Fungal Lactamases: Their Occurrence and Function

Minglu Gao1, Anthony E. Glenn2, Alex A. Blacutt1 and Scott E. Gold2*

1 Department of Plant Pathology, The University of Georgia, Athens, GA, United States, 2 Toxicology and Mycotoxin Research Unit, U.S. National Poultry Research Center, United States Department of Agriculture – Agricultural Research Service, Athens, GA, United States

Fungi are absorptive feeders and thus must colonize and ramify through their substrate to survive. In so doing they are in competition, particularly in the soil, with myriad microbes. These microbes use xenobiotic compounds as offensive weapons to compete for nutrition, and fungi must be sufficiently resistant to these xenobiotics. One prominent mechanism of xenobiotic resistance is through production of corresponding degrading enzymes. As typical examples, bacterial β-lactamases are well known for their ability to degrade and consequently confer resistance to β-lactam antibiotics, a serious emerging problem in health care. We have identified many fungal genes that putatively encode proteins exhibiting a high degree of similarity to β-lactamases. However, fungal cell walls are structurally different from the bacterial peptidoglycan target of β-lactams. This raises the question, why do fungi have lactamases and what are their functions? Previously, we identified and characterized one Fusarium verticillioides lactamase encoding gene (FVEG_08291) that confers resistance to the benzoxazinoid phytoanticipins produced by maize, wheat, and rye. Since benzoxazinoids are γ-lactams with five-membered rings rather than the four-membered β-lactams, we refer to the predicted enzymes simply as lactamases, rather than β-lactamases. An overview of fungal genomes suggests a strong positive correlation between environmental niche complexity and the number of fungal lactamase encoding genes, with soil-borne fungi showing dramatic amplification of lactamase encoding genes compared to those fungi found in less biologically complex environments. Remarkably, Fusarium species frequently possess large (>40) numbers of these genes. We hypothesize that many fungal hydrolytic lactamases are responsible for the degradation of plant or microbial xenobiotic lactam compounds. Alignment of protein sequences revealed two conserved patterns resembling bacterial β-lactamases, specifically those possessing PFAM domains PF00753 or PF00144. Structural predictions of F. verticillioides lactamases also suggested similar catalytic mechanisms to those of their bacterial counterparts. Overall, we present the first in-depth analysis of lactamases in fungi, and discuss their potential relevance to fitness and resistance to antimicrobials in the environment.

Keywords: soil, fungi, lactams, β-lactamases, Fusarium verticillioides
INTRODUCTION

The soil is one of the most complex habitats on earth due primarily to the diversity of microorganisms that inhabit it and the myriad biochemical products they secrete. Experiments utilizing metagenomic technologies estimate up to several million species of bacteria per gram in some naturally occurring soils (Gans, 2006). The majority of these species are, to date, uncultured. For fungi, less information is available. Earlier estimates suggested that there are approximately 1.5 million fungal species on the planet (Hawksworth, 1991), but more recent global estimates of six million soil fungi were suggested based on comprehensive molecular studies (Taylor et al., 2014). This microbial diversity creates a dynamic environment for microorganisms to communicate and compete for limited resources. Further, metabolic processes of microbes together with plants act as significant sources of chemical diversity, and microbes in the soil milieu are constantly and unavoidably exposed to foreign chemicals (xenobiotics). Some xenobiotics are easily tolerated and degraded, while others have inhibitory effects (Parkinson et al., 2001). Xenobiotics that are deleterious to the growth or metabolic activities of other microorganisms can be considered antibiotics, and play critical ecological roles in competitive interactions (Thomasow et al., 1997; Davelos et al., 2004; Kinkel et al., 2012). It has been posited that microorganisms and plants have adopted antibiotic production as offensive and/or defensive strategies to adjust to changing circumstances, allowing microbial colonization in the rhizosphere or persistence of plants in the environment (Lynch et al., 2004). Compared to the surrounding soil, the rhizosphere of plants can be particularly rich in nutrients (Marschner et al., 2004). This microbial oasis stimulates competitive and antagonistic relationships among would-be colonizers. For example, phenazine production by pseudomonads and trifolitoxin production from certain Rhizobium species correlate with soil survival and suppressive effects (Parkinson et al., 2001). In addition to transcriptional responses suggestive of a form of microbial communication (Goh et al., 2002; Davies, 2006). In addition to microbial sources, compounds with antibiotic activity are also found in plants (VanEtten et al., 1994; Bozdogan and Appelbaum, 2004; González-Lamothe et al., 2009). Maackiain is a plant-derived antibiotic extracted from red clover and alfalfa. Previous work has shown that maackiain is toxic to several genera of fungal pathogens of legume and non-gram negative hosts (Duczek and Higgins, 1976; Delserone et al., 1992). Maize, wheat, and rye can constitutively produce benzoaxazines and benzoaxazolinones, which help reduce insect damage and confer resistance to various fungal and bacterial pathogens (Couture et al., 1971; Baker and Smith, 1977; Glenn et al., 2016).

To combat antibiosis, bacteria have developed resistance mechanisms such as efflux pumps and hydrolytic enzymes. Notorious among the latter group, β-lactamases have been thoroughly studied due to the resistance they confer to the widespread clinically used β-lactam antibiotics. Parallel to the presence in bacteria, genes encoding “β-lactamases” are also abundant across different fungal families. In contrast to bacteria, almost nothing is known about the function of these genes in fungi. Previous work is limited to two studies on the hydrolytic function of lactamase (metallo-β-lactamase, MBL) encoding genes in Fusarium verticillioides and Fusarium pseudograminearum (Kettle et al., 2015b; Glenn et al., 2016). This evidence serves as a foundational paradigm for studying hydrolytic lactamases in fungi and prompts the hypothesis that, as in bacteria, many of these enzymes function in degradation and resistance to xenobiotic compounds. In this review, we will describe an initial look at the distribution of lactamase-encoding genes in fungi and speculate on their ecological roles. We will also describe current and planned approaches to decipher the roles of 46 lactamase-family genes in the F. verticillioides genome.

LACTAMS—THE ARCHETYPICAL CLASS OF ANTIBIOTICS

Bactericidal β-Lactams

β-Lactams comprise the largest group of antibiotics, and they have been extensively utilized for their antibacterial effect (Tipper, 1985). Beginning with Alexander Fleming's Nobel Prize-winning serendipitous discovery of a penicillin-producing mold, β-lactams and their semisynthetic derivatives have been the most impactful antibiotics in medicine (Demain and Elander, 1999; Lewis, 2013). Their mode of action is well characterized and involves a four-membered cyclic amide ring (Figure 1) that occupies the catalytic sites of transpeptidases, also referred to as penicillin-binding proteins. These proteins are essential for cross-linking peptidoglycan layers of bacterial cell walls, thus β-lactam antibiotics disrupt bacterial cell wall synthesis, resulting in cell lysis (Waxman and Strominger, 1983).

Lactam Production in Fungi

Fungi are the original source of two foundational β-lactam antibiotics: penicillin and cephalosporin. These drugs are still industrially produced, primarily using Penicillium chrysogenum and Acremonium chrysogenum (previously Cephalosporium), respectively (Brakhage et al., 2009). Lactam production in fungi is frequently coordinated through the activity of gene clusters containing necessary biosynthetic enzymes and pathway-specific transcriptional regulators (Brakhage et al., 2009; Khaldi et al., 2010; Osbourn, 2010; Brakhage and Schroechk, 2011).
Fungal gene clusters are hypothesized to assist in retention of biochemical functions by reducing gene loss due to recombination in highly dynamic genomes (Osborn, 2010). Fungal genomes provide enormous potential to produce many complex lactam-containing compounds (Figure 2), including higher order lactam compounds (e.g., five-membered, γ-lactam rings). Two new hetero-spirocyclic γ-lactams, azaspirofurans A and B, were isolated from a marine sediment-derived fungus Aspergillus sydowii (Ren et al., 2010). A maize seed-borne endophyte Sarocladium zeae (formerly Acremonium zeae) was found to produce γ-lactam compounds, named pyrrocidine A and B (He et al., 2002). Further, the cytotoxic awajanomycin from Acremonium species, cytochalasins from Rhinocladiella, and colletotrilactams A–D from endophytic Colletotrichum gloeosporioides all exemplify fungal production of higher order lactams (Wagenaar et al., 2000; Jang et al., 2006; Wei et al., 2016). Such lactam production among fungi diversifies xenobiotic composition in soil and may contribute to the discovery of new valuable antibiotics.

Antifungal Lactams
In addition to the fungal production of bactericidal lactams, emerging evidence indicates that certain atypical lactams can be fungistatic or fungicidal regardless of their origins (Figure 3; Brakhage et al., 2009). Novel monocyclic θ-thiolated β-lactams revealed varying degrees of in vitro antifungal activity against seven Candida species (O’Driscoll et al., 2008). The fungistatic mode of action against Candida was postulated to simulate what was observed against Staphylococcus aureus, where these lactams diffused through the cell membrane and interacted covalently with an unknown and possibly evolutionarily conserved target. Two synthetic azetidin-2-one compounds showed moderate antifungal activity against Botrytis cinerea, Colletotrichum lindemuthianum, and the oomycete Phytophthora infestans (Arnoldi et al., 1990). The previously mentioned pyrrocidine A and B from S. zeae are antagonistic to kernel rotting fungi including Aspergillus flavus and F. verticillioides (Wicklow et al., 2005). Interestingly, pyrrocidine A differs from B only in that it possesses a double bond in the γ-lactam ring, and pyrrocidine A shows inhibition at a lower concentration than does B, implying the relevance of the lactam ring to antibiosis.

Alternatively, structural conformation changes conveyed by the single vs. double bond could potentially play a role in the observed differential toxicity. Recent studies on synthetic bicyclic lactam analogs of natural plant derived lactones have also revealed their fungistatic effects against B. cinerea, Penicillium citrinum, and Aspergillus glaucus (Walczak et al., 2014). For example, by replacing an oxygen atom with nitrogen in the five-membered ring during a heteroatom analysis of cis-3-oxabicyclo[4.3.0]non-7-en-2-one, a novel γ-lactam compound was created with a significant increase in antifungal activity (Walczak et al., 2014). These discoveries should stimulate further exploration of antifungal lactams and their modes of action.

β-LACTAMASES

Lactam Resistance
The spread of antibiotic resistance among bacteria is one of today’s major world health concerns (Berendonk et al., 2015). In fact, many current publications in the popular press are predicting the end of the age of antibiotics in the near future (Sun and Dennis, 2016), and the World Health Organization recently held a conference on the subject entitled “The end of antibiotics?” Natural sources and clinical/agricultural overuse of antibiotics impose selection pressure for antibiotic resistance, leading to a rise in the number of resistant microbes and the spread of resistant genes regardless of their origins (Allen et al., 2010; Chang et al., 2015). Currently, three major mechanisms have been proposed to generate resistance to β-lactam antibiotics (Figure 4): (1) restricted access to drug targets either by (a) preventing drug entry or (b) enhanced drug efflux (Li et al., 1994), (2) alteration of drug targets (Malouin and Bryan, 1986), or (3) the presence of drug-degrading enzymes (Fernandes et al., 2013). Moderate lactam resistance may be developed by intragenic recombination, where genetically distinct alleles occasionally are produced. Such events generate, for example, new alleles of mosaic transpeptidase (penicillin target protein) genes with low penicillin-binding affinities (Zhang et al., 1990; Campos et al., 1992). HGT was proposed decades ago as another means of acquisition of lactam resistance. HGT appears responsible for the spread of both resistance-conferring transpeptidases and
FIGURE 2 | Examples of lactam-containing fungal compounds. Lactam bonds are highlighted in red. (1) Penicillin, the historically significant fungal lactam produced by Penicillium chrysogenum (Fleming, 1929); (2) cephalosporin, a group of bactericidal β-lactams from Acremonium chrysogenum (Harrison and Bratcher, 2008); (3) gliotoxin, a mycotoxin produced by Aspergillus fumigatus and several other species (Forseth et al., 2011); (4) sirodesmin PL, a phytotoxin produced by the fungus Leptosphaeria maculans causing blackleg disease of canola (Gardiner et al., 2004); (5) roquefortine C, a mycotoxin produced by Penicillium species (Kokkonen et al., 2005); (6) meleagrin, a bioactive alkaloid produced by deep ocean Penicillium (Nozawa and Nakajima, 1979); (7) cyclopiazonic acid, a toxic fungal secondary metabolite originally isolated from Penicillium cyclopium (Holzapfel, 1968); (8) equisetin, a Fusarium equiseti metabolite (Hazuda et al., 1999); (9) ilicicolin H is an NRPS-polyketide hybrid product discovered from Cylindrocladium iliciola MFC-870 and is a potent antifungal agent (Singh et al., 2011); (10) ergotamine, an ergopeptide and part of the ergot family of alkaloids from Claviceps purpurea (Schiff, 2006); (11) cytochalasin D, a cytostatically active metabolite isolated from Tubercularia species (Wang et al., 2003); (12) cytochalasin E from Rhinocladiella species (Wagenaar et al., 2005); (13) azaspirofuran A and (14) azaspirofuran B produced by Aspergillus sydowi (Ren et al., 2010); (15) awajanomicin produced by Acremonium species (Jiang et al., 2006); (16) fusarin C, a mycotoxin produced by several Fusarium species (Wiebe and Bjeldanes, 1981).

plasmid-encoded β-lactamases contributing to high-level lactam resistance and the appearance of “superbugs” with resistance to most or all current antibiotic therapies (Dowson et al., 1990; Coffey et al., 1993; Weldhagen, 2004; Davies and Davies, 2010).

### Bacterial β-Lactamases

β-Lactamase enzymes are the most common mechanism of resistance to β-lactam antibiotics, hydrolyzing the lactam bond in their four-membered ring structures to abolish activity (Livermore, 1998). As these antibiotics are classically active against peptidoglycan cell wall synthesis, the corresponding hydrolytic β-lactamases result in high prevalence of resistant strains and a potential increase in virulence. The first penicillin-hydrolyzing β-lactamase identified was an AmpC cephalosporinase in Escherichia coli in 1940, several years before the actual introduction of penicillin into clinical practice (Abraham and Chain, 1940).

Two primary schemes of classifying bacterial β-lactamases have been proposed based on functionality or molecular characteristics (Table 1). The functionality classification scheme divides bacterial β-lactamases into three major groups based on inhibitory specificities and the potential requirement of zinc ion for activity (Bush et al., 1995; Frère, 1995). Group 1 includes cephalosporinas that are not well inhibited by active site-directed β-lactamase inhibitors, such as clavulanic acid. Group 2 encompasses β-lactamases that are inhibited by clavulanic acid. Group 3 refers to MBLs that require zinc ions for activity. In addition to conventional hydrolases targeting β-lactams, the MBL superfamily includes lactonases that hydrolyze lactone bonds. A classic example is N-acyl homoserine lactonase produced by various bacteria. These lactonases are able to inactivate
N-acyl homoserine lactones by hydrolyzing the lactone bond, resulting in quenching of bacterial quorum-sensing signaling (Dong et al., 2001; Riaz et al., 2008). The necessity of zinc ions is suspected by the universal presence of a conserved di-nuclear zinc binding site in known lactonases and confirmed by zinc’s essential role during catalytic activity and protein folding (Thomas et al., 2005). The second scheme for classification of β-lactamases utilizes nucleotide and amino acid sequences to divide them into four molecular classes designated A–D (Bush et al., 1995). Enzymes belonging to class A, C, and D act by a serine-based mechanism, often containing Pfam domain PF00144. Those in class B are zinc-based MBLs with Pfam domain PF00753, equivalent to functional Group 3. Serine-based β-lactamases (SBLs) possess conserved motifs S-X-X-K, S/Y-X-N/V, and K-T/S-G in that order, where the serine in the first motif serves as the active site targeting the β-lactam ring. Class B β-lactamases contain a primary zinc-binding motif H-X-H-X-D-H followed by conserved amino acids of Gly, Leu, His, Gly, Asn, and His at specific positions. Except for these conserved amino acids, the rest of their sequences are generally divergent, with greatly differing tertiary structures and catalytic efficiencies (Ehmann et al., 2012).

**Fungal Lactamases**

Interestingly, genes encoding proteins with β-lactamase homology are widely distributed across major taxa. As of March 1, 2017, there were 1,096,469 manually and computationally annotated β-lactamase encoding genes reported in the National Center for Biotechnology Information (NCBI) protein database across all kingdoms of life. As depicted in Figure 5, 93% of them (1,021,177 genes) lie in the domain Bacteria. Although non-bacterial lactamases share similarities with those found in bacteria, less than 1% have been functionally characterized. It is very likely that many non-bacterial “β-lactamases” are not involved in degrading classic β-lactams, so we will refer to them simply as lactamases below. Interestingly, of the

![Figure 3](image-url)
FIGURE 4 | Three major resistant mechanisms present in bacteria against β-lactam antibiotics. Bacteria can develop resistance to β-lactams by (1) restricting their access to penicillin binding proteins; (2) altering penicillin binding proteins to avoid being recognized by β-lactams; (3) producing β-lactamases. Generally speaking, Gram-negative bacteria retain β-lactamases in the periplasmic space between inner and outer membranes. In contrast, Gram-positive bacteria do not possess an outer membrane, and they usually release β-lactamases to the extracellular environment. Fine details of the bacterial membranes and peptidoglycan layer are not shown in this simplified drawing.

TABLE 1 | Classification of bacterial β-lactamases.

| Functional classification | Molecular classification | Inhibition by clavulanic acid | Zinc requirement | Function |
|---------------------------|--------------------------|------------------------------|-----------------|----------|
| Group 1                   | Class C                  | No                           | No              | Cephalosporinase |
| Group 2                   |                          |                              |                 |           |
| 2a                        | Class A                  | Yes                          | No              | Penicillinas |
| 2be                       | Class A                  | Yes                          | No              | Extended-spectrum β-lactamases |
| 2br                       | Class A                  | Yes                          | No              | Inhibitor-resistant TEM-derivative enzymes |
| 2c                        | Class A                  | Yes                          | No              | Carbencillinase |
| 2d                        | Class A/D                | Yes                          | No              | Cloxacilanase |
| 2e                        | Class A                  | Yes                          | No              | Cephalosporinase |
| 2f                        | Class A                  | Yes                          | No              | Carbapenemase |
| Group 3                   | Class B                  | No                           | Yes             | Metalloenzyme |

roughly one million database entries with suspected lactamase homologs, 14,923 genes were found in fungi, which represents approximately half of the eukaryotic total (29,804 genes). Due to ever-increasing affordability and ease of sequencing, newly identified genes encoding putative lactamases are being added at an accelerating rate to databases.

Even though a large number of fungal genes have been identified that putatively encode lactamases with Pfam domains PF00144 or PF00753 similar to bacteria, only a few gene products have confirmed functions. For example, Saccharomyces cerevisiae possesses a small core set of highly conserved enzymes with lactamase domains, but they tend to have specialized functions not involving lactam hydrolysis (Table 2). The essential gene TRZ1 from S. cerevisiae encodes tRNase Z, involved in RNA processing (Chen et al., 2005; Zheklovsky et al., 2006). The essential endonuclease YSH1 in S. cerevisiae contains a MBL domain and plays key roles in pre-mRNA 3′ end formation, cooperating with other cleavage factors (Stumpf and Domdey,
Sunburst visualization of β-lactamase gene distribution by major taxon. Each node of the taxonomic hierarchy is represented as a separate arc, arranged radially with the domains at the center and the phyla arrayed around the outermost ring. The area of each arc is proportional to the number of β-lactamases reported in the NCBI protein database. I, M, and O refer to inner, middle, and outer arcs. The frequency of Fusarium lactamases among the Ascomycota is denoted in bar chart form.

TABLE 2 | Saccharomyces cerevisiae lactamase orthologs in three Fusarium species.

| Systematic name | Standard name | Function | Fusarium orthologs |
|-----------------|---------------|----------|-------------------|
|                 | Saccharomyces cerevisiae lactamase genes* |          | Fv | Fo | Fg |
|                 | **Saccharomyces cerevisiae has seven lactamase genes, two of which (TRZ1 and YSH1) are essential. **N/I represents no orthologs identified in these three Fusarium species. Fv, F. verticillioides; Fo, F. oxysporum; Fg, F. graminearum. |
|                 | FVEG_ | FOXG_ | FGSG_ |
| YDR272W         | GLO2  | Hydroxyacylglutathione hydrolase GLO2 | 08018/16907 | 01652/12249 | 13072 |
| YKR079C         | TRZ1  | tRNase Z | 05485 | 02309 | 06635 |
| YLR277C         | YSH1  | Cleavage polyadenylation factor subunit YSH1 | 14723 | 17946 | 00819 |
| YMR137C         | PSO2  | Pso2p nuclease | 00815 | 00696 | 00361 |
| YOL164W         | BDS1  | Sulfuric ester hydrolase | N/I** | N/I | N/I |
| YOR040W         | GLO4  | Hydroxyacylglutathione hydrolase GLO4 | 08018/16907 | 01652/12249 | 13072 |
| YPL103C         | PMP30 | N-acetylphtathidylethanolamine-hydrolyzing phospholipase D | 03849 | 05981 | 09261 |

1996). Filamentous fungi, including Fusarium (Table 2), possess YSH1 and TRZ1 orthologs in their genomes. Non-essential fungal lactamases appear to have diversified functions, not restricted to nucleases. The non-essential BDS1 in S. cerevisiae, presumably horizontally acquired from bacteria, possesses an MBL domain and functions as a sulfenic ester hydrolase (Hall et al., 2005). A discrete MBL type thioesterase in Aspergillus fumigatus was found to be required for biosynthesis of endocrocin, a simple anthraquinone commonly identified in fungal extracts (Lim et al., 2012). Asperthecin, a polyketide anthraquinone pigment, is produced by certain Aspergillus species (Howard and Raistrick, 1955), and disruption of the asperthecin biosynthetic gene cluster in Aspergillus nidulans revealed that a lactamase assisted the adjacent polyketide synthase to hydrolyze an aromatic polyketide into endocrocin-9-anthrone (Szewczyk et al., 2008). LovD, a SBL containing the PF00144 motif, in Aspergillus terreus was essential...
forLovastatinbiosynthesis, and it was also later described to beinvolvedinsynthesizingsimvastatin, a lipid-lowering agent, byacting on a protein-bound acyl substrate (Kennedy, 1999; Jiménez-Osés et al., 2014). Throughproteomic studies on both weakly and highly aggressive *Verticillium dahliae* isolates, it was inferred that a β-lactamase family protein might act as a pathogenicity factor that is recognized by the host plant immunesystem as an elicitor (El-Bebany et al., 2010). Thus, the functional diversity offungal lactamases is evident despite limited studies.

Recent literature has shown that two related fungal lactamases function in xenobiotic hydrolysis, similar to bacterial counterparts. In fact, our interest in fungal lactamases stems from the observation that the gene FVEG_08291 in *F. verticillioides* encodes a lactamase designated MBL1 that is responsible for the degradation of 2-benzoxazolinone (BOA) (Glenn et al., 2016). A similar but non-orthologous MBL in *F. pseudograminearum* (FPSE_08124) was also shown to be responsible for BOA degradation (Kettle et al., 2015a). BOA is a γ-lactam phytochemical produced by select graminaceous crops that is implicated in resistance to insect herbivory and microbial pathogens. The enzymatic capacity of *Fusarium* species to hydrolyze BOA is suggested to enhance colonization of the host, thus increasing the frequency and abundance of the species (Saunders and Kohn, 2008; Saunders et al., 2010). Interestingly, MBL1 is part of a gene cluster that is up-regulated in response to BOA, and this cluster, called the *FDB1* cluster, was also observed in the other maize pathogens *Fusarium subglutinans* and *Colletotrichum graminicola* (Glenn et al., 2016). The highly conserved synteny of the *FDB1* cluster between these fungi suggests *C. graminicola* acquired the cluster from *Fusarium* by HGT, and that the maize host and its phytochemicals, notably BOA and related lactams, are driving factors influencing the evolution and genomic content of these fungi.

### Fusarium LACTAMASES

#### Fusarium Lactamase Analysis as a Paradigm?

Our analysis suggests that soil-borne fungi tend to possess more lactamase encoding genes compared with the minimal sets from fungi predicted to live in environments of relatively

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**FIGURE 6** Lactamase genes are abundant within some fungi. The number of annotated ORFs possessing a lactamase domain is shown for the top 40 fungi among all annotated fungal species identified from the NCBI Protein Database. Species are ranked by the abundance of lactamases. Fungi known to be soil-borne are displayed with red bars, those not clearly cited in literature as soil-borne fungi are in blue, and yeast along with selected obligate plant pathogens are in gray. Numbers of *Fusarium* lactamases are highlighted in yellow.
low microbial diversity (Figure 6). Broadly distributed in soil, Fusarium species are likely in competition with diverse microbes and are presumably often exposed to xenobiotic compounds. Frequent confrontation with competing microorganisms inhabiting overlapping ecological niches is expected to hone genetic determinants of xenobiotic resistance. Fusarium interactions with soil competitors are complex, involving nutrient competition and chemical warfare (Kinkel et al., 2012). As noted in Figure 5, the majority (84.3%) of sequenced fungal lactamase encoding genes are from the phylum Ascomycota (12,771 genes), 11.6% of which belonged to the genus Fusarium (1479 genes). There were, on average, 37 lactamase encoding genes per Fusarium species, as opposed to 15 per species among non-Fusarium fungal genomes. The species noted with the highest number of lactamase encoding genes, 88, was the soil limited root pathogen Fusarium solani (Figure 6). Thus, we further propose that the abundance of Fusarium lactamasases is likely integral to the success of this genus as a soil competitor. Further analysis of the global and individual roles of lactamasases is important for more fully understanding Fusarium biology and its ecological interactions.

Detailed Analysis of F. verticillioides Lactamasases
To better understand molecular characteristics of Fusarium lactamasases, we cataloged the complete set of lactamase encoding genes from three representative and pathogenically important sequenced Fusarium genomes, F. verticillioides 7600 (Fv), Fusarium oxysporum 4287 (Fo), and Fusarium graminearum PH-1 (Fg), via homology-based protein reciprocal BLAST and bacterial β-lactamase HMMER sequence logo scanning (Wheeler and Eddy, 2013). We identified 46 lactamase domain-containing genes in Fv, 63 in Fo, and 38 in Fg, as listed in Table 3 by predicted enzymatic mechanisms (MBLs and SBLs). PSI-BLAST of each lactamase encoding gene in F. verticillioides helped uncover distant homologs and confirm domain integrity. Interestingly, some predicted SBL gene annotations (FVEG_03300, FVEG_14143, FVEG_15166, FVEG_17257, FVEG_17258) were missing core catalytic serine motifs or possessed only part of the conventional β-lactamase folds. Thus, these five genes were further evaluated for their open reading frames using the FGENESH program from Softberry (http://www.softberry.com) to refine gene predictions. Reannotated sequences suggested FVEG_17257 and FVEG_17258 should be merged as one lactamase encoding gene, while FVEG_03300, FVEG_14143, and FVEG_15166 remained unchanged, still missing the core serine and lacking canonical amino acids at the majority of conserved sites. These three were thus excluded from later syntenic and phylogenetic analyses. PSI-BLAST of MBLs in F. verticillioides also predicted several members could be involved in metabolizing RNA (FVEG_05485, FVEG_11466, FVEG_14723), degrading lipids (FVEG_11923, FVEG_03849),

| Species                      | MBL (FVEG) | Accession number | SBL (FVEG) | Accession number |
|------------------------------|------------|------------------|------------|-----------------|
| F. verticillioides 7600      | 00815      | 03849            | 04252      | 05261           | 05485 |
| 05734                        | 06854      | 06904            | 08291      | 09433           |       |
| 11466                        | 06854      | 06904            | 08291      | 09433           |       |
| 12347                        | 03000      | 14143            | 10753      | 10740           | 09057 |
| 13675                        | 15166      | 17257            |           |                 |       |
| F. oxysporum f. sp. lycopersici 4287 | 00696      | 01652            | 02309      | 02559           | 03076 |
| 03847                        | 03275      | 03924            | 05576      | 05981           | 07628 |
| 07119                        | 08711      | 10409            | 10816      | 10887           |       |
| 12727                        | 10911      | 10955            | 12166      | 12179           | 13106 |
| 14524                        | 13918      | 14363            | 15115      | 15429           |       |
| 15776                        | 17393      | 18438            | 18914      | 21695           | 22119 |
| 20403                        | 22149      | 22249            |           |                 |       |
| F. graminearum PH-1          | 00079      | 00361            | 00819      | 03085           | 04727 |
| 05331                        | 04656      | 04809            | 04813      | 05706           | 07314 |
| 10795                        | 07538      | 07702            | 07996      | 08136           | 08476 |
| 13173                        | 09143      | 09261            | 10267      | 10497           | 11664 |
| 03024                        | 13121      | 03457            |           |                 |       |
| 03303                        | 13493      | 03457            |           |                 |       |

MBL, metallo-β-lactamasases; SBL, serine-based β-lactamasases. Bolded accession numbers are those with signal peptides identified by SignalP 4.1 Server, suggesting likely secretion (Petersen et al., 2011).
repairing DNA (FVEG_00815, FVEG_04252), and hydrolyzing hydroxyacyl glutathione (FVEG_08018, FVEG_16907), which also require zinc ions for appropriate functions. See also Table 2.

Fv-oriented syntenic studies were performed such that corresponding orthologs and adjacent genes in Fg and Fo were examined. In terms of species phylogeny, Fv is more closely related to Fo than Fg. Thus, we naturally expected more orthologs identified in Fo. Except for those Fv lactamase encoding genes with no orthologs in the other two species, the rest of the genes generally retained syntenic clusters in the Fg and/or Fo genomes (Figure 7). Phylogenetic evaluation of 41 Fv genes having the core lactamase motifs revealed a high consistency with species evolution (Figure 8), where 37 genes fall into position A, clustering with orthologs in Fusarium fujikuroi in the respective phylograms. This suggests that Fv lactamases are most similar to those annotated in closely related species compared with other relatively distant species. Lactamase encoding genes in Fusarium species generally form a clade distinct from other Sordariomycetes. Interestingly, only 29% of the Fv MBLs had evidence of paralogy (Figure 7), whereas 63% of the Fv SBLs appeared to have paralogs. This suggests the two types of lactamases may have different evolutionary pressures impacting duplication and diversification. Only six Fv lactamase encoding genes lack possible orthologs in both Fo and Fg (Figure 7). Collectively the data indicate that some lactamase encoding genes originated before the divergence of Fusarium species, resulting in greater sequence diversity accompanying species divergence. Interestingly, FVEG_12347 was the only gene in the phylogenetic position B (Figure 8), suggesting that it is similar to Fg ortholog FGSG_04727 and lacks an ortholog in Fo (Figure 7). FVEG_08291, FVEG_09433, and FVEG_12457 notably exhibited more similarities to orthologs in other Sordariomycetes rather than in closely related Fusarium species. These fall into phylogenetic position C and are thus good candidates for HGT derivation. The FVEG_08291 protein sequence possessed 85% identity to an ortholog in C. graminicola (NCBI Reference Sequence: XP_008099767.1), another Sordariomycetes pathogen.
FIGURE 8 | Phylogenetic placement of predicted *Fusarium verticillioides* lactamase genes based on amino acid sequence alignment. This cartoon summarizes the phylogenetic pattern of lactamase genes with intact core motifs in *F. verticillioides*. Each query lactamase amino acid sequence was searched for its top 50 homologs using BLASTP in NCBI. Neighbor-joining trees using the Jukes–Cantor genetic distance model were constructed by Geneious Tree Builder (Ver. 8.1) for each individual homology search. All tree topographies complied with the configuration such that each *F. verticillioides* lactamase fell into one of three positions marked as A, B, and C. Collapsed clades are shown as triangles.

FIGURE 9 | Sequence alignment of motifs within *Fusarium verticillioides* metallo-β-lactamase proteins that are presumably associated with lactam hydrolysis. Amino acids matching at least 50% of all sequences are highlighted.

of maize, surpassing homology to other related genes in *Fusarium* species. This is the *MBL1* gene noted above as part of the *FDB1* cluster conferring resistance to BOA. A similar case was observed for FVEG_09433, where it was more closely related to orthologs in *C. graminicola* and other genera than to those of most other *Fusarium* species, even though there are apparent
FIGURE 10 | Sequence alignment of motifs within Fusarium verticillioides serine-based β-lactamases proteins that are presumably associated with lactam hydrolysis. Amino acids matching at least 75% of all sequences included were highlighted.

orthologs in Fusarium mangiferae (GenBank ID: CVL02248.1) and F. fujikuroi (GenBank ID: CCT69225.1). One possible explanation is that FVEG_09433 was introduced to Fusarium species within the F. fujikuroi species complex prior to the divergence of these three species, but its orthologs among other species of the complex were somehow lost. The serine-based lactamase encoded by FVEG_12457 was most similar to its orthologs in A. terreus (NCBI Reference ID: XP_001217058.1) and Penicillium roqueforti (GenBank ID: CDM29397.1) with a sequence identity of over 70%, exceeding the 60% average identity among related Fusarium genes.

Multiple Alignment using Fast Fourier Transform (MAFFT) analysis of presumed hydrolysis-related lactamase protein sequences was performed separately for MBLs and SBLs, presenting two distinctive patterns of conserved motifs (Figures 9, 10). Although these Fv lactamases exhibited considerable sequence diversity, conserved motifs were still observed. As to MBLs, the conserved motif His-X-His-X-Asp-His-X-Gly resembled that in classic bacterial MBLs (Figure 9). However, compared to typical cases in bacteria, the overall conserved motif pattern is different in Fv MBLs, and additional motifs were identified, including Pro-X-Gly-His in Motif 3, Gly-Asp in Motif 4, and Pro-Gly in Motif 5 (Figure 9).

A retrospective scrutiny of conserved motifs of bacterial PSI-BLAST hits revealed that these sites in Fv lactamases are also present in certain bacterial β-lactamases (data not shown). Fv SBLs demonstrate an interesting molecular pattern that is not present in bacteria (Figure 10). A total of nine motifs were identified in all intact Fv SBLs that are predicted to be hydrolysis-associated. Besides the catalytic core motif shared with bacteria (Ser-X-X-Lys as Motif 1), Fv lactamases contain conserved amino acids Leu-X-X-X-Gly in Motif 2, Pro-Glu-Leu in Motif 3, Leu-X-X-His-X-X-Gly in Motif 4, Pro-X-X-X-X-X-X-Tyr in Motif 5, Glu-X-X-X-Gly in Motif 6, a single conserved amino acid Pro in Motif 7, the Asp in Motif 8, and the Leu in Motif 9. However, none of these motifs (Motifs 2–9) are represented in bacterial species.

Phyre2 predictions of tertiary structures reflected an interesting discovery that the majority of Fv MBLs were similar to bacterial β-lactamases, presenting an α–β/β–α sandwich
structure composed of two β sheets at the core and α helices on the external surfaces (Kelley et al., 2015). Those conserved residues are generally located at flexible loops connecting different secondary structures. It can be inferred that the spatial adjacency of histidines would facilitate the coordination of zinc ions and that the aspartic acid residues participate in the hydrolysis reaction. As exemplified in Figure 11, FVEG_08291 was predicted to have the signature sandwich conformation with a flap structure (the flexible mobile loop), which is situated at the bottom of a wide shallow groove between two β-sheets (Figure 11A). This structure has proven to be critical in substrate binding in bacteria (Materon and Palzkill, 2001). Superimposition of protein structures revealed that FVEG_08291 resembles a quorum-quenching lactonase (AiiB) from Agrobacterium tumefaciens (Figure 11B). The conserved zinc-coordinated residues on the flexible loop as well as the easily accessible groove placement suggest the potential to accommodate various lactam or lactone molecules (Figures 11C,D). Other conserved amino acids not directly predicted to be associated with catalytic reactions may be involved in structure maintenance or substrate recognition, and overall the catalytic mechanisms of fungal lactamases require further exploration.

CONCLUSION AND FUTURE DIRECTIONS

The complexity of soil environments, particularly those with nutrient-driven competition in the rhizosphere, has led to diverse organisms capable of antimicrobial activity. Plants also contribute to rhizospheric antimicrobial content, either proactively (phytoanticipins), or reactive to pathogen contact (phytoalexins) (Morrissey and Osbourn, 1999; Kato-Noguchi et al., 2008). Thus, the soil environment contains high antibiotic diversity including β-lactams, tetracyclines, sulfonamides, aminoglycosides, imidazoles, etc. (Thiele-Bruhn, 2003). Competitive relationships among soil microflora exert
selective pressure on genes for antibiotic production and resistance. These genes in turn shape microbial populations and diversity, largely through development of antibiotic resistance mechanisms, such as the enzymatic degradation of β-lactam-containing compounds. Xenobiotic degradation in soil is propelled by enzymatic processes such as hydrolysis, oxidative decarboxylation, and hydroxylation (Chen et al., 1997; McGrath et al., 1998; Al-Ahmad et al., 1999; Halling-Sørensen, 2000; Thiele-Bruhn, 2003). Interestingly, functional metagenomics have revealed that, as the major resistance source against β-lactams, β-lactamase encoding genes were abundant even in undisturbed soil absent of anthropogenic selective pressure, contributing to a massive reservoir for genetic exchange among soil microflora (Allen et al., 2009).

Given our examination of fungal hydrolytic lactamases, we propose an ecological model (Figure 12) centering on the production and function of both lactams and lactamases produced by plants, bacteria, and fungi. We expand the conventional focus beyond that of solely bacterial β-lactamases and instead propose a more generic ecological model linking lactam production with hydrolytic functions of organismal lactamases. Lactam antibiotics presumably benefit their producers by securing ecological niches, whereas numerous lactam producers have also developed hydrolytic lactamases postulated to combat antibiosis. For example, soil-associated fungi typically possess more lactamase encoding genes than those from environments with lower microbial diversity since soil environments contain significant antibiotic diversity. Analysis of Fusarium species provides the foundation for our hypothesis that soil fungi frequently utilize lactamases in detoxification of xenobiotics, especially given Fusarium species’ wide soil distribution, lactamase-rich genomes, and recent functional characterization of lactamase encoding genes. The general abundance and persistence of lactamase genes in fungal genomes suggests a significant role for these enzymes in the soil environment, presumably in protection from many as yet unknown xenobiotics. We have generated a large set of lactamase mutants in F. verticillioides and are conducting transcriptional and phenotypic analyses upon exposure to various lactam compounds in order to more thoroughly evaluate the role and activity of these lactamases, thus broadening our appreciation of both the lactam compounds and corresponding lactamases in

![Figure 12](image-url)
terms of their diversity and impact on both bacterial and fungal communities. This work also has the potential to broaden our appreciation of environmental sources of antimicrobial resistance to include both bacteria and fungi, especially with regard to use of antibiotics in agriculture.

AUTHOR CONTRIBUTIONS
All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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