spectrometry profiles of indole-diterpenes from a subset of endophyte-infected perennial ryegrass showed that endophytes that contained functional genes present in *ltm* clusters 1 and 2 were capable of producing simple indole-diterpenes such as paspaline, 13-desoxypaxilline, and terpendoles, compounds predicted to be precursors of lolitrem B. Analysis of toxin biosynthesis genes by PCR now enables a diagnostic method to screen endophytes for both beneficial and detrimental alkaloids and can be used as a resource for screening isolates required for forage improvement.

Epichloë endophytes systemically colonize cool-season grasses, and comprise sexual *Epichloë* spp. and their asexual derivatives, the *Neoptyphodium* spp. Collectively, they produce a range of bioprotective alkaloids, including the ergot alkaloids, pyrrolopyrazine (peramine), aminopyrrolizidines (lolines), and indole-diterpenes (including lolitrems) (5, 63). Most *Neotyphodium* spp. are of hybrid origin, with two or three *Epichloë* or *Neotyphodium* ancestors (11, 44, 59, 66). The alkaloids produced by epichloë endophytes enhance the competitive ability of endophyte-infected grasses by protecting the grass host from insect and mammalian herbivory. Alkaloids such as peramine and the lolines are known for their anti-insect properties (5, 55, 63), while ergovaline and the lolitrems are detrimental to grazing livestock, causing fescue toxicosis and ryegrass staggers, respectively, in pastoral ecosystems (17, 18, 31, 47, 52). Many epichloë endophytes are able to produce multiple classes of alkaloids but, to date, no plant-endophyte combination has been identified that produces lolitrems, lolines, peramine, and ergovaline (5, 10).

A number of naturally occurring endophytes have been identified that do not produce the mammalian toxins, ergovaline and lolitrem B, but retain beneficial agronomic properties (26, 27), such as tolerance to abiotic stress (32). Artificially inoculated associations of these endophytes with elite tall fescue (4) and perennial ryegrass cultivars (16) have been commercially established and shown to enhance animal productivity, while alleviating the negative responses such as fescue toxicosis and ryegrass staggers (1, 2, 50, 51, 68).

The indole-diterpene, lolitrem B, is the major mammalian toxin responsible for ryegrass staggers, a syndrome found in animals grazing *N. lolii*-infected perennial ryegrass (*Lolium perenne*) (17, 18). To date, lolitrem production has only been identified in *Epichloë festucae*-, *Neotyphodium lolii*-, and *Neotyphodium* sp. strain FaTG-2-infected grasses (9, 10). The absence of lolitrems in many epichloë endophyte-grass associations does not appear to be due to a grass host effect but rather to the inability of the endophyte to produce the compound (5, 63). Until recently, genetic analysis of the genes required for the production of lolitrem B was not available, so a large effort was put into the chemical analysis of endophyte-infected grasses that resulted in the identification of indole-diterpene compounds, such as lolitriol, lolicines A and B, lolilline and terpendole M, predicted to be precursors or by-products of lolitrem B biosynthesis (5, 19, 37, 38, 40, 46–49). These analyses showed that the ability to produce the alkaloids was dependent on the presence of the endophyte within the plant. The plant genotype and environmental conditions are also known to influence the amount and relative abundance of the alkaloids produced by the endophyte (5, 12, 13, 30), making alkaloid chemotyping of endophyte-infected grasses difficult.

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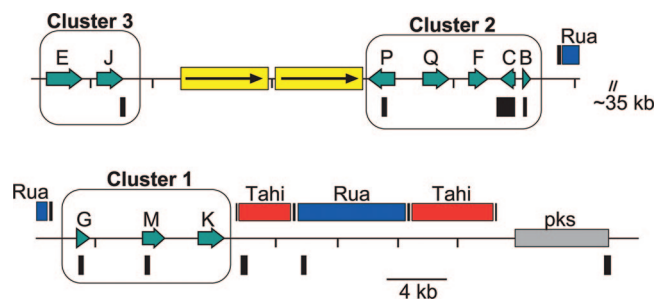† Present address: AgResearch Grasslands, Grasslands Research Centre, Private Bag 11008, Palmerston North, New Zealand.

FIG. 1. Physical map of the *N. lolii LTM* locus. The three clusters required for indole-diterpene biosynthesis are boxed and numbered. The regions amplified as probes used for the Southern hybridizations shown in Fig. 2 and 3 are represented by the black boxes below the map. The low-copy direct repeat is indicated as arrows boxed in yellow. The retrotransposons, Tahi and Rua, are depicted as red and blue boxes, respectively. The *pks* pseudogene is shown as a gray box. The primers used to amplify each fragment can be found in Table 3. (Adapted from reference 75 with permission from Elsevier.)

For example, loline production was recently shown to be a wound-inducible response in *Neotyphodium coenophialum*-infected tall fescue (64) and *Epichloë glyceriae*-infected *Glyceria striata* (22), where the level of lolines detected in undamaged *E. glyceriae*-infected plants was minimal compared to the wounded-infected plant (22).

Recently, a cluster of genes at the *LTM* locus involved in indole-diterpene biosynthesis was isolated from *N. lolii* and *E. festucae* (74, 75). The *LTM* locus is complex in that it contains three clusters separated by retrotransposon relics and other highly repetitive sequences (Fig. 1). The gene cluster consists of at least 10 genes, which encode LtmG, a geranylgeranyl diphosphate synthase; LtmM, an FAD-dependent monooxygenase; LtmC, a prenyl transferase; LtmB, a hypothetical protein of unknown function; LtmF, a prenyl transferase with similarity to dimethylallyl tryptophan synthase; four P450 monooxygenases (LtmJ, LtmK, LtmP, and LtmQ); and LtmE, a multifunctional enzyme that contains two prenyl transferase domains with homology to LtmC and LtmF. The 10 *ltm* genes are coregulated and highly expressed by the endophyte when associated with the grass host (74, 75).

Based on our knowledge of the biochemical pathway responsible for the production of the indole-diterpene paxilline and its precursors, we predict that LtmG, LtmM, LtmC, and LtmB are required for the biosynthesis of paspaline, the proposed indole-diterpene intermediate that forms a structural backbone essential for the production of more complex indole-diterpenes (56, 57, 73–75). LtmP and LtmQ are predicted to be required for demethylation and hydroxylation of paspaline, respectively, and therefore, are essential for the production of the lolitrem class of indole-diterpenes. LtmF, LtmJ, LtmK, and LtmE appear unique to lolitrem biosynthesis and are predicted to be required for the elaboration of the additional rings that define this class of molecules (75).

Previous studies of the *LTM* locus (74, 75) have focused on three epichloë endophytes with known chemotypes: the lolitrem B producing *E. festucae* (isolate Fl1) and *N. lolii* (isolate Lp19) and nonproducing *E. typhina* (isolate E8). The observation that lolitrem B production correlated with the presence of the ten *ltm* genes contained within the *LTM* locus led us to

hypothesize that the capability to produce lolitrem B and indole-diterpene precursors by epichloë endophytes can be predicted by PCR profiling of the essential pathway genes. The aim of the present study was to determine the distribution of the *ltm* genes among epichloë endophytes, including representative isolates of 10 sexual *Epichloë* species and a taxonomically diverse set of *Neotyphodium* isolates. We then utilized the PCR analysis and liquid chromatography-tandem mass spectrometry (LC-MS/MS) data to elucidate the symbiotum indole-diterpene chemotype.

MATERIALS AND METHODS

Fungal strains and growth conditions. *Epichloë* and *Neotyphodium* strains (Table 1) were grown on potato dextrose medium and maintained as previously described (41, 43, 74). Endophyte-infected plant material is listed in Table 2.

Molecular biological techniques. Genomic DNA from *Epichloë* and *Neotyphodium* species were isolated from freeze-dried mycelium by using the methods of Byrd et al. (6) and Yoder (70) and a Plant DNeasy extraction kit (Qiagen, Hilden, Germany).

DNA restriction digestion, Southern blotting, and probe labeling were carried out as previously described in Young et al. (74). Standard PCR amplifications of genomic DNA templates were carried out as previously described (74) using primer pairs listed in Table 3. The primers for each gene were designed to conserved domains identified in peptide sequence alignments of known *ltm* homologs and sequences predicted to have a role in indole-diterpene production. The primer sequence was subsequently designed with a bias toward the *ltm* sequence (71). Primers were also designed to the known housekeeping genes, *ggsA* (which encodes a geranylgeranyl diphosphate synthase) and *chsV* (which encodes a class V chitin synthase), and used to test the integrity of the genomic DNA stocks used in the PCR screen. PCR products were purified by using a PCR purification kit (Qiagen). DNA for probes were radiolabeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham) by random primed synthesis using a High Prime kit (Roche, Basel, Switzerland), and probes were purified by using ProbeQuant columns (Pharmacia). Standard hybridizations were carried out at 65°C overnight as previously described (72). Low-stringency hybridization was performed with a 4 h prehybridization in 10× Denhardt solution (58) and then hybridized for 48 h at 37°C in 43% (vol/vol) formamide, 5× Denhardt solution, 5× SSC (0.75 M NaCl, 0.075 M trisodium citrate), 0.1% sodium dodecyl sulfate (SDS), 50 mM sodium phosphate (36), and 10 μg of phenol-extracted herring sperm DNA/ml. Posthybridization, the membrane was washed at room temperature in two changes of 2× SSC (0.3 M NaCl, 0.03 M trisodium citrate)–0.1% SDS for 10 min and then with three 15-min washes in 2× SSC–0.1% SDS at 50°C. Membranes that were hybridized multiple times were stripped of their radioactive signal by three to four washes in boiling 0.1% SDS and checked for residual radioactive signal prior to each hybridization.

PCR products were sequenced by using BigDye chemistry (v31.; Applied Biosystems, Foster City, CA) using gene-specific primers, and the products were separated on an ABI 3730 sequence analyzer. Sequences were aligned by using Sequencher version 4.8 (Gene Codes, Ann Arbor, MI) and annotated using MacVector version 9.5 (MacVector, Inc., Cary, NC). The GenBank accession numbers for Fg1 and Frc7 *ltmF* are EU530694 and EU544671, respectively.

Plant inoculations and lolitrem analysis. Inoculation of endophyte-free perennial ryegrass (cv. Nui) seedlings with *E. festucae* isolates Fr1, Frr1, Frc5, Frc7, Fg1, Fl1, and E189; *N. lolii* isolates Lp19 and AR1; and *Neotyphodium* sp. isolate Lp1 were performed as previously described (74) (Table 2). For each association, three independent plants were analyzed for lolitrem B (74) and other indole-diterpenes. Pseudostem fractions from each plant were freeze-dried and milled in a modified (reduced volume) domestic coffee mill. Pseudostem material was chosen for alkaloid analysis since prior experience had shown that alkaloids tend to accumulate to higher levels in this tissue. Plant fractions (50 mg) were extracted in 2-ml polypropylene capped tubes with 1 ml of propan-2-ol for 2 h at ambient temperature and mixed by inversion on a rotary mixer. After centrifugation to sediment residual solids, aliquots of the extract were transferred to septum capped vials for subsequent fluorescence high-pressure LC (HPLC) (74) and LC-MS/MS analysis.

LC-MS/MS. Alkaloid analysis and identification was performed by using a linear ion trap system and associated liquid chromatography modules (Thermo LTQ and Thermo Surveyor, San Jose, CA). Samples (10 μl) were injected onto a Prodigy ODS-3 column (5-μm pore size, 150 by 4.6 mm; Phenomenex, Torrance, CA) with solvent flow of 1 ml/min at 25°C. The solvents were 40%

TABLE 1. Biological material

Biological material	Isolate	Closest nonhybrid ancestor(s)[a]	Predicted chemical phenotype[b]	Grass host	Tribe	Culture no.[c]	Source or reference(s)
E. amarillans	E52	*E. amarillans*	No IDT	*Sphenopholis obtusata*	Aveneae	ATCC 200743	44, 60, 61, 62
E. amarillans	E57	*E. amarillans*	No IDT	*Agrostis hiemalis*	Aveneae	ATCC 200744	44, 60, 61, 62
E. baconii	E248	*E. baconii*	No IDT	*Agrostis stolonifera*	Aveneae	ATCC 76552	29, 44, 61, 62
E. baconii	E1031	*E. baconii*	No IDT	*Calamagrostis villosa*	Aveneae	ATCC 200745	29, 44, 60, 61
E. brachyelytri	E1040	*E. baconii*	No IDT	*Brachyelytrum erectum*	Brachyelytreae	ATCC 200752	44, 60, 61
E. bromicola	E501	*E. bromicola*	No IDT	*Bromus erectus*	Bromeae	ATCC 200749	29, 44, 61
E. bromicola	E799	*E. bromicola*	No IDT	*Bromus benekenii*	Bromeae	ATCC 201559	29
E. clarkii	E422	ETC	No IDT	*Holcus lanatus*	Poeae	ATCC 90168	29, 60, 61
E. elymi	E56	*E. elymi*	No IDT	*Elymus canadensis*	Triticeae	ATCC 201551	44, 60, 61, 62
E. elymi	E184	*E. elymi*	No IDT	*Elymus virginicus*	Triticeae	ATCC 200850	44, 60, 61
E. festucae	**E189**	**E. festucae**	**Lolitrems***	**Festuca rubra subsp. rubra**	**Poeae**	**ATCC 90661**	**29, 44, 62**
E. festucae	E2368	*E. festucae*	No IDT	*Festuca* spp.	Poeae		C. L. Schardl, University of Kentucky
E. festucae	**Fg1**	**E. festucae**	**Lolitrems**	**Festuca glauca**	**Poeae**		**41**
E. festucae	**Fl1**	**E. festucae**	**Lolitrems***	**Festuca longifolia**	**Poeae**	**ATCC MYA-3407**	**28, 41**
E. festucae	**Fr1**	**E. festucae**	**No IDT**	**Festuca rubra**	**Poeae**		**28, 41**
E. festucae	**Frc5**	**E. festucae**	**No IDT**	**Festuca rubra subsp. commutata**	**Poeae**		**41**
E. festucae	**Frc7**	**E. festucae**	**IDT**	**Festuca rubra subsp. commutata**	**Poeae**		**41**
E. festucae	**Frr1**	**E. festucae**	**No IDT**	**Festuca rubra subsp. rubra**	**Poeae**		**41**
E. glyceriae	E277	*E. glyceriae*	No IDT	*Glyceria striata*	Meliceae	ATCC 200747	44, 60, 61
E. glyceriae	E2772	*E. glyceriae*	No IDT	*Glyceria striata*	Meliceae	ATCC 200755	44, 60, 61
E. sylvatica	E354	ETC	No IDT	*Brachypodium sylvaticum*	Brachypodieae	ATCC 200748	29, 44, 60, 61
E. sylvatica	E503	ETC	No IDT	*Brachypodium sylvaticum*	Brachypodieae	ATCC 200751	29, 44, 60, 61
E. typhina	E8	ETC	No IDT	*Lolium perenne*	Poeae	ATCC 200736	29, 41, 44, 60, 61, 62
E. typhina	E1022	ETC	No IDT	*Poa nemoralis*	Poeae	ATCC 201668	29, 44
E. typhina	E2463	ETC	No IDT	*Dactylis glomerata*	Poeae		C. D. Moon and C. L. Schardl, University of Kentucky
E. typhina	E348	ETC	No IDT	*Phleum pratense*	Aveneae	CBS 102648	29, 61
E. typhina	E425	ETC	No IDT	*Phleum pratense*	Aveneae	ATCC 200851	29, 44, 60, 61
E. typhina	E505	ETC	No IDT	*Brachypodium pinnatum*	Brachypodieae	ATCC 200739	29, 44, 60, 61
N. aotearoae	e899	Nao	No IDT#	*Echinopogon ovatus*	Aveneae	ATCC MYA-1229	42
N. australiense	e938	*E. festucae*, ETC	No IDT	*Echinopogon. ovatus*	Aveneae	CBS 109347	42
N. coenophialum	e19	*E. festucae*, ETC, LAE	No IDT	*Lolium arundinaceum*	Poeae	ATCC 90664	44, 65
N. coenophialum	Tf28	*E. festucae*, ETC, LAE	IDT	*Lolium arundinaceum*	Poeae		9
N. funkii	e4096	*E. festucae*, *E. elymi*	IDT	*Stipa robusta*	Stipeae	ATCC MYA-2583	45
N. gansuense var. *inebrians*	e818	*N. inebrians*	No IDT	*Achnatherum inebrians*	Stipeae	ATCC MYA-1228	44, 45
N. lolii	**AR1**	**E. festucae**	**IDT**	**Lolium perenne**	**Poeae**		**41**
N. lolii	Lp14	*E. festucae*	IDT*	*Lolium perenne*	Poeae		9, 41, 62
N. lolii	Lp19	*E. festucae*	Lolitrems*	*Lolium perenne*	Poeae		9, 41
N. lolii	Lp5	*E. festucae*		*Lolium perenne*	Poeae		9, 41, 62
N. lolii	Lp7	*E. festucae*		*Lolium perenne*	Poeae		9, 41
N. lolii	Lp9	*E. festucae*		*Lolium perenne*	Poeae		9, 41, 62
N. melicicola	e822	*E. festucae*, *N. aotearoae*	IDT	*Melica racemosa*	Meliceae	CBS 108340	42
N. siegelii	e915	*E. festucae*, *E. bromicola*	IDT	*Lolium pratense*	Poeae	ATCC 74483	44
Neotyphodium sp. strain FaTG-2	Tf15	*E. festucae*, LAE		*Lolium arundinaceum*	Poeae		9, 41
Neotyphodium sp. strain FaTG-3	Tf20	*E. festucae*, LAE		*Lolium arundinaceum*	Poeae		9, 41
Neotyphodium sp. strain HboTG-2	Hd1	*E. bromicola*, ETC	No IDT	*Hordeum bogdanii*	Triticeae		M. Christensen, AgResearch, New Zealand
Neotyphodium sp. strain LpTG-2	**Lp1**	**E. festucae, ETC**	**IDT**	**Lolium perenne**	**Poeae**		**9, 41, 62**
Neotyphodium sp. strain LpTG-3	Lp2	*E. festucae*, ETC		*Lolium perenne*	Poeae		9, 41, 62
N. tembladerae	e1169	*E. festucae*, ETC	IDT	*Poa huecu*	Poeae	ATCC 200844	44
N. tembladerae	e4055	*E. festucae*, ETC	IDT	*Festuca arizonica*	Poeae	ATCC MYA-2564	44
Neotyphodium sp.	Poa	ETC[d]	IDT?	*Poa* sp.	Poeae		M. Christensen, AgResearch, New Zealand

[a] Abbreviations: ETC, *E. typhina* complex; LAE, *Lolium*-associated endophyte as documented in Moon et al. (44).

[b] The predicted phenotype is based on the PCR screen for the presence of the *ltm* genes. Phenotypes that were confirmed by chemical analysis in this study are indicated in boldface. Chemical phenotypes confirmed in other publications are indicated by an asterisk. No IDT, no indole-diterpene production; IDT, indole-diterpene production; lolitrems, lolitrem production; #, isolate positive as shown by Miles et al. (39).

[c] ATCC and CBS refer to isolates deposited at the American Type Culture Collection (Manassas, VA) or the CentraalBureau voor Schimmelcultures (Utrecht, The Netherlands), respectively.

[d] The possible hybrid status is undetermined.

TABLE 2. Endophyte-infected plant material[a]

Plant material	Isolate contained	Closest nonhybrid ancestor(s)	Analyzed IDT chemotype[b]	Grass host/endophyte inoculation
G1730-G1732	Fr1	*E. festucae*	No IDT	*L. perenne*/PN2207
G1733-G1735	Frr1	*E. festucae*	No IDT	*L. perenne*/PN2212
G1736-G1738	Frc5	*E. festucae*	No IDT	*L. perenne*/PN2209
G1739-G1741	Frc7	*E. festucae*	IDT	*L. perenne*/PN2132
G1742-G1744	Fg1	*E. festucae*	Lolitrems	*L. perenne*/PN2211
G1745-G1747	Fl1	*E. festucae*	Lolitrems*	*L. perenne*/PN2278
G1748-G1750	E189	*E. festucae*	Lolitrems*	*L. perenne*/PN2241
G1751	AR1	*E. festucae*	IDT	*L. perenne*/PN2279
G1752-G1754	Lp1	*E. festucae*, ETC[c]	IDT	*L. perenne*/PN2197

[a] All plant materials were derived from *L. perenne*. The tribe for all plant materials was Poeae, and the reference for all plant materials was the present study.

[b] *, chemotype confirmed in other publications. No IDT, no indole-diterpene production; IDT, indole-diterpene production; lolitrems, lolitrem production.

[c] ETC, *E. typhina* complex.

aqueous acetonitrile with 0.1% acetic acid (by volume) (solvent A) and acetonitrile with 0.1% acetic acid (solvent B) in proportions beginning with 20% solvent B, rising linearly to 50% solvent B at 20 min, and then to 100% solvent B at 40 min and recycling after 60 min.

The ion trap was operated in positive atmospheric pressure chemical ionization mode with an N_2 sheath and auxiliary gas (set to 40 and 10, respectively), the source voltage at 6 kV, the capillary temperature at 200°C, and the atmospheric pressure chemical ionization vaporizer temperature at 450°C. The ion trap was tuned to maximum sensitivity with an infusion of paxilline diluted in ca. 65% aqueous acetonitrile with 0.1% acetic acid at 1 ml/min. A parent ion list for targeted indole-diterpenes was programmed to permit data-dependent observation of tandem mass spectrometry (MS^2 and MS^3) spectra for compound identification purposes and comparison with authentic standards.

For retention time and mass spectral comparison purposes, paxilline was obtained from Sigma (St. Louis, MO), and lolitriol, lolitrem B, lolitrem E, terpendole M, 13-desoxypaxilline, terpendole C, and paspaline were obtained from Sarah Finch (AgResearch, Ltd., New Zealand).

RESULTS

Defining *ltm* gene profiles of epichloë endophytes by PCR. Genomic DNA of 44 isolates from both sexual and asexual epichloë endophytes was subjected to PCR to detect the pres-

ence of *ltm* genes (Fig. 2). As expected, amplification products resulted from the 10 *ltm* primer pairs with genomic DNA from *N. lolii* Lp19 and *E. festucae* Fl1 (Fig. 2). Ten PCR products also were amplified from genomic DNA of one other isolate, *E. festucae* Fg1, while products corresponding to nine, eight, and five *ltm* genes were amplified from genomic DNA of *E. festucae* E189, Frc7, and E2368, respectively. Although E189 did not yield an amplification product with primers designed for *ltmE*, Southern analysis of E189 genomic DNA showed hybridization with a PCR fragment of *ltmE*, amplified from Fl1 with primers ltmE356 and ltmE341, which indicated the presence of this gene (data not shown). There was no evidence that the remaining *E. festucae* isolates—Fr1, Frr1, and Frc5—contained *ltm* genes apart from a faint band with the *ltmB* primers with Frc5 DNA. Of the *Epichloë* spp. included in the analysis, PCR products corresponding to four *ltm* genes were clearly amplified from *E. bromicola* strain E799. PCR products corresponding to at least one *ltm* gene were detected in five *Epichloë* spp., which included *ltmG* from *E. amarillans* E52 but not *E. amarillans* E57 (Fig. 2). However, most of the sexual *Epichloë* spp. appeared to lack *ltm* genes. Faint bands were detected in some samples but these were not analyzed further to determine whether they were from nonspecific amplification. Amplification of the eight genes that comprise clusters 1 and 2 was the most frequently observed pattern, especially among the *Neotyphodium* species examined that included *N. lolii* isolates AR1 and Lp14. *N. coenophialum* e19 has an *E. festucae* ancestry, but just a single PCR product corresponding to *ltmP* was amplified from the DNA of this strain (Fig. 2).

Southern analysis was used to assess the robustness of detecting the epichloë endophytes *ltm* genes by PCR (Fig. 3), since we could not exclude the possibility of false-negative or false-positive results arising from incompatibility or mispriming of the PCR primers. The two geranylgeranyl diphosphate synthase genes, *ggsA*, a housekeeping gene and *ltmG* required for indole-diterpene production, were hybridized to EcoRI-digested genomic DNA isolated from a range of the epichloë endophyte isolates tested by PCR (Fig. 3 and data not shown).

TABLE 3. Primers used for probes and PCR analysis

| Gene	Primer 1		Primer 2		Size (bp)	Application
	Name	Sequence (5′–3′)	Name	Sequence (5′–3′)		
chsV	ChsV-312	CTGGCCAGTCGTTTCCACGT	ChsV-289	GTCGCCGATGTATCGTATTG	483	PCR
ggsA	CYLp19-16	CGTCGCCCACAACATCTTTG	CYLp19-1	CACCATTTCGAGGTAGTC	214	PCR
ltmC	ltmC-216	AGATGACATCTGGAGCATGG	ltmC-236	CTTAAGCGAATTCTACCTTGTGGGTC	1236	Probe
ltmC	ltmC-278	GAAACTGCCAATCGAGCATA	ltmC-279	TTCTTGCAATCATTTTGCAATTG	403	PCR
ltmE	ltmE-356	CCGAGTTTGATGACCTGCTG	ltmE-341	TTCCGCTTCCGAGTAGACTC	687	Probe/PCR
ltmF	ltmF-359	GAATTATGTTACTCTTGGGG	ltmF-360	AAGTTGGCACATAGGTCTTC	227	PCR
ltmG	ltmG-3	ACCGACGCCATTAATGAG	ltmG-1	TGGATCATTCGCAGATAC	407	Probe
ltmG	ltmG-156	GCACAAACAATAAATTCGGCCAA	ltmG-157	AATTTGCCCTCTGTTAAATCCTC	383	PCR
ltmJ	ltmJ-205	CCAAGCATCGATTTGTCACC	ltmJ-206	AATCTGATCGCCATCTTTGC	242	Probe/PCR
ltmK	ltmK-160	ATATTGAATTGCTGCGTGAGGAG	ltmK-161	AGAGGCCAAGAAGCGGCCTGGACA	568	PCR
ltmM	ltmM-7	ACTGGGCATCTTCCATAG	ltmM-35	GTTCGGTGCCTCTAATAC	448	Probe
ltmM	ltmM-158	GTGATCGGTGCTGACGGGGTCCA	ltmM-159	TATCGCCATATTTGCTCCTTGCCC	669	PCR
ltmP	ltmP-191	CCAAGGAGGTTTTGAATGTA	ltmP-192	TTGGATGAGCTCAATCATGC	374	Probe
ltmP	ltmP-280	ATGGCTGTCATTCATACAACAGCTATG	ltmP-281	AGCGTCCCGGACAGGCATATCTCCCA	508	PCR
ltmQ	ltmQ-313	CTACCAGGACAGGCGTGACGTCC	ltmQ-282	CAGAGGTTTAACCCTCTTGACGC	334	PCR
Rua	rua-4	ATAGTCTAACTAGAGGGC	rua-23	TAGATAGGGAAGTTATGC	382	Probe
Tahi	tahi-16	AGTCTTTCCTAGCGTAGG	tahi-95	GTAAGCGGTTAAAAGGGA	522	Probe
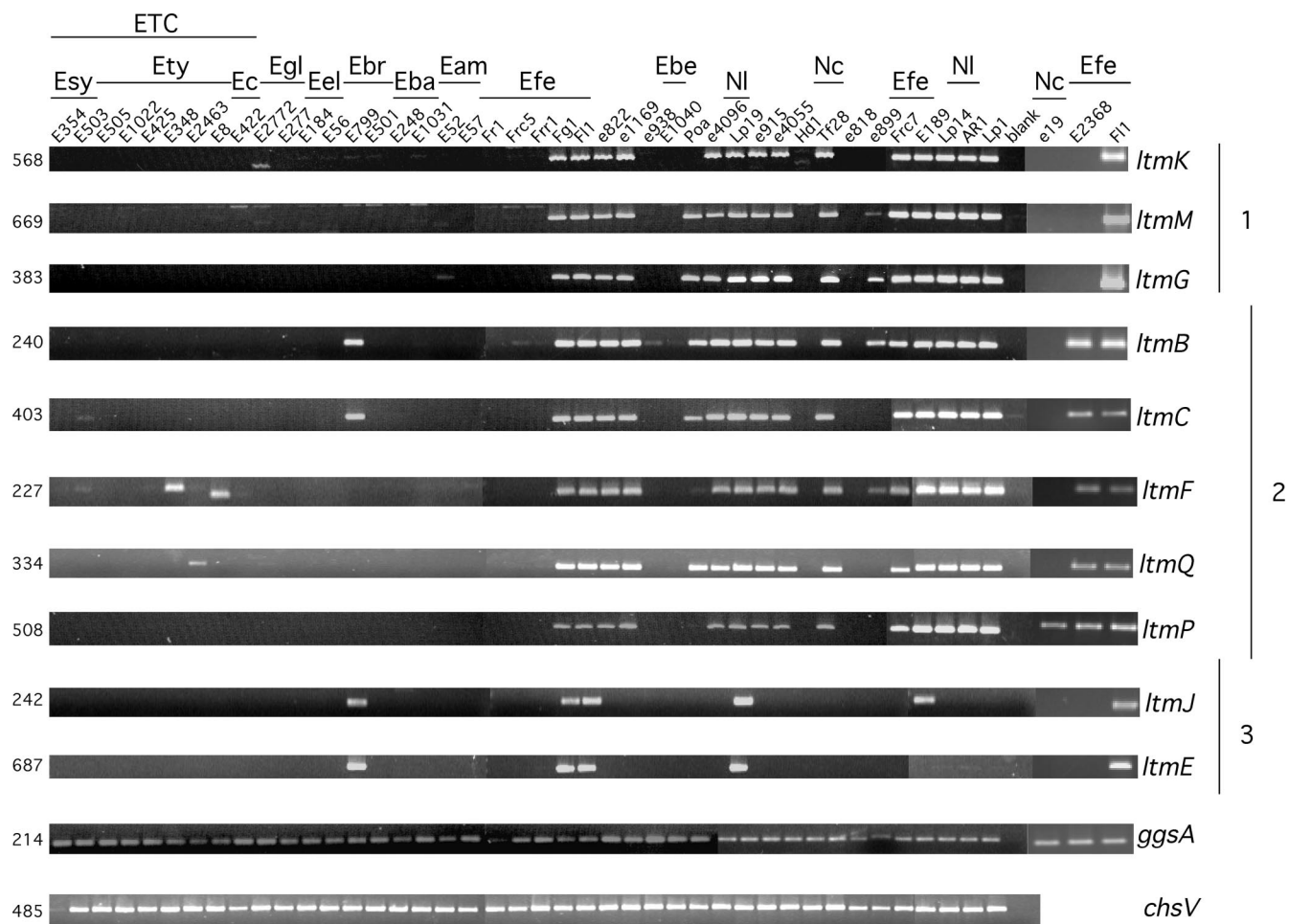
FIG. 2. PCR screen for *ltm* genes. PCR products were amplified with primer pairs designed to conserved regions of the designated gene. The cluster of each gene is indicated as 1, 2, or 3 on the right of the samples. The primers used to amplify each fragment are listed in Table 3. The PCR product size is indicated in base pairs to the left of the samples. Eam, *E. amarillans*; Eba, *E. baconii*; Ebe, *E. brachyelytri*; Ebr, *E. bromicola*; Ec, *E. clarkii*; Eel, *E. elymi*; Efe, *E. festucae*; Egl, *E. glyceriae*; Esy, *E. sylvatica*; Ety, *E. typhina*; Nc, *N. coenophialum*; Nl, *N. lolii*; ETC, *Epichloë typhina* complex. The remaining species can be found in Table 1.

A direct correlation was observed between strains that hybridized with *ltmG* (Fig. 3) and DNA fragments amplified using *ltmG* primers from genomic DNA of the strains (Fig. 2). Furthermore, the *ggsA* probe hybridized to genomic DNA from all of the epichloë endophytes, with a common 4.5-kb band seen in *E. festucae* isolates, or those isolates with *E. festucae* ancestry (data not shown). Given DNA from all strains hybridized with the *ggsA* probe and resulted in amplification products with the *ggsA* primers, the lack of amplification of the *ltm* genes observed for some strains (Fig. 2 and data not shown) was not due to the quality of the DNA.

In further support of the reliability of the PCR analysis for the *ltm* gene detection, the *ltmC* gene was chosen for low-stringency hybridization to EcoRI-digested genomic DNA from a subset of epichloë endophytes (Fig. 4A). The *ltmC* gene was selected because it is an essential pathway gene (57) that is not considered to belong to a major family of closely related genes such as the P450- and FAD-dependent monooxygenases. This was supported by the analysis of the *E. festucae* genome sequence (E2368), which showed there was no significant

match to *ltmC* other than to the gene itself (data not shown). The Southern analysis showed the *ltmC* probe did not hybridize to *E. amarillans* E52 or *E. festucae* Fr1, Frr1, and Frc5, strains that were previously scored as negative for *ltmC* by PCR (Fig. 4A). However, all of the other isolates tested did show hybridization, which is consistent with the PCR results. The Southern hybridization was repeated under standard hybridization conditions to detect *ltm* genes from each cluster: *ltmM*, *ltmP*, and *ltmJ* (Fig. 4B). The hybridization patterns for *ltmM* and *ltmP* were consistent with the results obtained for *ltmC*, where the genes were not detected in isolates E52, Fr1, Frr1, and Frc5. The hybridization pattern for *ltmP* resulted in a greater size variation of hybridizing fragments (Fig. 4B). This result is likely indicative of *ltmP* being adjacent to an AT-rich repetitive region, as is seen in Lp19, and would therefore reflect the genomic differences found in each strain adjacent to *ltm* cluster 2 (75). The *ltmJ* hybridization suggested that only four isolates—E189, Fg1, Fl1, and Lp19—contained sequences from *ltm* cluster 3 (Fig. 4B).

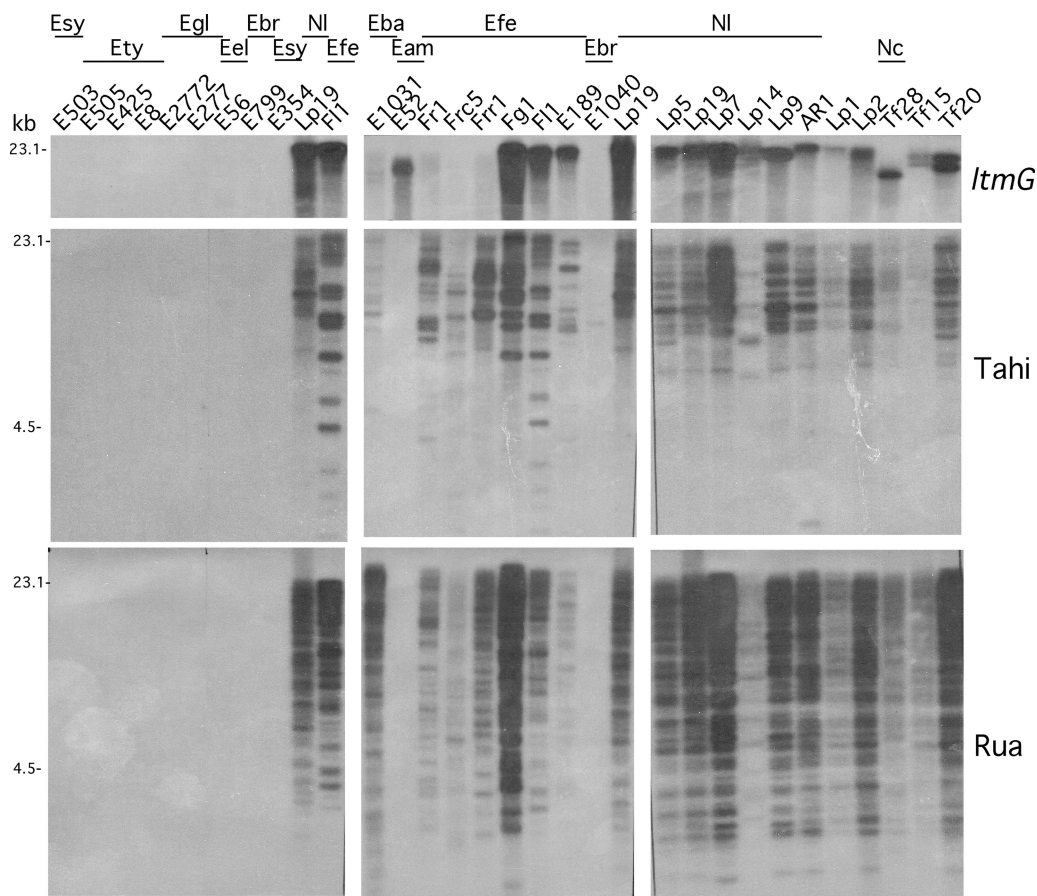To determine whether the retrotransposon sequences pre-

FIG. 3. Detection of *ltmG* and retrotransposon sequences flanking the *LTM* locus in epichloë endophytes using Southern analysis. All genomic DNA (2 μg/lane) was digested with EcoRI and hybridized with ^{32}P-labeled fragments. Each probe is indicated to the right of the figure. The location of *ltmG*, Tahi, and Rua probes at the *LTM* locus can be found in Fig. 1. The primers used to amplify each fragment are listed in Table 3. Each blot was stripped and checked for residual radiolabel between hybridizations. Lp19 and Fl1 were included on each blot as hybridization controls. The species abbreviations are as for Fig. 2.

viously found to be located at the *N. lolii LTM* locus (74, 75) were present in other epichloë endophytes, Southern blots of genomic DNA were hybridized with probes to the Tahi and Rua elements (Fig. 3). These retrotransposon probes hybridized to DNA from *E. festucae* and *E. baconii* strains but did not hybridize to genomic digests of other sexual *Epichloë* isolates tested. The apparent copy number of the retrotransposons varies between isolates. *E. baconii* E1031 and *Neotyphodium* sp. strain FaTG-2 Tf15 (of *E. baconii* and *E. festucae* hybrid origin) have fewer Tahi elements than are found in *E. festucae* Fl1 and *N. lolii* Lp19. *E. festucae* E189 and Frc5 and *N. lolii* Lp14 have fewer Tahi and Rua elements than either *E. festucae* Fl1 or *N. lolii* Lp19. Hybridization of the *pks* pseudogene found adjacent to *ltm* cluster 1 in Fl1 (74) shows no obvious phylogenetic distribution, being present in several different *Epichloë* species but not necessarily in all isolates tested within a species (data not shown). *E. amarillans* E52 contains both *ltmG* and *pks* sequences, but the retrotransposon probes did not hybridize to genomic digests of this strain.

Correlating indole-diterpene chemotypes with *ltm* gene PCR profiles. The PCR and Southern analysis of the *LTM* locus showed a significant difference between *E. festucae* isolates with respect to *ltm* gene composition, ranging from the absence

of all genes to the presence of all 10 genes (Fig. 2, 3, and 4). To test the hypothesis that lolitrem biosynthesis requires all 10 *ltm* genes, a selection of isolates from *E. festucae* (Fr1, Frr1, Frc5, Frc7, Fg1, Fl1, and E189), *N. lolii* (AR1, of *E. festucae* ancestry), and *Neotyphodium* sp. (Lp1, of *E. festucae* and *E. typhina* ancestry) were inoculated into perennial ryegrass (cv. Nui), and tissue extracts from these symbiota were analyzed for indole-diterpenes. The HPLC analysis of these symbiota showed that only Fl1 and E189 endophyte-infected plant material contained lolitrem B (Table 4). LC-MS/MS analysis showed that paspaline, 13-desoxypaxilline, and paxilline were present in perennial ryegrass plants infected with Frc7, Fg1, AR1 (*E. festucae* ancestry), and Lp1 (*E. festucae* and *E. typhina* ancestry). Fl1- and E189-infected plants also contained paspaline and 13-desoxypaxilline, but they did not contain paxilline. In addition, several unknown indole-diterpenes were detected in plant extracts of these isolates (Table 4). Indole-diterpenes were not detected in symbiota infected with Fr1, Frr1, and Frc5, a result consistent with the complete absence of *ltm* genes in these strains (Fig. 2, 3, and 4 and Table 4). Endophyte-infected perennial ryegrass that contained Fg1, which was positive by PCR for all ten *ltm* genes, did not produce lolitrem B but did produce lolitriol, and compounds tentatively
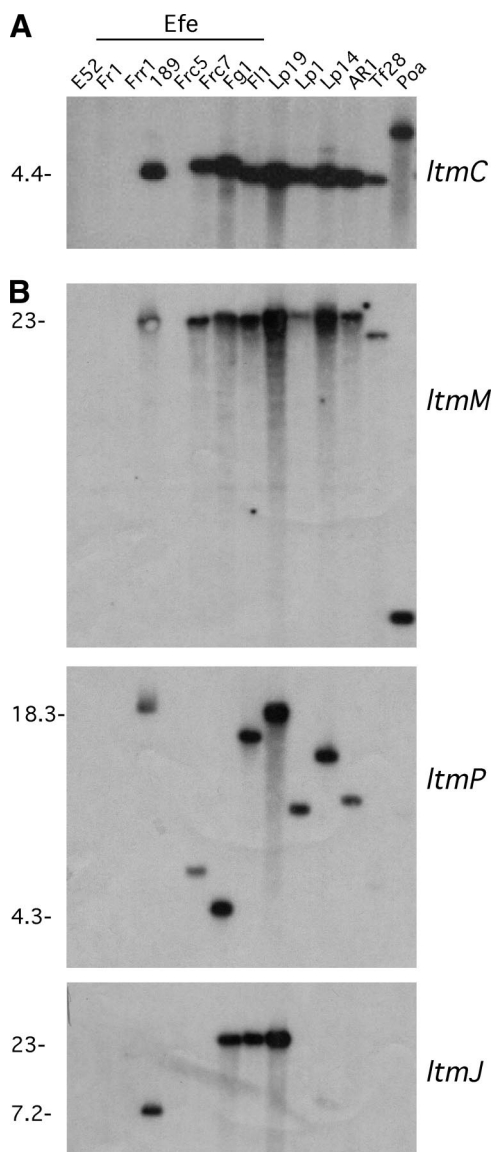
FIG. 4. Southern analysis of endophyte strains for *ltmC*, *ltmM*, *ltmP*, and *ltmJ* genes. Southern analysis of EcoRI-digested genomic DNA hybridized with ^{32}P-labeled probes (Table 3). (A) Autoradiograph of low-stringency hybridization with the complete *ltmC* gene. (B) Standard hybridization with the probes labeled to the right of the figure. Each blot was stripped and checked for residual radioactivity between hybridizations.

identified as lolitrem K and lolitrem J. These indole-diterpenes lack the isoprene group needed to form ring I. Frc7, which has a similar *ltm* gene profile to Lp1 and AR1, did not produce terpendole C and the unknown indole-diterpenes with *m/z* values of 534 and 518.

Two asexual perennial ryegrass endophytes, AR1 and Lp1, did not produce detectable levels of lolitrems in planta but were shown to produce the less complex indole-diterpenes, paspaline, 13-desoxypaxilline, paxilline, and terpendole C, a result that is consistent with the presence of functional gene products from *ltm* clusters one and two. A summary of the

predicted chemotype of the species screened to date is shown in Tables 1 and 2.

The presence of lolitriol, lolitrem K, and lolitrem J in the Fg1-perennial ryegrass symbiota is consistent with the hypothesis that this strain has a nonfunctional copy of *ltmF*, which encodes a prenyl transferase (75). A comparison of the Fg1 *ltmF* nucleotide sequence with that of Fl1 (Fig. 5) and Lp19 (accession no. DQ443465) indicated that Fg1 has a single base deletion within the coding sequence that results in a premature stop codon and, thereby, a nonfunctional gene. Sequence analysis of the Lp19 intergenic region spanning *ltmQ* and *ltmF* showed the presence of a 277-bp sequence with hallmarks of a miniature inverted-repeat transposable element (MITE) that was also identified as a repeat sequence within the promoter of *ltmP* (75). Within this element we identified 35-bp terminal inverted-repeats and a putative TA target site duplication. Two copies of the element, called EFT-14 (for *E. festucae* transposable element) (14), are positioned 363 and 344 bases upstream from the predicted translational start sites of *ltmF* and *ltmP*, respectively. PCR of genomic DNA using primers designed to span the intergenic region from the 3' end of *ltmQ* to the beginning of *ltmF* produced a larger sized band in Fg1 compared to Fl1 and Lp19 (data not shown). Comparison of the nucleotide sequence for the *ltmQ-ltmF* intergenic promoter region of Fg1 against the previously published sequence from Lp19 showed that the EFT-14 element was not present in Fg1 but three additional repetitive sequences that consisted of two possible MITEs, EFT-24 and EFT-25, and an 18-bp minisatellite sequence (consensus sequence of AYACCCCTAWAR AATRCY) (Fig. 5). *E. festucae* Frc7, a strain that contains the genes from *ltm* clusters 1 and 2, also has the repetitive sequences and the frameshift within the *ltmF* coding sequence (Fig. 5).

DISCUSSION

The recent cloning of a cluster of genes for lolitrem biosynthesis has allowed us to determine the *ltm* gene profiles of 44 epichloë endophytes, representing 22 different species, and deduce their indole-diterpene biosynthetic capability. The PCR-based gene analysis allowed us to group these different strains into three main classes: (i) lolitrem producers that have all 10 genes; (ii) indole-diterpene producers that are missing *ltmE* and *ltmJ* (of cluster 3) but have at least the core set of genes *ltmG*, *ltmM*, *ltmC*, and *ltmB* and thus are able to make paspaline and other intermediates; and (iii) indole-diterpene nonproducers without any *ltm* genes or without the full complement of the four core genes. The majority of the sexual isolates (24 of 28) were predicted to be nonproducers consistent with the absence of the *ltm* genes from the isolates tested. The presence of remnant *ltm* sequences in some of these isolates (e.g., E799) suggests that an ancestor of these strains once had the ability to synthesize indole-diterpenes but presumably lost the sequences because they no longer provided an adaptive advantage. Therefore, the inability of these strains to produce lolitrem B is attributed to the absence of the genes rather than mutations within the genes. A similar observation has been made for *E. festucae* strain E189, a non-loline producer that lacks the genes required for loline production (25). Loss of secondary metabolite production has been identified in other

TABLE 4. Occurrence of some indole diterpenes in ryegrass plants infected with selected endophytes

Compound[a]	m/z M+H+	Retention time (min)	MS ID[b]	Result obtained with endophyte isolate(s)[c]:					
				Fr1, Frr1, Frc5	Lp1, AR1	Frc7	Fg1	Fl1	E189
Paspaline*	422	32.5	1	−	+	++	++	+	+
13-Desoxypaxilline*	420	21.6	2	−	++	++	++	++	+
Paxilline*	436	15	2	−	+	++	++	Ld	−
Unknown	454	9.2		−	+	++	+	+	+
Unknown	518	26.1		−	++	−	−	++	+
Unknown	534	21.8		−	++	−	−	++	++
Terpendole C*	520	27.1	1	−	++	−	−	+	+
Lolitriol*	620	9.5	3	−	−	−	+	++	++
Lolitrem N	620	8.6		−	−	−	−	++	Ld
Lolitrem K	602	16.1		−	−	−	++	+	−
Lolitrem J	662	14.3		−	−	−	+	++	Ld
Lolitrem E*	688	30.1	3	−	−	−	−	++	+
Lolitrem B*	686	28.7	3	−	−	−	−	++	++

[a] *, compound identified by relative retention and mass spectral comparison to authentic standards. The remaining compounds were tentatively identified by mass, relative retention, and possible relationship(s) to known compounds. Unknown, the mass indicates an as-yet-uncharacterized indole-diterpene.

[b] Order of mass spectral fragmentation used to identify compound compared to authentic standard for occurrences marked "++".

[c] −, Compounds not detected; Ld, ions at the limit of detection where confirmation of the peak could not be confirmed by mass spectral fragmentation; +, compounds observed at low ion intensity; ++, compounds observed at medium or high intensity. The *ltm* genes and clusters present in the isolates were as follows: Lp1 and AR1, KMGBCFQP and *ltm* 1, 2; Frc7, KMGBCF∧QP and *ltm* 1, 2; Fg1, KMGBCF∧QPJE and *ltm* 1, 2, 3; Fl1 KMGBCFQPJE and *ltm* 1, 2, 3; and E189, KMGBCFQPJE and *ltm* 1, 2, 3. F∧ indicates a nonfunctional *ltmF* gene. For E189, *ltmE* was detected by Southern analysis only.

groups of closely related fungi, such as aflatoxin in *Aspergillus* species (8, 67), and gibberellin (3, 33, 35) and fumonisin (21, 54) biosynthesis in *Fusarium* (teleomorph *Gibberella* spp.) species. Analysis of secondary metabolite gene clusters at the gibberellin and fumonisin loci in closely related *Fusarium* species and for aflatoxin biosynthesis in *Aspergillus* spp. revealed examples of partial or absent gene clusters (3, 8, 21, 35, 54, 67).

FIG. 5. Schematic comparison of the *ltmF* gene between Lp19, Fl1, Fg1, and Frc7. The sequence chromatograms show the missing A in the Fg1 and Frc7 *ltmF* sequence that results in a frameshift. The schematic maps were drawn from the sequence contained in accession numbers DQ443465 (Lp19), EU530694 (Fg1), and EU544671 (Frc7). The MITE sequences are labeled as EFT14, EFT24, and EFT25 with the terminal inverted-repeats (TIRs) shown as arrows. The 18-bp minisatellite sequence is labeled mini. The red circle represents the stop codon in the truncated *ltmF*.

However, in some cases, a lack of secondary metabolite production correlated with little or no gene expression and/or sequence variations within the coding and promoter regions of genes within the cluster, which can result in naturally occurring pathway variants (21, 33–35, 53). The *LTM* locus from the *Epichloë* and *Neotyphodium* species we screened contain examples of gene absence (partial or complete cluster) and in three cases, sequence variation in a gene (*ltmF* and *ltmE*) that blocks or alters the encoded biosynthetic step.

The members of the *E. festucae* clade were represented in all three classes of *ltm* gene distribution, where only some isolates (five of eight) contained *ltm* genes, and of those with *ltm* genes, only four isolates had the four core genes responsible for paspaline production. The *ltm* gene profile for these *E. festucae* isolates was predicted by PCR and supported by Southern analysis. In turn, the ability of seven *E. festucae* to produce indole-diterpenes in planta was confirmed by HPLC and LC-MS/MS. Three *E. festucae* isolates—Fr1, Frr1, and Frc5—lack *ltm* genes and did not produce indole-diterpenes when in symbiosis with perennial ryegrass. Of the remaining *E. festucae* isolates, only Fg1 and Fl1 contained the 10 *ltm* genes, while E2368, Frc7, and E189 contained 5, 8, and 9 genes, respectively. The alkaloid analysis for Fg1 and E189 was inconsistent with predictions from the *ltm* gene profile, as determined by PCR. Strain Fg1 appears to contain all 10 *ltm* genes, and yet in planta it produced lolitrems J and K but not lolitrem B (Table 4). This result was subsequently explained based on the presence of a frameshift in *ltmF*, resulting in a nonfunctional gene. PCR analysis of E189 detected nine *ltm* genes and did not detect *ltmE* (encoding a multifunctional prenyl transferase with similarity to LtmC and LtmF at the N- and C-terminal domains, respectively), and yet this strain was still able to produce lolitrem B in planta, albeit at a consistently lower level than did Fl1. However, Fl1 and E189 had different indole-diterpene profiles where in E189-infected plant material only trace amounts of lolitrem N and J were detected, and lolitrem
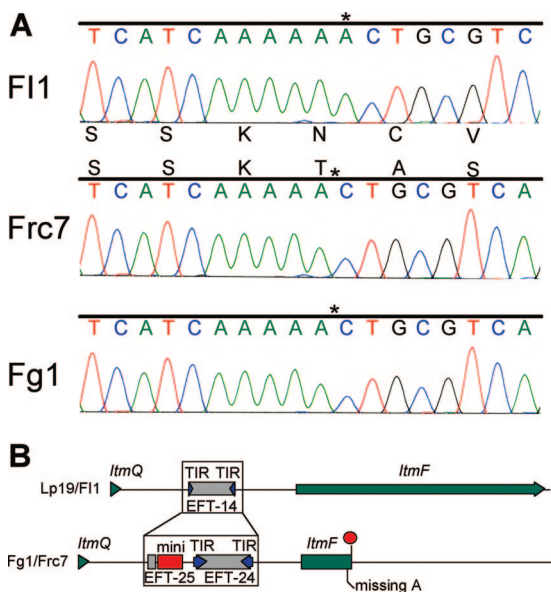
K was not detected at all. Southern analysis determined that at least a partial copy of *ltmE* is present in E189 (data not shown), but this result does not indicate whether one or both prenyl transferase domains are complete and functional. Sequence analysis of *ltmE* from E189 and complementation analysis with each *ltmE* domain in a deletion background will determine whether just one domain is required for lolitrem B production. We also cannot discount the influence that the host genotype has on the alkaloid profile of E189-infected material.

E. festucae is a progenitor to many asexual species (see Table 1) (11, 20, 44), so greater variation of the *ltm* gene distribution was expected among the *Neotyphodium* hybrids, which would reflect the *ltm* amplification patterns identified from *E. festucae*. Analysis of the eight *E. festucae* isolates resulted in five different *ltm* PCR profiles (0, 5, 8, 9, and 10 *ltm* genes). Of the 16 *Neotyphodium* endophytes screened by PCR, 12 have an *E. festucae* ancestor and, of these, 11 contained *ltm* sequences. *N. lolii* Lp19 contained all 10 genes, nine isolates were positive for 8 genes, and just *ltmP* was amplified from *N. coenophialum* e19 (a hybrid with ancestry from *E. festucae*, *Lolium*-associated clade and *E. typhina* complex). Only three *Neotyphodium* isolates—*N. australiense* (e938; Efe x ETC), *Neotyphodium* isolate Hd1, and *N. gansuense* var. *inebrians* (e818)—did not yield amplification products with the *ltm* primers and, of these, only e938 has *E. festucae* ancestry (42). Although these data could suggest that a common, predominantly lolitrem producing *E. festucae* ancestry was involved with the hybridization events that produced *Neotyphodium* spp. with *E. festucae* lineage, we must also consider the possibility that there is greater diversity within the *Neotyphodium* species than tested here. This present study has a strong bias toward endophytes associated with grasses in the genus *Lolium* and does not present comprehensive analyses from the recently characterized *N. tembladerae* (also with *E. festucae*- and *E. typhina*-like ancestry) identified in host tribes Poeae, Aveneae, Bromeae, and Meliceae in Argentina (20). The two *N. tembladerae* isolates used in this study, e1169 from *Poa huecu* (from Argentina) and e4055 from *Festuca arizonica* (from Arizona), have eight *ltm* genes and were both isolated from grasses in the Poeae tribe. *N. australiense* (e938) and *N. aotearoae* (e899) isolated from *Echinopogon ovatus* of the tribe Aveneae provided examples of variation in *ltm* gene composition with respect to their phylogenetic origins, with no evidence for *ltm* genes in e938 (which has *E. festucae* ancestry) but at least four—*ltmM*, *ltmG*, *ltmB*, and *ltmF*—in e899, which resides on a distinct and deeply rooted clade within the epichloë phylogeny (42, 44). In a previous study, alkaloid analysis of an endophyte-infected *Echinopogon ovatus* from New Zealand did not indicate the presence of lolitrem B by HPLC, but analogs of paxilline were detected by enzyme-linked immunosorbent assay (39). The small quantity of DNA that was isolated from e899, due to its slow growth rate, was not enough to include in Southern analysis to determine whether an *ltmC* orthologue could be detected, which would provide support that a paxilline analogue could be made by this isolate.

The biosynthetic pathway for lolitrem is predicted to act as a metabolic grid rather than a linear pathway where the A and B rings form independently of the I ring (see the lolitrem B structure in Fig. 6) (19, 46, 48, 49, 69). By comparing the lolitrem intermediates identified in Fl1, Fg1, Frc7, AR1, and

Lp1 to the corresponding gene profiles of these strains, we can begin to better define the function of the gene products in the biosynthetic pathway (Fig. 6). However, since the biochemical pathway is so complex, the functions of each gene product required for lolitrem biosynthesis will be confirmed by gene disruption and chemical analysis of the resulting mutants (D. Takemoto and B. Scott, unpublished data). In agreement with the chemotype predicted from the gene composition of the strains Fl1, Fg1, Frc7, E189, AR1, and Lp1, these strains were able to produce at least paspaline and 13-desoxypaxilline. This is consistent with the genes *ltmG*, *ltmM*, *ltmB*, *ltmC*, and *ltmP* from Fl1, Fg1, Frc7, E189, AR1, and Lp1 encoding functional gene products. Although paxilline was detected in Lp1, AR1, Frc7, and Fg1, it was absent from Fl1- and E189-infected plant material. Our proposed lolitrem pathway (Fig. 6) indicates that a minor pathway route for the production of paxilline is present for some isolates; thus, we speculate that paxilline is formed due to a diversion of intermediates in isolates that do not contain the complete complement of *ltm* genes. AR1 and Lp1 lack genes, *ltmE* and *ltmJ*, found in *ltm* cluster 3 but are both able to produce terpendole C that is prenylated to give rise to the I ring but lacks the A and B rings found in lolitrem B (Fig. 6 and Table 4). These observations are consistent with the roles of LtmJ and LtmE catalyzing the formation of the A and B ring structures and roles for LtmF and LtmK in the formation of the I ring structure (Fig. 6). Analysis of Fg1 by PCR indicated the presence of 10 *ltm* genes, and yet this isolate is not able to produce terpendole C or lolitrem B in planta. Instead, lolitriol and putative lolitrem K and J were detected in Fg1-infected plant material, indicating that gene products for formation of the A and B rings are present and functional but that those required for formation of the I ring are not. Analysis of the *ltmF* promoter and coding region in Fg1 indicated that this isolate did not contain a functional *ltmF* gene. These data provide further support that LtmE and LtmJ are likely candidates for the formation of the A and B rings (Fig. 6). Lolitrem J is an acetylated product, accumulating in the absence of isoprenylation required for the formation of an I ring, via action of a further oxidation step. Frc7 lacks *ltmE* and *ltmJ* and would be predicted to have a similar indole-diterpene profile to AR1 and Lp1 with the production of terpendole C. However, unlike AR1 and Lp1, Frc7 is unable to produce the terpendole metabolites due to the same mutation in *ltmF* that was identified in Fg1.

This research has established, by PCR and LC-MS/MS analysis, that agriculturally important endophytes such as AR1 that have been reported to lack lolitrems (16) have the machinery and ability to produce other indole-diterpenes, such as paspaline, 13-desoxypaxilline, and terpendole C. Paxilline and terpendole C are tremorgenic in mice, but the tremors they induce do not last as long as those caused by lolitrems A, B, and F (48). Field trials with AR1-infected perennial ryegrass showed improved animal productivity over common toxic endophyte (lolitrem B producer)-infected perennial ryegrass, with no evidence of animal toxicity such as ryegrass staggers (1, 2). Indole-diterpene precursors of lolitrem B could provide a selective advantage to the grass that is not related to animal toxicity but may involve protection against insects, such as that found with the nontremorgenic indole-diterpene nodulisporic acid from *Nodulisporium* species (7).
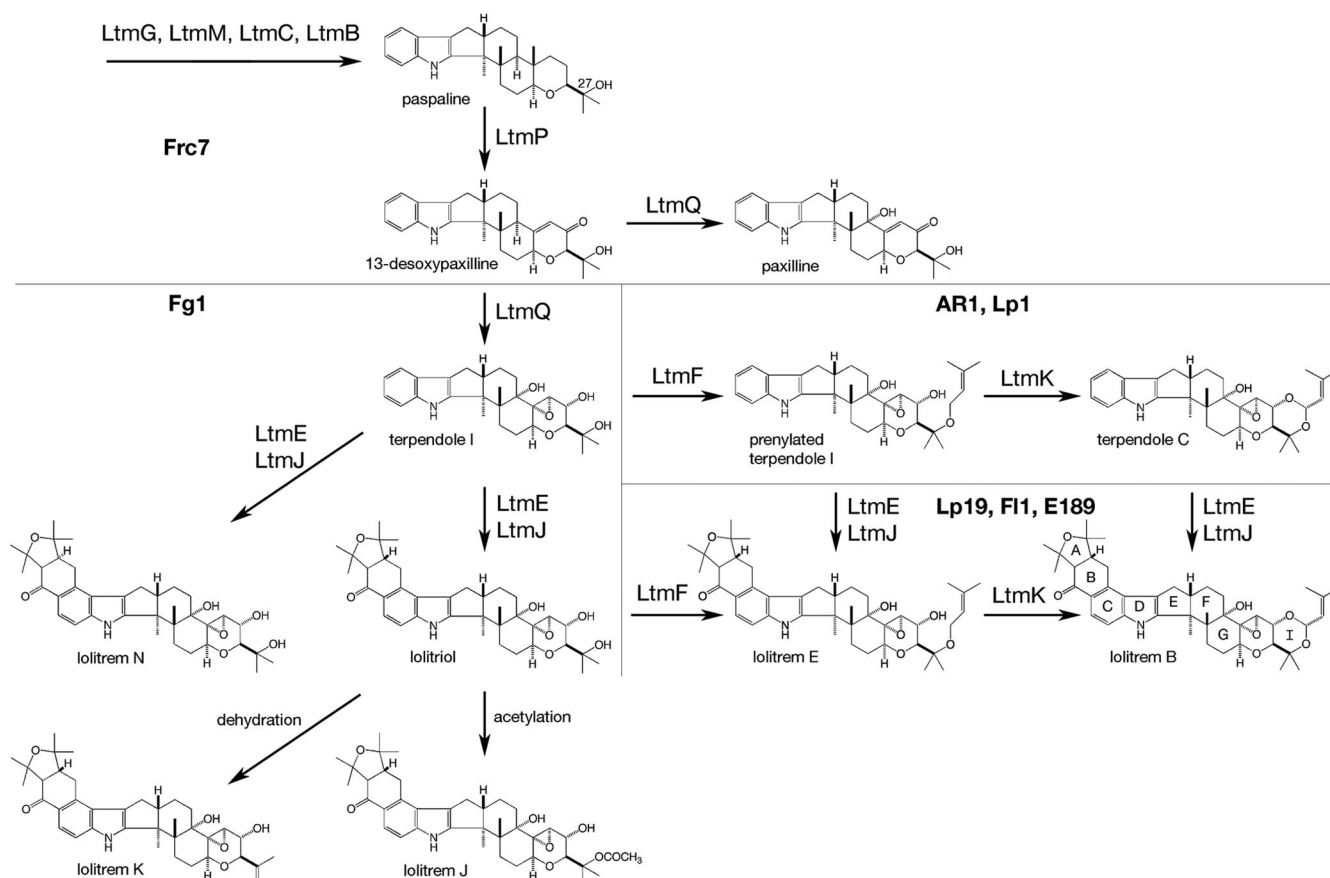
FIG. 6. Proposed framework for indole-diterpene biosynthesis in epichloë endophytes. The scheme proposed is a working model to explain the chemical diversity of indole-diterpenes found in different epichloë isolates. The identities of the rings in the lolitrem B structure are denoted by letters. Single arrows do not necessarily denote single enzymatic steps.

The *LTM* loci within *E. festucae* Fl1 and *N. lolii* Lp19 are very complex in that the *ltm* genes are present on three clusters separated by AT-rich remnants of transposable elements that appear to have undergone a repeat-induced point mutation-like mechanism (74, 75). The majority of *Epichloë* species tested lacked both *ltm* genes and the transposable elements Rua and Tahi found associated with the *LTM* locus identified in *N. lolii* and *E. festucae* (74, 75). The *pks* pseudogene found adjacent to cluster 1 of Fl1 was present in some *Epichloë* species. However, some *E. festucae* isolates that lacked the *ltm* genes contained these retrotransposons, indicating that the *LTM* locus and the retroelements had a separate evolutionary origin. The discontinuous distribution of the *ltm* genes within epichloë endophytes suggested that a common ancestor has contributed to the evolution of this cluster, as was established within ascomycetes with respect to polyketide synthases (24) and recently proposed with the nonribosomal peptide synthetase genes among epichloë endophytes (23). The Tahi and Rua transposable elements are present in high copy numbers within the *E. festucae* genome and within *Neotyphodium* species with an *E. festucae* progenitor. Both elements are also present in *E. baconii*, a close relative of *E. festucae* (11), but absent from other sexual species such as *E. amarillans*. Analyses show that *E. amarillans*, *E. baconii*, and *E. festucae* cluster together in a well-supported clade within the epichloë phylog-

eny (11, 44, 45); thus, these data could suggest that the retro-transposon elements, Tahi and Rua, invaded *Epichloë* after *E. amarillans* diverged but before the split of *E. baconii* and *E. festucae*. However, the rapid evolution of these elements may preclude detection by hybridization in the more distant relatives. The presence of other repetitive elements, such as MITEs, at the *LOL* locus of *N. uncinatum*, a hybrid of *E. bromicola* and *E. typhina* origin, suggests that transposons may be widespread in epichloë endophytes (15).

The data presented here show that many epichloë endophytes are unable to produce the neurotoxin lolitrem B likely due to a lack of the required biochemical machinery. There is evidence that *Epichloë* ancestors possessed the *ltm* gene clusters and that these have been lost over time, a situation similar to that proposed for the *LOL* locus required for loline production (25). PCR profiling of the *ltm* genes can be utilized as a tool to predict the indole-diterpene biosynthetic capability of an isolate before it is inoculated into a pasture grass. Additional DNA sequences from epichloë endophytes will allow for further refinement of primer sequences, thereby making the PCR screen even more robust. This technique can provide an advantage over current chemotyping methods that rely solely on the chemical analysis of an expected compound. Although amplification of an *ltm* gene does not necessarily predict synthesis of a functional gene product in the host grass, the ab-

sence of a gene or set of genes provides a robust method for predicting alkaloid compounds in pasture grasses that is independent of host genotype and wound-inducible responses, thereby providing a more targeted approach to chemotyping endophyte-grass associations. Extension of this technique to the other alkaloid biosynthesis genes such as those required for the synthesis of the beneficial lolines and peramine, and detrimental ergot alkaloids will provide the plant breeding industry with a "toolkit" to identify epichloë endophytes of agronomic importance.

ACKNOWLEDGMENTS

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