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Genomic, transcriptomic, and ecological diversity of *Penicillium* species in cheese rind microbiomes

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ABSTRACT

Although *Penicillium* molds can have significant impacts on agricultural, industrial, and biomedical systems, the ecological roles of *Penicillium* species in many microbiomes are not well characterized. Here we utilized a collection of 35 *Penicillium* strains isolated from cheese rinds to broadly investigate the genomic potential for secondary metabolism in cheese-associated *Penicillium* species, the impact of *Penicillium* on bacterial community assembly, and mechanisms of *Penicillium*-bacteria interactions. Using antiSMASH, we identified 1558 biosynthetic gene clusters, 406 of which were mapped to known pathways, including several mycotoxins and antimicrobial compounds. By measuring bacterial abundance and fungal mRNA expression when culturing representative *Penicillium* strains with a cheese rind bacterial community, we observed divergent impacts of different *Penicillium* strains, from strong inhibitors of bacterial growth to those with no impact on bacterial growth or community composition. Through differential mRNA expression analyses, *Penicillium* strains demonstrated limited differential gene expression in response to the bacterial community. We identified a few shared responses between the eight tested *Penicillium* strains, primarily upregulation of nutrient metabolic pathways, but we did not identify a conserved fungal response to growth in a multispecies community. These results in tandem suggest high variation among cheese-associated *Penicillium* species in their ability to shape bacterial community development and highlight important ecological diversity within this iconic genus.

1. Introduction

Filamentous fungi in the genus *Penicillium* are ubiquitous and diverse, spanning a wide range of habitats and occupying integral roles in several industries, including agriculture, food, and drug production (Visagie et al., 2005). Several species are devastating plant pathogens, leading to moldy growth and toxin accumulation in economically important crops (Filtenborg et al., 1996; Frisvad et al., 2004; Perrone and Susca, 2017). Other species have been used to develop antibiotics (Toghueo and Boyom, 2020; Guzmán-Chávez et al., 2018), including the eponymous antibiotic penicillin that helped spark the golden age of antibiotic discovery (Chain et al., 1940; Abraham et al., 1941; Weber et al., 2012; Fleming, 1929). Some species can enhance properties of artisanal food products, for example by shaping the quality and aesthetics of rinded cheeses (Kinsella and Hwang 1976; Karahadian et al., 1985; Ropars et al., 2012) and fermented meats (Magistà et al., 2017).

Despite their industrial and economic importance, we know surprisingly little about the ecology of *Penicillium* species, including how they interact with other species in microbial communities. Studies have predominantly focused on host-pathogen interactions including *P. expansum*, the causal agent of blue rot in apples (Sanderson and Spotts, 1995; Prusky et al., 2004; Kumar et al., 2018; Luciano-Rosario et al., 2020) as well as *P. digitatum* and *P. italicum* in citruses and other fruits (Errampalli, 2014; Costa et al., 2019; Papoutsis et al., 2019). In an attempt to inhibit *Penicillium* rot in food systems, studies have examined how yeasts and bacteria could serve as inhibitory biocontrol agents of *Penicillium* in crops such as fruits (Wilson and Chalutz, 1989; Ma et al., 2019; Chen et al., 2021) and wheat grains (Belkacem-Hanfi et al., 2014). However, such examples are not only focused on a small range of pathogenic *Penicillium* species, but are also typically limited to pairwise interactions among microbes that do not naturally co-occur.

When growing in multispecies communities, the presence of a

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Penicillium species could shift bacterial community composition due to divergent ecological impacts on individual community members. Co-occurring species that are stimulated by *Penicillium* may increase in abundance and become dominant community members (Wang et al., 2018). Alternatively, community members that are inhibited by *Penicillium* may decrease in abundance or go extinct within communities (Larsen and Knechel, 1997; Hossain et al., 2008). Discovering the genomic and ecological traits underlying *Penicillium* impacts on community assembly outcomes may help develop new tools to manage *Penicillium*-dominated microbial communities.

The cheese rind microbiome, a low complexity microbial community consisting of only a handful of species (Wolfe et al., 2014), offers an ideal system to understand the diversity and mechanisms of *Penicillium*-bacteria interactions. *Penicillium* species are abundant in many cheese rinds, where they can either be starter cultures that are inoculated into the cheese (e.g. *P. camemberti* for Camembert or Brie production) or "wild" molds that naturally colonize caves and can be considered normal (e.g. natural rinds that form on clothbound Cheddars and tomme style cheeses) or nuisance microbes (e.g. blue spots of *P. biforme* on some bloomy rind cheeses (Wolfe et al., 2014; Marcellino and Benson 2013; Ropars et al., 2012; Martin and Cotter 2023; Irlinger et al., 2015). Past work by our group and others have focused on the diversity and distributions of *Penicillium* species in cheese rinds (Wolfe et al., 2014; Bodinaku et al., 2019; Ropars et al., 2012, 2020). A few studies have characterized the mechanisms of interactions between a few cheese-associated *Penicillium* species and select bacteria (Pierce et al., 2021; Tannous et al., 2023; Wolfe et al., 2014; Cleary et al., 2018; Cosetta et al., 2020), and have demonstrated both positive and negative interactions. We are not aware of a comprehensive survey of both the genomic and ecological diversity of the full range of *Penicillium* species that can live in cheese rind microbiomes.

To characterize how a broader diversity of *Penicillium* species impacts cheese rind bacterial communities, we isolated 35 *Penicillium* strains from a single cheese aging facility in the United States. While many of these isolates are from section *Fasciculata* of the genus *Penicillium* where cheese-associated species are well-known, we also obtained isolates that spanned other parts of the *Penicillium* phylogeny. We first determined the genomic potential for secondary metabolite production of isolated *Penicillium* strains using biosynthetic gene cluster (BGC) prediction in antiSMASH. We compared these results to their antibacterial ability both in co-culture with cheese bacterial communities as well as in secreted metabolite experiments. Finally, we used RNA-sequencing to identify conserved and unique responses of species of *Penicillium* isolated from cheese against a standard bacterial community commonly found on cheeses.

2. Materials and methods

2.1. Sampling and isolation of Penicillium strains

Penicillium strains were isolated from cheese rinds as well as the surrounding air within a single cheese aging facility in the eastern United States. This facility was aging natural and washed rind cheeses on wooden shelves produced from both raw and pasteurized milk. The combined air and rind sampling approach is part of a larger unpublished study of the fungal diversity of a newly established cheese aging facility. In this current experimental work, we are only using a subset of *Penicillium* molds that were isolated in the larger descriptive study.

To isolate fungi from rind samples, approximately 6.25 cm^2 of the rind surface was swabbed with a sterile cotton applicator, swirled in five mL 1x phosphate buffered saline (PBS) for ten seconds, shipped to the lab within 48 h, and plated on plate count agar with milk and salt (PCAMS; Cosetta and Wolfe 2020) supplemented with chloramphenicol (50 mg/L) to inhibit bacterial growth. After five to seven days of growth on PCAMS at room temperature, morphologically unique fungal colonies were isolated and re-streaked on PCAMS with chloramphenicol (50 mg/L). After seven days of growth, fungal spores were harvested and stored at −80 °C in 15% glycerol.

For air samples, lids were removed from Petri dishes containing 20 mL cheese curd agar (CCA; Cosetta and Wolfe 2020) and were left statically on shelves for five minutes to allow spores to settle onto the surface of the agar plates. These plates were then shipped to the lab within 48 h, and incubated at room temperature upon arrival. After five to seven days of growth, morphologically distinct fungal colonies were re-streaked onto PCAMS with chloramphenicol (50 mg/L). After seven days of growth, fungal spores were harvested and stored at −80 °C in 15% glycerol.

2.2. Whole genome sequencing and biosynthetic gene cluster prediction

For whole genome sequencing, DNA was extracted from putative *Penicillium* isolates using the Powersoil DNA Isolation Kit (Qiagen, Germany). Spores were scraped with a sterile wooden dowel from the surface of one-week old cultures growing on PCAMS agar and added to the solution in the Powerbead Tubes. DNA was then extracted following the manufacturer instructions, with only 50 μL of Solution C6 used to elute the final DNA (instead of 100 μL). DNA was prepared for Illumina sequencing using the NEBNext® Ultra™ II for DNA Library Prep kit (New England Biolabs, Ipswich, MA, USA) following manufacturer instructions. Libraries were paired-end sequenced on a NextSeq550 at the Tufts University Core Facility with a read length of 150 bp. Reads were trimmed to remove low-quality bases (ends trimmed using the modified-Mott trimming algorithm with a limit of 0.05 and ambiguous nucleotides trimmed with a maximum number of ambiguities set to 2) and failed reads were removed from libraries. Genomes were assembled using the *de novo* assembler in CLC Genomics Workbench version 8.0 (Qiagen, Aarhus, Denmark) and annotated using GenSAS (https://www.gensas.org/) with gene model prediction using AUGUSTUS (Stanke and Stephan, 2003). Draft genomes were submitted to antiSMASH fungal version (v.5.0) using the "relaxed" detection setting for BGC predictions (Blin et al., 2019). Due to a processing error in the initial dataset, *Penicillium* strain 231 was run on antiSMASH fungal version (v.6.0).

2.3. Phylogenomic analysis

To reconstruct the evolutionary history of populations and species in the genus *Penicillium*, we employed a pipeline previously used to determine the relationship of species in the genera *Penicillium* and *Aspergillus* (Bodinaku et al., 2019; Steenwyk et al., 2019) with slight modifications. To do so, we first downloaded 73 publicly available *Penicillium* genomes and three outgroup *Aspergillus* genomes from NCBI's GenBank database (Table S1; date of retrieval January 31, 2020). To identify single-copy orthologous genes, we employed the BUSCO, v3.0.2 (Waterhouse et al., 2018), pipeline, using the Pezizomycotina database of single-copy orthologous genes (n = 3,156) from OrthoDB, v9 (Waterhouse et al., 2013). Examination of the percentage of single-copy BUSCO genes revealed high gene content completeness across all genomes. Importantly, newly sequenced *Penicillium* genomes had nearly as many single-copy complete BUSCO genes as publicly available *Penicillium* genomes (94.15 ± 14.01% and 96.44 ± 9.71%, respectively). Multiple sequence fasta files were constructed from BUSCO genes that were found in 50% or more of taxa. The resulting 3,210 BUSCO genes were aligned using MAFFT, v7.402 (Katoh and Standley 2013), using parameters previously described (–bl 62, –op 1.0, –maxiterate 1000, –retree 1, –genafpair) (Steenwyk et al., 2019). Nucleotide sequences were then mapped onto the amino acid sequence alignments using a custom Python (v3.5.2) script with BioPython, v1.7 (Cock et al., 2009); this script is now the "thread_dna" function in PhyKIT, a broadly applicable toolkit for processing and analyzing phylogenomic data (Steenwyk et al., 2021). Nucleotide sequences were then trimmed using trimAl, v.1.2rev59 (Capella-Gutiérrez, Silla-Martínez, and Gabaldón 2009), with the 'gappyout' parameter, which was shown to be a top-

performing trimming strategy in benchmarking studies (Tan et al., 2015; Steenwyk et al., 2020).

To reconstruct the evolutionary history across the *Penicillium* species, we employed concatenation- and coalescence-based approaches (Rokas et al., 2003; Edwards 2009). The concatenation approach uses the total evidence of all single-gene loci by combining their multiple sequence alignments into a supermatrix (Rokas et al., 2003). The resulting matrix is used for tree inference. In contrast, the coalescence-based approach relies on first inferring single-gene trees and then infers a species tree from the individual single-gene trees using the multispecies coalescent framework, which accounts for single-gene tree variability (Edwards 2009). Both methods are widely accepted, but can occasionally result in different species tree topologies (Steenwyk et al., 2023). Current opinion is divided on when it is best to use concatenation or coalescence approaches (Steenwyk et al., 2023). Thus, we implemented both approaches and report both phylogenies.

For concatenation-based inference, we concatenated the 3,120 aligned and trimmed sequences into a single data matrix (9,089,226 sites). The original script to generate a supermatrix is now incorporated into the "create_concat" function in PhyKIT (Steenwyk et al., 2021). The single matrix was used as input into IQ-TREE, v1.6.11 (Nguyen et al., 2015). The "GTR + F + I + G4" model was inferred to be the best fitting substitution model by IQ-TREE. To expand our search of tree space, we conducted five independent tree searches using default starting trees and five independent searches using the approximated maximum likelihood tree inferred with the 'fast' parameter implemented in IQ-TREE. The resulting 10 trees were compared and the tree with the best log likelihood value was chosen as the 'best' tree. For coalescence-based inference, we first inferred single gene trees on each individual single copy orthologous gene with the 'nbest' parameter set to 10 and used the best fitting model for each gene tree. The resulting 3,210 gene trees were used as input into ASTRAL, v5.6.3 (Mirarab and Warnow, 2015). Bipartition support was examined using 5,000 ultrafast bootstrap approximations (Hoang et al., 2018) in concatenation-based inference and using local posterior probability values in coalescence-based inference. Both phylogenies were visually compared for incongruence.

2.4. Community assembly and plug-on-lawn experiments

A standard set of four bacterial species (*Staphylococcus equorum* strain BC9, *Brevibacterium aurantiacum* strain JB5, *Brachybacterium alimentarium* strain JB7, and *Psychrobacter* sp. strain JB193) was used as a model cheese rind community to study *Penicillium*-bacteria interactions. These bacteria were isolated from a different cheese aging facility in the United States, but are representative of the diversity of bacteria that are typically found on natural cheese rinds (Wolfe et al., 2014). These four particular strains have been well-characterized in our past work (Wolfe et al., 2014; Tannous et al., 2023; Niccum et al., 2020; Cosetta et al., 2020; Kastman et al., 2016) and by using this standard set of cheese rind bacteria, we can compare responses to these new *Penicillium* strains with bacterial-fungal interactions we have characterized previously.

Bacterial strains used in experiments were from cultures with a known CFU concentration and stored frozen at −80 °C in 15% glycerol in brain–heart infusion (BHI) liquid media. *Penicillium* strains used in experiments were derived from spores harvested dense streaks on PCAMS. Experimental glycerol stocks of *Penicillium* strains had a known concentration of CFUs and were frozen at −80 °C in 15% glycerol in 1x PBS.

For community assembly experiments, 200 CFUs of all four bacterial strains were inoculated into 1.5 mL microcentrifuge tubes (USA Scientific, Ocala, FL, USA) containing 150 μL of CCA without a fungus or with 200 CFUs of one of eight *Penicillium* strains. *Penicillium* strains that were used in experiments were selected to both represent phylogenetic breadth as well as include several representatives from section *Fasciculata*, where taxa that were previously isolated from cheese and other proteinaceous foods are found (Houbraken et al., 2016; Ropars et al., 2012). Communities were allowed to incubate at 24 °C in the dark for seven days, diluted in 15% glycerol in 1x PBS, homogenized by pestling, and plated out onto PCAMS with chloramphenicol (50 mg/L) and PCAMS with natamycin (21.6 mg/L) to assess fungal and bacterial abundance, respectively (Cosetta and Wolfe 2020). Community assembly experiments were completed three independent times (three biological replicates), with five technical replicates per experiment.

Statistical analyses of community assembly data were conducted with the vegan package (Oksanen et al., 2022) in R (version 4.1.1). "N" values refer to the number of independent experimental replicates conducted over separate occasions with separate materials and cultures and "n" values refer to the and the number of technical replicates within each experiment. To quantify how *Penicillium* species differentially influenced bacterial community composition, we created a distance matrix using the Bray-Curtis dissimilarity index to compare abundance and composition of the four bacterial strains associated with different *Penicillium* neighbors. Differences in community composition were visualized using non-metric multidimensional scaling (NMDS). Differences in community composition were assessed using a permutational analysis of variance (PERMANOVA).

We used plug-on-lawn experiments to determine if metabolites that can be secreted by the fungi have antibacterial properties. To create samples of CCA media with fungal metabolites, 100 μL of inoculum of *Penicillium* strains at a density of 500 CFUs/μL was spread onto the surface of CCA and incubated at 24 °C in a dark incubator for seven days. A flame-sterilized cork borer was used to punch out a circular plug (18-mm diameter) into the CCA where the fungi had grown. The media side of the plug (under where the fungal mycelium had grown) was carefully placed to make agar-to-agar contact with the surface of a PCAMS + natamycin (21.6 mg/L) plate previously inoculated with 100 μL of an overnight liquid culture (in liquid BHI shaking at 450 rpm at 24 °C) of one of the four bacterial strains diluted to an OD_{600} of 0.01 in 1xPBS. Each of the four bacterial strains were tested separately. The plug-on-lawn approach allows for the metabolites secreted by the *Penicillium* strains grown on CCA to diffuse into the solid PCAMS medium and affect the growth of the responding bacteria. Plates were incubated at 24 °C for three to five days before measuring the diameter of zones of inhibition in the bacterial lawns observed around the fungal plugs.

2.5. RNA extraction and sequencing

To measure global changes in mRNA expression in the *Penicillium* species when co-cultured with the cheese rind bacteria, we spotted 20 μL of an inoculum containing a *Penicillium* strain (10 CFUs/μL) alone or with the bacterial community described above (each at 10 CFUs/μL) onto a 100 mm Petri dish containing 20 mL of cheese curd agar and incubated in a dark incubator at 24 °C for three days. Two slower-growing strains, strain 200 and 285, were grown in identical conditions for five days to synchronize *Penicillium* growth and development (until sporulation began and spots were similar size as the faster growing strains that were grown for three days). After incubation, fungal cells were harvested by slicing off the top layer of the spot with a flame-sterilized scalpel, submersed in RNALater (Thermofisher Scientific, Waltham, MA, USA), and frozen at −80 °C. After at least 24 h at −80 °C, cells were removed from the RNALater, frozen in liquid nitrogen, and manually ground to a fine powder with a pre-sterilized and pre-cooled mortar and pestle. Up to 200 mg of the powder was then processed using the RNeasy Plant Mini Kit (Qiagen, Germany) according to manufacturer instructions with the following addition: the column was incubated with 80 μL of RNase-free DNase (Qiagen, Germany) for 15 min at room temperature between the two RW1 wash steps. There were three biological replicates per treatment.

Fungal mRNA was isolated following manufacturer instructions for the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA, USA). The mRNA was then prepared for Illumina sequencing with the NEBNext Ultra II RNA Library Prep Kit for Illumina

following NEB protocol instructions for preparation of intact RNA (New England Biolabs, Ipswich, MA, USA), then pooled and sequenced on a NextSeq 550 at Tufts University Core Facility Genomics (single-end sequencing, 150 cycles) to achieve approximately 4,000,000 reads per biological replicate.

2.6. Differential expression analysis

Raw Illumina reads were mapped to assembled *Penicillium* genomes using Geneious Prime mapper (version 2020.2.5) at medium–low sensitivity, with reads mapping to multiple best matches randomly assigned. Differential expression between the alone and + bacteria treatments was calculated with DESeq2 (Love, Huber, and Anders 2014). Genes were considered differentially expressed if expression in the + bacteria treatment was greater than doubled (log2 ratio > 1) or less than halved (log2 ratio < -1) compared to growth alone at a p-value < 0.05, adjusted for false discovery rate using the Benjamini–Hochberg procedure (Love, Huber, and Anders, 2014). To identify pathways that were enriched in differentially expressed genes, we used the KOBAS-i Gene-list Enrichment tool (Bu et al., 2021) with *Penicillium*

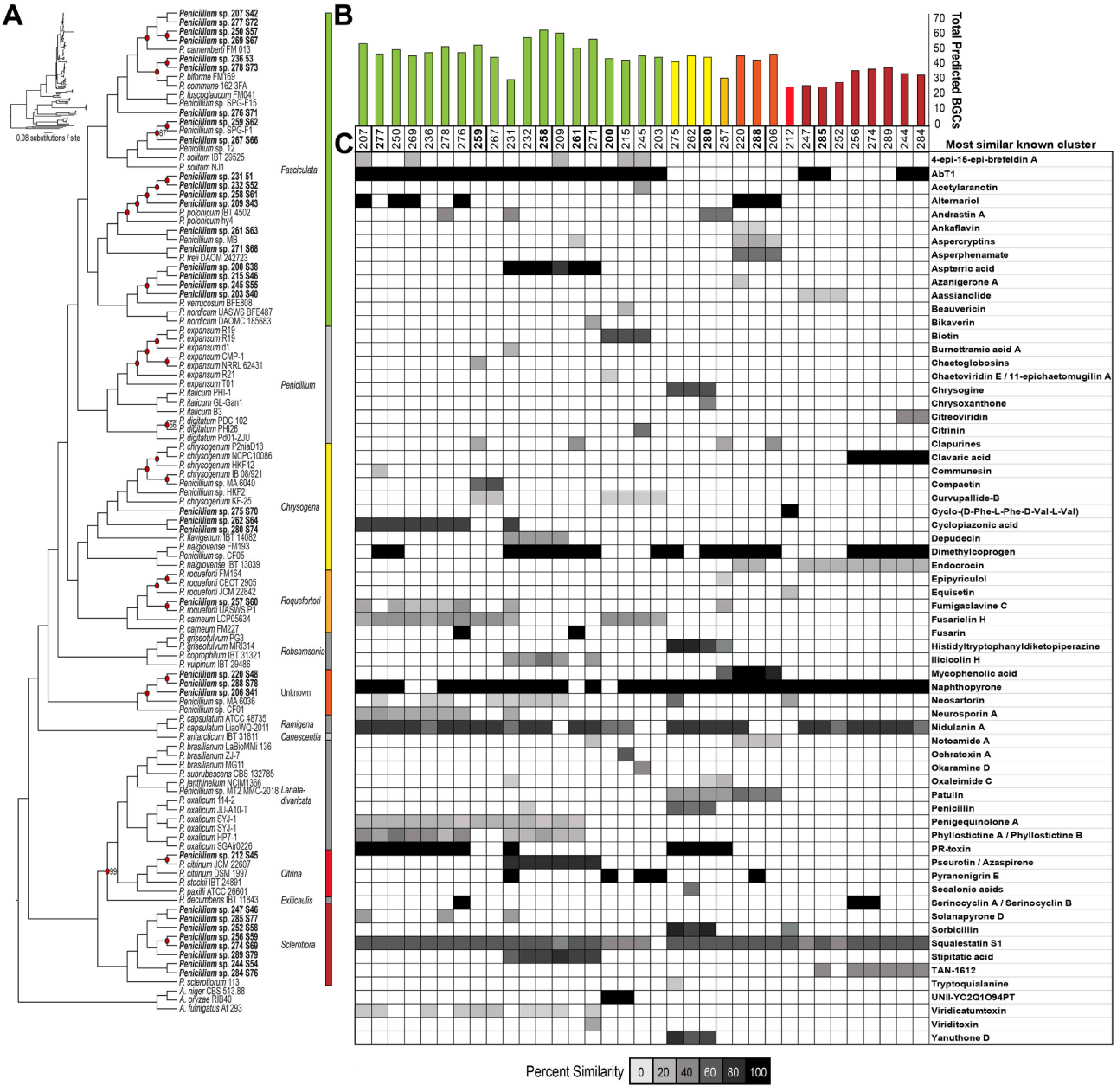

Fig. 1. Isolation of *Penicillium* molds from a cheese aging facility identified 35 *Penicillium* strains with variable biosynthetic gene clusters. (A) Concatenation-based phylogenetic tree of 35 *Penicillium* strains isolated from cheese and 73 publicly available *Penicillium* genomes. Genomes sequenced in this study are in bold. Section names are shown to the right of the phylogeny. Red dots indicate bipartitions that were not supported in the coalescence-based phylogeny. Differences between the concatenation- and coalescence-based phylogenies are likely due to both biological and analytical factors (e.g., rapid speciation events and errors in single-gene tree estimation, respectively) (Steenwyk et al., 2023). A phylogeny with branch lengths that represent substitutions per site is shown in the top left corner. Only bipartition support values below 100 are shown. **(B)** Total number of BGCs in cheese rind *Penicillium* strains as predicted by antiSMASH. Bar colors represent the section each *Penicillium* strain falls under, as shown in panel A. Strain labels in bold indicate strains chosen for ecological and transcriptomic studies. **(C)** Overview of characterized BGCs within each strain as predicted by antiSMASH, with cell color intensity representing increasing percent similarity of the predicted region to the most similar known cluster.

digitatum chosen as the background genome to test for enrichment.

2.7. Data availability

All raw Illumina reads and *de novo* assembled *Penicillium* genomes have been deposited in NCBI in BioProject PRJNA943062. Raw Illumina reads from RNA-sequencing have been deposited in NCBI in BioProject PRJNA943161.

3. Results and discussion

3.1. Isolation of 35 cheese Penicillium strains with a broad range of secondary metabolite potential

To better understand *Penicillium*-bacteria interactions in the cheese rind microbiome, we sampled cheese rinds from a single cheese aging facility in the United States and successfully isolated 35 morphologically unique *Penicillium* strains spanning the phylogeny of the genus (Fig. 1A). Many of the *Penicillium* strains (19 of the 35, or 54%) were from section *Fasciculata* and were closely related to taxa including *P. biforme*, *P. camemberti*, *P. verrucosum*, and *P. solitum*. The second-most sampled section, with eight strains (23%) was section *Sclerotiora*, which is less commonly reported in cheese. Three strains (262, 275, and 280) were clustered in section *Chrysogena* close to *P. chrysogenum*. One strain each was in sections *Roquefortori* (257) and *Citrina* (212). The remaining three strains (206, 220, and 288) were clustered in an unknown section, but has a beta-tubulin sequence that is highly similar to *P. brevicompactum*.

Several studies that have isolated *Penicillium* from cheese identified similar species, also with a dominance of strains clustered in section *Fasciculata*, particularly strains identified as *P. biforme* and closely related species (Lund et al., 1995; Kure et al., 2001; Ozturkoglu Budak et al., 2016; Ramos-Pereira et al., 2019). *P. citrinum*, of which one of our strains (strain 251) clusters closely with, is not commonly associated with standard cheese communities but has been isolated in other studies (Decontardi et al., 2017). We did not, however, isolate any *Penicillium* strains in section *Penicillium* (Fig. 1**A**), of which *P. expansum* has been considered associated with cheese contamination (Pitt and Hocking 2009).

To elucidate their potential for secondary metabolite production and potential impacts on co-occurring bacterial communities, we ran all 35 genomes through antiSMASH (fungal version) to predict BGCs within the *Penicillium* genomes. antiSMASH predicted 1558 BGCs (Table S2), averaging 44.5 BGCs per genome, slightly lower than the 55 BGCs per genome calculated in a previous *Penicillium* genomic study with 24 *Penicillium* strains (Nielsen et al., 2017), but on par with a large-scale study analyzing 1037 fungal genomes, in which they found Eurotiomycetes genomes contained on average 48 BGCs per genome (Robey et al., 2021), and higher than their overall average of 35 BGCs per fungal genome. The 35 species ranged from 26 to 64 predicted BGCs per genome, with slightly more BGCs found in strains in section *Fasciculata* and *Chrysogena* and less in *Roquefortori* and *Sclerotiora* (Fig. 1B). These higher values highlight sections *Fasciculata* and *Chysogena* as strong candidates for future genome mining efforts; as many common cheese *Penicillium* species are categorized in section *Fasciculata*, these data also underscore the potential of cheese-derived *Penicillium* in natural products discovery.

Of the 1558 clusters, 1158 (74%) were uncharacterized. The remaining 406 BGCs were identified as being most similar to one of 66 BGCs producing a known secondary metabolite (Fig. 1C), 44 of which were unique to one section of the genus and 18 of those unique to a single strain. Only two BGCs were found in all six represented sections of the genus, squalene synthase inhibitor squalestatin S1 (34/35 strains) (Dawson et al., 1992) and pigmented spore intermediate naphthopyrone (31/35 strains) (Fujii et al., 2001), both commonly found across ascomycetes (Bergstrom et al., 1995; Xu et al., 2019). Squalestatin S1 is known for its biomedical applications (Baxter et al., 1992), but its potential ecological roles are not known. Naphthopyrone pigments have been described as defense mechanisms against predators in fungi (Xu et al., 2019). How these putative compounds impact bacterial communities has not been assessed.

Other commonly found BGCs were for nidulanin A (29 strains), a secondary metabolite of unknown function (Raffa and Keller, 2019), and dimethyl coprogen (21 strains), an iron siderophore responsible for scavenging iron in the environment (Jalal, Love, and van der Helm, 1988) (Fig. 1C). The ecological roles of these secondary metabolites in microbial community dynamics are not well established, but their commonality and conserved nature indicates a potentially important biological role among this ubiquitous and diverse genus. A previous study in the cheese rind system found that coprogen siderophores are produced by *Penicillium* and other fungi and can reduce bacterial dependence on their own siderophores for iron uptake (Pierce et al., 2021).

The antiSMASH results highlighted two putative BGCs that could inhibit bacterial growth in cheese rinds. The antibiotic penicillin is widely appreciated for its ability to inhibit bacterial growth, and the penicillin BGC was only found among strains clustered in section *Chrysogena* (Fig. 1C). Six strains closely related to *P. polonicum* were predicted to contain the BGC encoding for pseurotins, a group of fungal secondary metabolites that can have antibacterial activity and were previously demonstrated to shape bacterial community composition in one *Penicillium* strain (Tannous et al., 2023).

Penicillium species are known to produce mycotoxins that are harmful to human and animal health (Pitt 2002). We identified a few strains that contained BGCs associated with mycotoxin production (Fig. 1C). Eight *Penicillium* strains contained BGCs for cyclopiazonic acid in section *Fasciculata*, and 7 strains within sections *Chryosgena*, *Roquefortí*, and the uncharacterized section contained BGCs for PR-Toxin, mycophenolic acid, and/or patulin (Fig. 1C). We also identified one strain containing the BGC for ochratoxin A (Fig. 1C) and another closely related strain that contains the BGC for citrinin. Both strains were most closely related to *P. verrucosum,* which has been flagged as an ochratoxin A and citrinin producer (Lund and Frisvad, 2003).

3.2. Penicillium strains have divergent impacts on cheese rind bacterial community assembly

To determine the ecological impact of *Penicillium* species in the cheese rind microbiome, we chose eight of the 35 strains that spanned the *Penicillium* phylogeny and measured their interactions with a standard cheese rind bacterial community (Fig. 2A). These included strain **277** (near *P. biforme/camemberti* in the phylogeny), strain **259** (near *P. solitum*), strain **258** (near *P. polonicum*), strain **261** (closely related to *Penicillium* sp. strain MB from Tannous et al., 2023), strain **200** (near *P. verrucosum*), strain **280** (near *P. chrysogenum*, and produces a yellow pigment characteristic of *P. chrysogenum*), strain **288** (in a group of *Penicillium* without a clear taxonomic identity), and strain **285** (also in a group of *Penicillium* without a clear taxonomic identity). We grew four bacterial species (*Staphylococcus equorum* strain BC9, *Brevibacterium aurantiacum* strain JB5, *Brachybacterium alimentarium* strain JB7, and *Psychrobacter* sp. strain JB193) either alone (without a fungus) or with the addition of one of the eight *Penicillium* strains. After ten days of incubation, we measured abundance of all four bacterial members compared to growth without *Penicillium*.

We found highly variable impacts of different *Penicillium* species on total bacterial community growth with strains 258, 200, and 280 as strong inhibitors of the community and the remaining five *Penicillium* strains as having slight to no impact on bacterial growth or community composition (PERMANOVA, $F_{8,18} = 26.22$, $p < 0.001$; Fig. 2B-2C; Table S3). Although other sections were not densely represented, we observed a wide variety of community outcomes from the four strains within section *Fasciculata* alone (277, 259, 258, 261), suggesting that taxonomic relatedness per se does not predict how *Penicillium* species

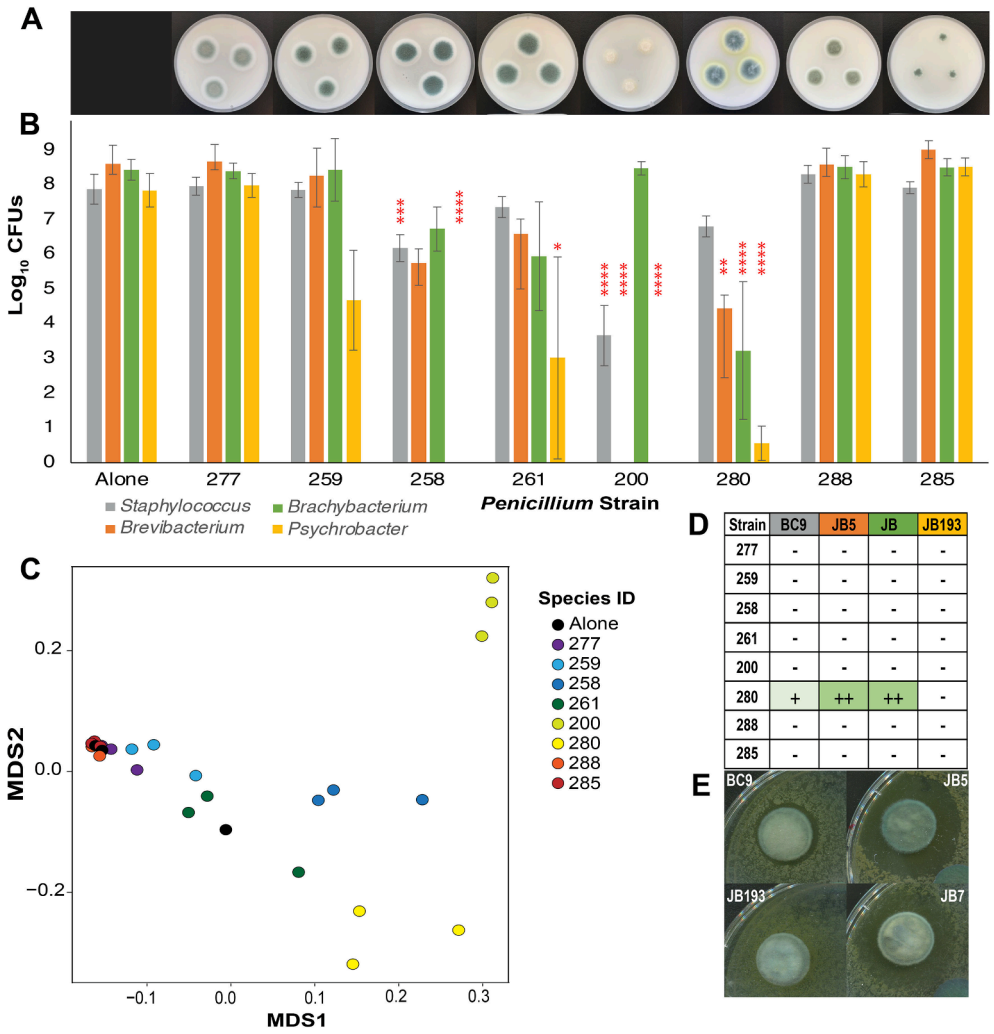

Fig. 2. Divergent impacts of eight *Penicillium* strains on the cheese rind bacterial community. (A) Eight *Penicillium* isolates chosen for ecological experiments, imaged after three to five days of growth at 24 °C on cheese curd agar. **(B)** Bacterial abundance (log_{10} CFUs) of four standard cheese rind bacteria after ten days of growth at 24 °C on cheese curd agar (N = 3, n = 5) either with no fungus or with one of eight *Penicillium* isolates (PERMANOVA, $F_{8,18} = 26.22$, $p < 0.001$) with red asterisks representing statistical significance via Dunnett's test for multiple comparisons within each bacterial species (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). All abundance values are reported in Table S3. **(C)** NMDS plot associated with bacterial abundance, with each point representing an independent experimental replicate mean. Colors represent the eight *Penicillium* strains. $R^2 = 0.92$. **(D)** Plug-on-lawn results, with columns representing the bacterial 'lawn' species and rows representing the fungal 'plug' strain. – indicates no zone of inhibition, + indicates a zone of inhibition less than 25 mm in diameter, and ++ indicates a zone of inhibition greater than 25 mm. **(E)** Representative zones of inhibition created by strain 280 on (from top left clockwise) *Staphylococcus equorum* (BC9, 22 mm diameter), *Brevibacterium aurantiacum* (JB5, 35 mm diameter), *Brachybacterium alimentarium* (JB7, 31 mm diameter), and *Psychrobacter* sp. (JB193, no zone of inhibition).

will interact with bacterial communities. Strain 261, which only significantly inhibited growth of *Psychrobacter* (Fig. 2B), is very closely related to another strain previously studied in the lab (strain MB, Fig. 1A) that inhibited all four bacteria species (Tannous et al., 2023), which suggests ecological impact can both species-specific and strain-specific. Strain 261 and MB, putatively both *P. cyclopium*, contain the BGC encoding for pseurotin production, an antimicrobial compound previously established to be involved in shaping the cheese rind bacterial community (Tannous et al., 2023), but strain 261 did not upregulate this cluster in the presence of the community (see Section 3.3), nor was the strain considered a strong inhibitor of bacterial growth (Fig. 2B-2C). While growth with strains 258 and 280 resulted in relatively similar bacterial communities by strongly inhibiting *Psychrobacter* and slightly reducing *Staphylococcus, Brevibacterium,* and *Brachybacterium* abundance, strain 280 selected for a *Brachybacterium*-dominant community (Fig. 2B), suggesting strain 280 utilizes a different mechanism of inhibition from the other two strains.

To better characterize potential mechanisms of inhibition, 'plug-on-lawn' experiments were done to test antibacterial activity of the eight *Penicillium* species (Fig. 2D-2E). Only one isolate, strain 280, inhibited bacterial growth enough to create a zone of inhibition (Fig. 2D-2E). Surprisingly, plugs of strain 280 resulted in slight inhibition of *S. equorum*, strong inhibition of both Actinobacteria (*Brevibacterium* and *Brachybacterium*) strains, and no inhibition of *Psychrobacter* (Fig. 2D-2E), the opposite of what we would have expected based on the results in the community (Fig. 2B). Furthermore, strains 200 and 258, which significantly inhibited the bacterial community in co-culture, did not create a zone of inhibition.

The discrepancies between our community experiments and observations from the plug-on-lawn experiments could be due to several possibilities. The plug-on-lawn approach cultures the fungal inhibitor and bacterial responder separately, eliminating the opportunity for bacteria to induce production of fungal metabolites. Only the effects of metabolites that are produced when the *Penicillium* was growing alone on CCA will be measured in these experiments. Previous studies have demonstrated that co-culturing fungi with other microbes induces the

production of certain metabolites (Netzker et al., 2018). In addition, the bacterial 'lawns' consisted of a single bacterial species, while the synthetic community consisted of a four-membered cheese rind bacterial community, so differences between the two experiments could be a result of interspecific bacteria-bacteria interactions occurring in the synthetic community experiment.

3.3. Transcriptomic diversity of Penicillium species in co-culture is species-specific and driven by nutrient metabolism

To further characterize mechanisms of *Penicillium*-bacteria interactions, we used RNA-sequencing to measure differential mRNA expression in eight *Penicillium* strains grown either alone or with a standard four-member bacterial community. Growth in co-culture with the bacterial community resulted in anywhere from 2 to 757 differentially expressed genes (DEGs) across the *Penicillium* isolates, a range of 0.02–7% of all the genes within the genome (Fig. 3A). A handful of studies have measured global differential mRNA expression in *Penicillium*, including comparing *P. citrinum* grown with sucrose or glucose as a sugar source that identified 1085 DEGs (T. Li et al., 2017), higher than our highest *Penicillium* expression change of 757 genes. Another study, with a more conservative cut-off of $\log_2$ ratio ≥ 2, identified 434 DEGs in *P. expansum* grown in co-culture with the yeast *Meyerozyma guilliermondii* (Yang et al., 2020), much higher than our highest value of 151 DEGs (strain 288) using the same cut-off parameters. Overall, muted expression profiles across most *Penicillium* species when grown with bacteria suggests *Penicillium* are not strongly affected by bacterial neighbors on cheese rinds in the conditions and at the time points that we used.

In our pathway enrichment analysis, we only detected a few pathways that were significantly differentially expressed (boxed boxes in Fig. 3B-C) due to low percentages of the total number of differentially expressed genes within each pathway. Only three pathways were significantly upregulated in multiple strains: butanoate metabolism in

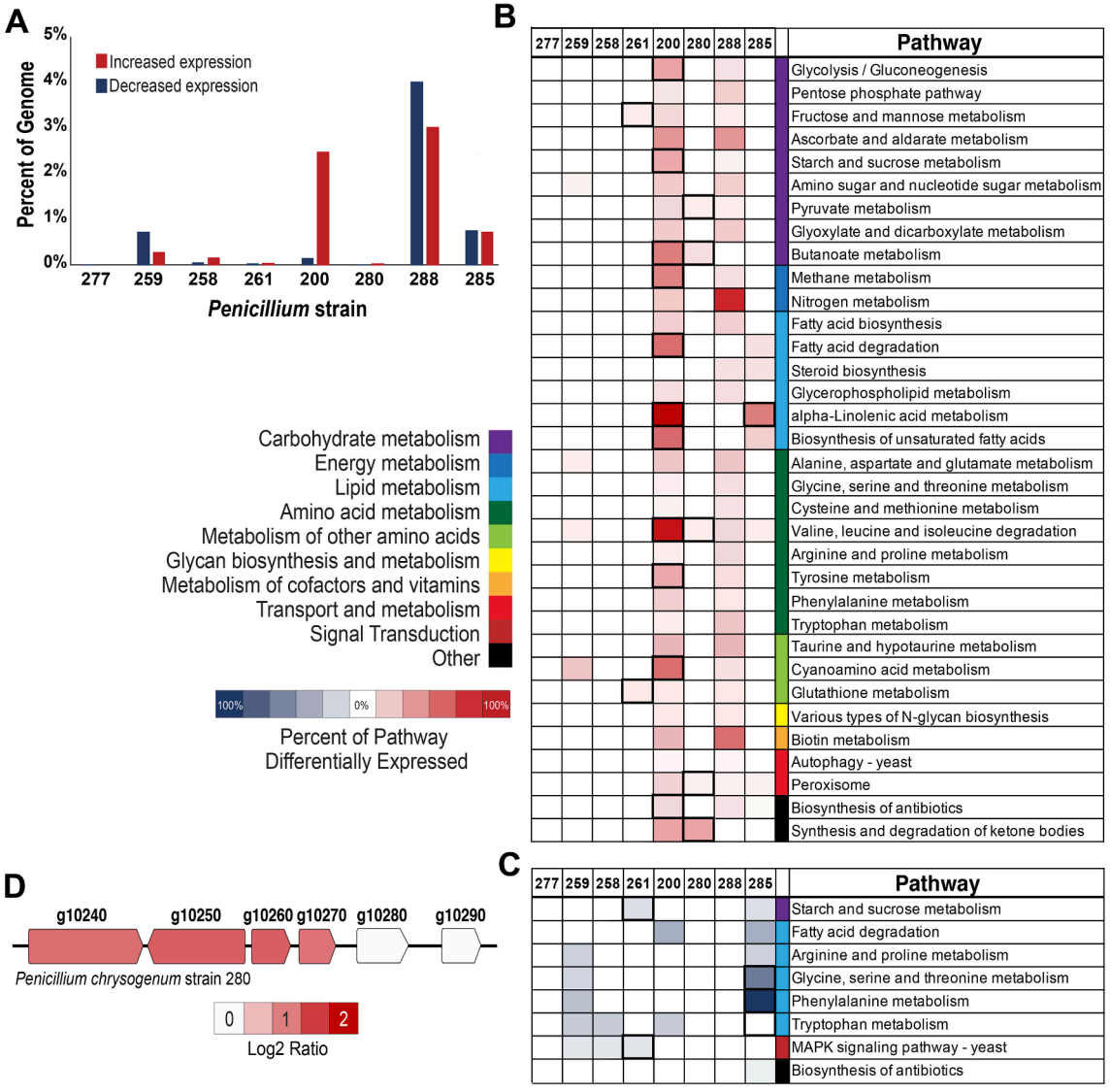

Fig. 3. Differential mRNA expression of *Penicillium* species when co-cultured with a cheese rind bacterial community. Significant differential expression is defined as $\log_2$ fold change greater than 1 or less than −1 when grown with a four-member bacteria community compared to growth in monoculture on cheese curd agar. **(A)** Percentage of genes within each *Penicillium* genome significantly upregulated (in red) or downregulated (in blue) when grown with a bacterial community. **(B-C)** Percentage of genes within a KEGG functional pathway significantly upregulated (**B**, in red) or downregulated (**C**, in blue) in at least two strains. Increasing cell color intensity corresponds with increasing percentage of the pathway that was differentially expressed. **(D)** Schematic of a putative BGC that was upregulated in the presence of a bacterial community in *P. chrysogenum* strain 280, with color intensity corresponding to differential expression $\log_2$ ratio. See Tables S4-S5 for a full overview of differential expression outputs.

strains 200 and 280, alpha-linolenic acid metabolism in strains 200 and 285, and valine, leucine, and isoleucine degradation pathways in strains 200 and 280. Butyrates, alpha-linolenic acids, and branched amino acids are all abundant in cheese (McSweeney, 2017; Karahadian et al., 1985; Molimard and Spinnler, 1996) and the increased expression of these fungal pathways in response to bacteria may be due to metabolic interactions between the fungi and bacteria. Further research is needed to understand the mechanism by which only a few of the eight *Penicillium* strains upregulated catabolic breakdown of these products in the presence of a bacteria community.

The transcriptional responses that we observed across the different strains did not correspond with how they impacted the bacterial community. For example, two fungal strains that had the strongest transcriptional responses when co-cultured with bacteria, strains 200 and 288 (Fig. 3A-B), had contrasting effects on the bacterial community. Strain 200 caused one of the strongest shifts in bacterial community composition, while strain 288 had no effect on the bacterial community (Fig. 2B-C).

Despite strong interactions between bacteria and *P. chrysogenum* strain 280 in co-culture and clear zones of inhibition in the plug-on-lawn assays (Fig. 2B, 2D), only six genes were differentially expressed when *P. chrysogenum* was grown with bacteria compared to growth alone (Fig. 3D). This included four upregulated genes (gene IDs g10240, g10250, g10260, and g10270) adjacent to one another within the genome, a spatial structure indicative of a potential BGC. AntiSMASH analysis of this region returned a six-gene BGC encoding proteins putatively involved in the production of an uncharacterized beta-lactone, including two genes immediately downstream of the set of four upregulated genes (Fig. 3D). The cluster returned a strong NCBI BLAST hit to *Penicillium chrysogenum* strain Wisconsin 54–1255 with 98% sequence identity, but no other *Penicillium* species, suggesting this cluster may be unique to *P. chrysogenum*.

Beta-lactones are a broad group of four-membered cyclic esters that resemble beta-lactams, a well-known class of antibiotics including penicillin, in both structure and biosynthesis mechanism. The group includes toxins (Lane et al., 1952; Yamada et al., 1965), antibiotics (Mori et al., 1985; Koomsiri et al., 2017), and compounds with anticancer (Gill et al., 2015) or antiobesity activity (Weibel et al., 1987; Chen et al., 2016; Kang and Kim, 2016) and are produced by plants, insects, bacteria, and fungi (Robinson et al., 2019). Beta-lactones have been isolated from at least two *Penicillium* species, *P. polonicum* (Wen et al., 2020) and *P. chrysogenum* (Li et al., 2022; Cen et al., 2023), although neither the genetic basis nor antibacterial activity were tested in both cases.

4. Conclusion

In our study, we identified 1558 putative BGCs from 35 *Penicillium* strains isolated from cheese rinds. antiSMASH was unable to identify a strong match for 1152 putative BGCs (74%), suggesting a large potential for natural product discovery in cheese rind *Penicillium* strains. Despite the density at which we sampled several regions of the *Penicillium* phylogeny (Fig. 1A), 18 of the 66 characterized BGCs were only detected once in a single strain, highlighting the diversity of secondary metabolism within the genus as well as the importance of strain-level diversity in fungal secondary metabolism. Future metabolomic studies will be needed to confirm that the genomic potential we have identified here corresponds with actual production of predicted metabolites and will also potentially identify the products of uncharacterized BGCs predicted by antiSMASH.

The *Penicillium* strains had fairly limited transcriptional responses when co-cultured with a standard cheese bacterial community. This is in striking contrast with previous work by our group that has shown generally strong transcriptional responses of various cheese rind bacteria when they were co-cultured with cheese fungi (Ye et al., 2023; Kastman et al., 2016; Cleary et al., 2018). Collectively, these results align with previous observations that while cheese rind bacteria have strong responses when co-cultured with fungi, the converse is not true and fungi have limited responses to the presence of cheese rind bacteria (Wolfe et al., 2014). This is possibly due to the reliance of cheese rind bacteria on fungi to alter the cheese environment and promote their growth, including the deacidification of the cheese (Wolfe et al., 2014; Bonaïti et al., 2004), release of free amino acids (Kastman et al., 2016), and production of siderophores to increase iron access (Kastman et al., 2016; Pierce et al., 2021).

Despite the prevailing belief that fungi produce antibacterial secondary metabolites in response to microbial competitors in their environment (Molloy and Hertweck, 2017; Netzker et al., 2018; Keller, 2019), we observed just one potential signature of this in our transcriptomic data. Strain 280, most closely related to *P. chrysogenum*, upregulated just four genes in co-culture with cheese rind bacteria. These four genes were positioned adjacent to one another and were predicted to produce a beta-lactone. Future work is needed to characterize this beta-lactone and determine the role of this putative secondary metabolite in mediating microbiome composition and interactions.

We view the characterization work in this manuscript as a first step in understanding the ecology and metabolomics of diverse cheese-associated *Penicillium* species. Given some of the limitations of our study, there is a lot more work that needs to be done to help contextualize our results and provide mechanistic insights. Our sampling of *Penicillium* molds is only from a single cheese aging facility and may not reflect the full diversity of *Penicillium* molds that can be found on surface-ripened cheeses. Including more species and more strains of *Penicillium* in future studies might reveal additional potential metabolites relevant to this system and interaction outcomes. Adding more taxa across the phylogenetic breadth of *Penicillium* may also help identify if the ecology of these molds in cheese rinds can be explained by their phylogenetic relationships. The transcriptional responses of the *Penicillium* strains were only from a single time point and a single condition. Including additional time points or including additional community members that can have a stronger effect on *Penicillium* growth, such as *Glutamicibacter arilaitensis* that can inhibit fungal growth (Cleary et al., 2018), might elicit different and potentially stronger transcriptional responses from the fungi.

Proper cheese rind development is important for both the aesthetics and flavors of naturally aged cheeses. The *Penicillium* molds studied here are especially important for the development of natural rinds on cheeses such as clothbound cheddars, some blue cheeses, and tomme style cheeses (Ropars et al., 2012; Marcellino and Benson, 2013). Cheesemakers often report unwanted molds as challenges in managing rind microbial communities (Biango-Daniels and Wolfe, 2021), but the ecology of spoilage molds in cheese rinds has been largely understudied. Our work demonstrates that different *Penicillium* species can have unique impacts on rind development. We acknowledge that our synthetic cheese rind communities in the lab may not perfectly represent the more diverse and dynamic communities that form in cheese aging facilities. For example, the presence of other yeasts or filamentous fungi may alter the outcomes of *Penicillium*-bacteria interactions. However, the strong inhibition of typical cheese rind bacteria that we observed with some *Penicillium* species could impact the aesthetics, shelf-life, and possibly the safety of these products. Future studies aimed at determining how to favor relatively benign molds (such as *P. biforme*) over some of the strongly inhibitory molds (such as *P. chrysogenum*) in cheese aging environments can help develop approaches to better manage the ecology of cheese rind *Penicillium* species.

CRediT authorship contribution statement

Ruby Ye: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Megan Biango-Daniels:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review

& editing. **Jacob L. Steenwyk:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing, Writing – original draft. **Antonis Rokas:** Funding acquisition, Methodology, Supervision, Writing – review & editing. **Nicolas L. Louw:** Formal analysis, Methodology, Writing – review & editing. **Robert Nardella:** Data curation. **Benjamin E. Wolfe:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: JLS is a scientific advisor for WittGen Biotechnologies. JLS is an advisor for ForensisGroup Inc. AR is a scientific consultant for LifeMine Therapeutics, Inc.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fgb.2023.103862.

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