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Great diversity of KSα sequences from bat-associated microbiota suggests novel sources of uncharacterized natural products

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Abstract

Polyketide synthases (PKSs) are multidomain enzymes in microorganisms that synthesize complex, bioactive molecules. PKS II systems are iterative, containing only a single representative of each domain: ketosynthase alpha (KSα), ketosynthase beta and the acyl carrier protein. Any gene encoding for one of these domains is representative of an entire PKS II biosynthetic gene cluster (BGC). Bat skin surfaces represent an extreme environment prolific in Actinobacteria that may constitute a source for bioactive molecule discovery. KSα sequences were obtained from culturable bacteria from bats in the southwestern United States. From 467 bat bacterial isolates, we detected 215 (46%) had KSα sequences. Sequencing yielded 210 operational taxonomic units, and phylogenetic placement found 45 (21%) shared <85% homology to characterized metabolites. Additionally, 16 Actinobacteria genomes from the bat microbiome were analyzed for biosynthetic capacity. A range of 69–93% of the BGCs were novel suggesting the bat microbiome may contain valuable uncharacterized natural products. Documenting and characterizing these are important in understanding the susceptibility of bats to emerging infectious diseases, such as white-nose syndrome. Also noteworthy was the relationship between KS α homology and total BGC novelty within each fully sequenced strain. We propose amplification and detection of KSα could predict a strain's global biosynthetic capacity.

Keywords: PKS II (type two polyketide synthases), bats, microbiota, natural products, Actinobacteria, *Streptomyces*

Introduction

In the four-decade (1981–2019) review of pharmaceuticals by Newmann and Cragg (2020), they found 32% of new drugs were produced from natural products. Despite the medical necessity of new therapies to combat emerging and drug-resistant pathogens, and notwithstanding the effort in the last decade to approve new drugs, there continues to be an ever-growing need for drug discovery. Analogs of known small molecule drugs can be found by modifying cultivation procedures or by manipulating biosynthetic pathways (Jabes and Donadio 2010). High-throughput screening and predictive computational techniques have unlocked a new realm of processing complex natural products and their derivatives for innovative drug design and discovery (Wang *et al.* 2016, Thomford *et al.* 2018, Newman and Cragg 2020). However, these advancements are often dependent on the current taxonomic databases, which comprise a small fraction of total bacterial/archaeal diversity. Despite the shift in strategies from traditional bioactivity-guided discovery to genome mining and more recent approaches enabled by multiple 'omics' techniques (Bachmann *et al.* 2014, Navarro-Muñoz *et al.* 2020), evidence exists that readily cultivatable bacteria harbor the potential to produce new natural products. One such example is an assessment of cultivation techniques used to recover natural product biosynthetic gene clusters (BGCs) from sediment by Elfeki *et al.* (2018), who found that 76–91% have yet to be characterized in known databases. Expanding discovery efforts to include rare source taxa and environments could reveal rare natural products (Bérdy 2012, Hu *et al.* 2015, Katz and Baltz 2016).

The era of 'Modern Actinobacteria' was coined to describe the interest in exploring special and extreme environments in search of natural products from Actinobacteria known for their prolific bioactive compounds (Law *et al.* 2020). One such extreme environment is caves and the bats within them. Metagenomic analyses have estimated that Actinobacteria comprise 13–35% of the bat skin microbiome (Avena *et al.* 2016, Winter *et al.* 2017). These percentages strongly correlate to proportions of bats each group caught from caves, suggesting that the external microbiomes of bat skin are to some degree acquired from local environments, an observation corroborated in recent bat microbiome studies (Avena *et al.* 2016, Lemieux-Labonté *et al.* 2016, 2017). Based on culture-independent studies in cave ecosystems, Actinobacteria are readily abundant on the cave walls and soils (Northup *et al.* 2011, Hathaway *et al.* 2014, Riquelme *et al.* 2015, Wu *et al.* 2015, Wiseschart *et al.* 2018) and have been shown to be a rich reservoir for novel

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species (Groth *et al.* 2002, Gutierrez-Patricio *et al.* 2014, Ko *et al.* 2015, Hamm *et al.* 2019, 2020).

Many bacteria, particularly those within the phylum Actinobacteria, are known to produce secondary metabolites synthesized by polyketide synthase (PKS) pathways (Shen 2003). A considerable diversity exists for polyketide metabolites produced by these multifunctional enzymatic systems, many of which are clinically valuable antibiotics or chemotherapeutic agents (Shen 2000, Manivasagan *et al.* 2014). Three types of bacterial PKSs are known to date, classified by structure and mechanistic differences. Despite the intricacies of these assemblies, all PKS II pathways contain a set of three genes that encode the so-called minimal PKS: two ketoacyl synthase subunits (KSα and KSβ) and an acyl carrier protein (ACP) (Seow *et al.* 1997). One KSα domain can be considered representative of one BGC (Hertweck *et al.* 2007). This is unlike other biosynthetic systems where correlations are complicated by multiple domains in a single BGC (Reddy *et al.* 2012).

Actinobacteria within the external microbiome of bats may play a vital role in host health. Since the emergence of white-nose syndrome (WNS), an invasive fungal disease that threatens numerous bat species across the United States and Canada (Turner *et al.* 2011, Coleman and Reichard 2014), studies suggest that bat microbiomes may provide host protection, leading to greater bat survival from infection (Lemieux-Labonté *et al.* 2017, 2020). WNS-infected bats suffer severe wing damage, affecting thermoregulation, blood electrolyte concentration and gas exchange (Meteyer *et al.* 2009, Cryan *et al.* 2010). Bats also show increased frequency of arousals from torpor, depleting fat stores during hibernation, ultimately leading to starvation (Warnecke *et al.* 2013). WNS has recently been confirmed in New Mexico in 2021 (https://www.whitenosesyndrome.org/where-is-wns), where caves exhibit the appropriate temperatures and relative humidity to support the growth of *Pseudogymnoscus destructans*, the disease's causative agent (Cunningham and LaRock 1991, Forbes 1998, Torres-Cruz *et al.* 2019).

In this study, we document PKS II gene diversity and novelty by sequencing KSα genes known to synthesize bioactive small molecules. Sequences were derived from culturable bacteria isolated from bats in the southwestern United States. We targeted the PKS II pathway because it is arguably simpler than other antibiotic pathways (Wawrik *et al.* 2005) and is very common in Actinobacteria, which comprised a large proportion of the culture collection. Genomic analyses of a 16-member subset of bat bacterial isolates were additionally explored to document total BGC diversity and novelty of the bat microbiome. Preliminary data suggest that KSα gene homology within individual isolates could serve as a proxy for its total biosynthetic capacity.

Materials and methods

Isolates for KSα screening

We selected six bat species from New Mexico (*Corynorhinus townsendii*, *Eptesicus fuscus*, *Myotis ciliolabrum*, *Myotis evotis*, *Myotis velifer* and *Myotis thysanodes*) and three bat species from Arizona (*Parastrellus hesperus*, *C. townsendii* and *Myotis californicus*) as sources for bacterial isolation. Because mortality rates from WNS vary among bat species (Langwig *et al.* 2012, 2016), these bat species were chosen based on potential vulnerability to WNS, or evidence that they may carry the fungus, but not acquire the disease. For example, *P. destructans* has been detected on *C. townsendii*, but there have been no diagnostic symptoms of WNS documented (https://www.whitenosesyndrome.org/, accessed August 12, 2021). *Eptesicus fuscus* is considered susceptible to WNS and is symptomatic, but it has a lower mortality rate than other species (Pettit and O'Keefe 2017). Myotis bats in the Southwest are congeners of *Myotis lucifugus*, one of the most heavily impacted species by WNS (Turner *et al.* 2011) and are presumed to be susceptible to infection because of their life histories and hibernation behaviors. The susceptibility to WNS for *P. hesperus* is unknown at this time but may be similar to its eastern analog of *Perimyotis subflavus*, which is affected greatly by the disease.

Procedures for identification and swabbing of the WNS-negative bats obeyed guidance by Ellison *et al.* (2013) and were previously reported by Hamm *et al.* (2017). Capturing, handling and swabbing were overseen by expert bat biologists under the following collection permits: 2014 Arizona and New Mexico Game and Fish Department Scientific Collecting Permit (SP670210, SCI#3423 and SCI#3350), National Park Service Scientific Collecting Permit (CAVE-2014-SCI-0012, ELMA-2013-SCI-0005, ELMA-2014-SCI-0001 and PARA-2012-SCI-0003), USGS Fort Collins Science Center Standard Operating Procedure (SOP) 2013-01, and an Institutional Animal Care and Use Committee (IACUC) permit from the University of New Mexico (protocol #12-100835-MCC) and from the National Park Service (protocol #IMR-ELMA.PARA-Northup-Bat-2013.A2). Bat skin and fur surfaces were thoroughly swabbed with a sterile applicator and Ringer's solution. Swabs were streaked on three media types: actinomycete isolation agar (Difco, Sparks, MD), gellan gum (7.0 g/L gellan gum, 7 mM calcium chloride) or humic acid-vitamin agar (Hayakawa and Nonomura 1987) supplemented with cycloheximide (50 mg/L), nalidixic acid (50 mg/L), trimethoprim (50 mg/L) and a vitamin solution (Hayakawa and Nonomura 1987). Cultures were grown at 20°C and subcultured for purification on Reasoner's 2A medium (R2A; Difco, Sparks, MD).

Taxonomic classification of bacteria was determined with BLAST (Altschul *et al.* 1990) using sequences previously reported in Hamm *et al.* (2017) or replicating their 16S rRNA molecular procedure (Tables S1 and S2, Supporting Information). Because of our interest in the connection to WNS, 17 additional antagonists of *P. destructans* previously reported by Hamm *et al.* (2017) were screened. These isolates were associated with the selected bat species and two additional bat species, *Tadarida brasiliensis* (eight isolates) and *Antrozous pallidus* (one isolate). This brought the grand total to 467 Actinobacteria isolates screened for PKS II genes (Table S3, Supporting Information). Of these, there were 50 isolates from each bat species, except for *C. townsendii*, which was the source of 100 isolates (50 from New Mexico and 50 from Arizona). Forty-two of the isolates, including multiple *Streptomyces* species, had been noted to inhibit *P. destructans* (Hamm *et al.* 2017, Salazar-Hamm *et al.* unpublished).

Molecular methods used for KSα sequencing

DNA was extracted from pure cultures using the MoBio UltraClean microbial DNA isolation kit (MoBio, Carlsbad, CA), according to the manufacturer's protocol, except for the replacement of the vortexing step with 1.5 min of bead beating at a medium speed. Polymerase chain reaction (PCR) was used to amplify a portion of the KS α domain of the PKS II pathway using KSα-F (5′-TSGCSTGCTTCGAYGCSATC-3′) and KSα-R (5′-TGGAANCCG CCGAABCCGCT-3′) (Berry *et al.* 2002). Degenerate primers were selected for their known efficacy of recovering diverse PKS II gene sequences, especially from the genus *Streptomyces* (Metsä-Ketelä *et al.* 2002). Reactions were carried out with 0.75 μL of filter sterilized dimethyl sulfoxide, 15 μM of each primer, 20 μM each din-

ucleoside triphosphate (Applied Biosystems, Foster City, CA), 1 μg bovine serum albumin (Ambion, Austin, TX) and 0.5 U of AmpliTaq (Applied Biosystems, Foster City, CA) in a final volume of 25 μL. All PCR reactions began with an initial step at 94°C for 5 min. This was followed by 30 cycles of 98°C for 10 s, 70°C for 30 s and then 72°C for 30 s, with a final extension at 72°C for 5 min. Based on resource constraints, between 13 and 17 isolates positive for KSα genes from each bat species were selected for sequencing to estimate the diversity of KSα genes. Those isolates known at the time to be *P. destructans* inhibitors that amplified a KSα gene were sequenced, and the rest were selected at random from those possessing the KSα gene. PCR products were cleaned using ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA) before sequencing using Big Dye Terminator v1.1 (Applied Biosystems, Foster City, CA) on an ABI 3130 sequencing machine (Molecular Biology Facility, University of New Mexico, Albuquerque, NM). Those isolates that did not return clean sequences were considered to have multiple variants of the KSα gene and were then cloned. Cloning was performed using the TOPO TA cloning kit with the pCR 4 TOPO vector (Invitrogen, Carlsbad, CA). Twelve of the clones from each isolate were chosen as a representative sample to be sequenced at Genewiz (Boston, MA).

Identification of KSα gene homology

Sequences were verified in Sequencher v5.1 and grouped at 97% similarity into operational taxonomic units (OTUs). Gene variant number was determined by the number of OTUs per isolate. Nucleotide sequences were confirmed as KSα using BLASTn with default parameters (https://blast.ncbi.nlm.nih.gov/) and translated into protein sequences using Prodigal (Hyatt *et al.* 2010). Sequences were then manually vetted to ensure proper frame translations. A reference set of 134 sequences was curated by comparing experimental protein sequences with PKS II secondary metabolite BGCs from the antiSMASH 5.1.2 database (Blin *et al.* 2019). Additionally, 94 sequences were added to the reference set by comparing the experimental protein sequences to the GenBank database using BLASTp (https://blast.ncbi.nlm.nih.gov/) with default parameters. This resulted in a combined reference set of 228 sequences. Experimental protein sequences were aligned to this reference set using Clustal Omega (Sievers *et al.* 2011). Aligned sequences were trimmed using BioEdit (https://bioedit.software.informer.com/), and identity matrices were made to compare experimental sequences generated in this study with themselves and against the reference sequences, with 85% sequence dissimilarity being considered potentially novel compounds. Good's coverage was calculated using the equation 1-(F1/*N*) where F1 is the number of single variant sequences and *N* is the total number of sequences recovered for an isolate.

Phylogenetic analysis of bat KSα genes

The web server version of WebPrank (Löytynoja and Goldman 2010; https://www.ebi.ac.uk/goldman-srv/webprank/) was used to align 210 experimental KSα sequences and 228 reference sequences using default parameters. A maximum likelihood phylogenetic tree was then inferred in IQTREE with the default setting of auto detection for substitution models (Trifinopoulos *et al.* 2016). The resulting tree was annotated using the Interactive Tree of Life (iTOL v.6; Letunic and Bork 2019). Sequences that were uncharacterized (i.e. share <85% identity to described metabolites) or from isolates that can inhibit *P. destructans* are indicated.

Bat microbiota genomic analyses

Fourteen bacteria isolated from *M. velifer*, *M. thysanodes* or *T. brasiliensis* bats in Carlsbad Caverns National Park were chosen for genome sequencing and secondary metabolite analyses because of their ability to inhibit *P. destructans* as reported in Hamm *et al.* (2017). DNA was extracted from pure cultures using the MoBio UltraClean microbial DNA isolation kit (MoBio, Carlsbad, CA) and sent to MR DNA (Shallowater, TX) for genomic sequencing. The initial concentration of DNA was evaluated using the Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA). Libraries were prepared with 50 ng of DNA from each sample using Nextera DNA Sample Preparation Kit (Illumina, San Diego, CA) following the manufacturer's guide. The samples underwent the simultaneous fragmentation and addition of adapter sequences. These adapters are utilized during a limited-cycle (five cycles) PCR in which unique indices were added to the samples. Following the library preparation, the final concentration of the libraries was measured using the Qubit® dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA), and the average library was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The libraries were pooled and diluted (to 10.0 pM) and sequenced paired end for 500 cycles using the Illumina HiSeq system.

Raw genome data files were imported into Galaxy, a server hosted at the Center for Evolutionary & Theoretical Immunology (CETI) in the Department of Biology, University of New Mexico. All paired-end data files were trimmed with Trim Galore! (Galaxy Version 0.4.2) using default parameter settings. Trimmed read pairs were assembled using VelvetOptimiser (Galaxy Version 1.1.0) using a start *k*-mer value of 31 and an end *k*-mer value of 99 at a *k*-mer search step size of 4. Seven genomes did not assemble with 1000 contigs or less and were reassembled using Abyss (Galaxy Version 2.0.1.0). A *k*-mer length of 64 and the basic parameters in Abyss were used. Five genomes still did not assemble well with VelvetOptimiser (*k*-mer 31–99) or Abyss (*k*-mer 64) and were then assembled with VelvetOptimiser using a start *k*-mer value of 161 and an end *k*-mer value of 191 at a *k*-mer search step size of 2. These genomes were also reassembled with SPAdes (Galaxy Version 3.9.0) using *k*-mer values 91, 103 and 125. The best assembly for each genome based on N50, number of contigs, max and min length of contigs, housekeeping genes and the number of secondary metabolite gene clusters was used for analyses (Table S2, Supporting Information).

Two additional bat microflora genomes were gathered from NCBI under accession numbers QQNA00000000 (Hamm *et al.* 2019) and CP060404 (Hamm *et al.* 2020) from recently described novel species. Genome-wide identification, annotation and analysis of secondary metabolite biosynthesis gene clusters were performed on the 16 bat genomes using antiSMASH 5.1.2 database (Blin *et al.* 2019). We recognize the limitations of comparing BGC content between genomes using multiple assembly platforms. However, we were able to explore intragenomic correlations between KSα homology and total BGC novelty.

Accession numbers

Experimental KSα sequences were deposited in GenBank with the accession numbers MT830375–MT830584. Bacterial 16S rRNA gene sequences were acquired from Hamm *et al.* (2017) or deposited in a GenBank batch submission with the accession numbers MW568175–MW570505. GenBank numbers are listed in Table S1 (Supporting Information). Genomic sequences were deposited at DDBJ/ENA/GenBank with the accession numbers: JAGJJX000000000, JAGPOR000000000, JAGMUD000000000, JAGMUE000000000,

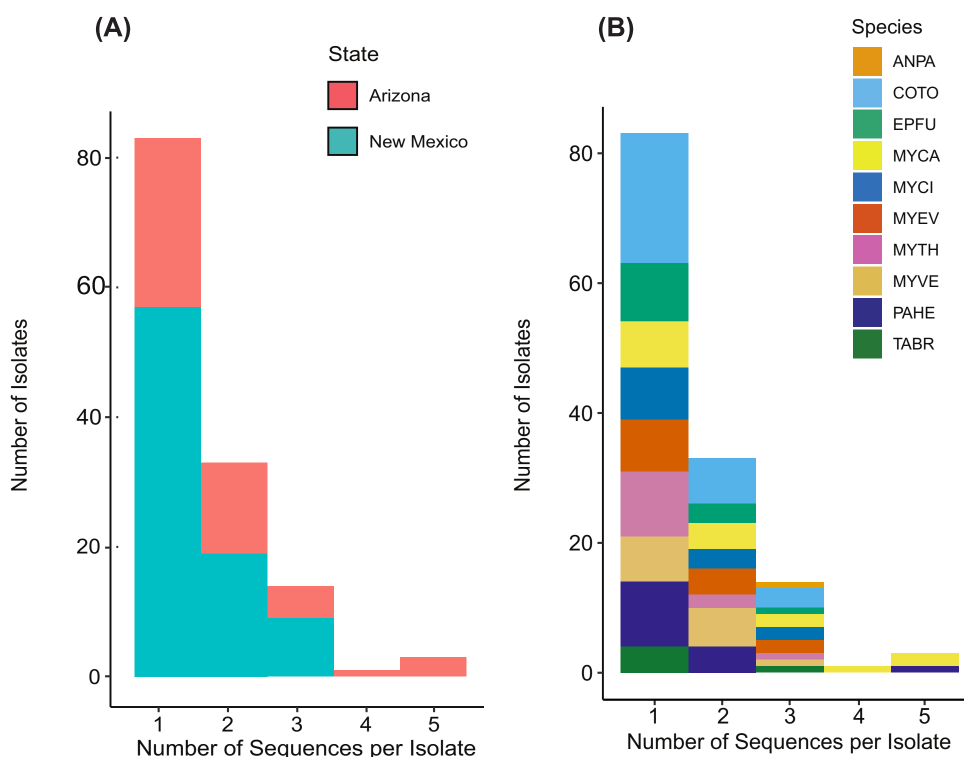

Figure 1. KSα gene variant number per isolate. **(A)** The number of amino acid sequences per isolate by state. **(B)** The number of amino acid sequences per isolate by bat species from which the isolate was cultured. Bat species abbreviations: ANPA, *Antrozous pallidus*; COTO, *Corynorhinus townsendii*; EPFU, *Eptesicus fuscus*; MYCA, *Myotis californicus*; MYCI, *Myotis ciliolabrum*; MYEV, *Myotis evotis*; MYTH, *Myotis thysanodes*; MYVE, *Myotis velifer*; PAHE, *Parastrellus hesperus*; TABR, *Tadarida brasiliensis*.

JAGMUF000000000, JAGMUG000000000, JAGMUH000000000, JAGMUI000000000, JAGMUJ000000000, JAGMUK000000000, JAGMUL000000000, JAGMUM000000000, JAGMUN000000000 and JAGMUO000000000 (Table S2, Supporting Information). The raw data in the NCBI database (https://www.ncbi.nlm.nih.gov/) serve as an acceptable digital repository by U.S. Geological Survey's (USGS) standards. Data generated during this study are available as a USGS data release (Salazar-Hamm *et al.* 2022).

Results

A total of 467 isolates collected from bats across New Mexico and Arizona were screened for KSα genes. *Eptesicus fuscus* bats had the greatest percentage of isolates containing KSα sequences (60%) followed by *M. thysanodes* (54%) (Fig. S1, Supporting Information). For all other bat species, KSα sequences were detected in 34–48% of the isolates. Of the 215 isolates (46%) with KSα sequences present, 134 isolates (between 13 and 17 from each bat species) were sequenced for KSα. A total of 85 isolates were sequenced from bats sampled in New Mexico, yielding 57 isolates with a single variant of the KSα gene and 28 with multiple variants (Fig. 1A). A total of 49 isolates were sequenced from bats sampled in Arizona, resulting in 26 isolates with a single variant and 23 with multiple variants (Fig. 1A). There were no observed patterns between bat species and KSα gene presence (Fig. 1B). Those with multiple variants were cloned and sequenced. To ensure enough sequencing depth in the clones, Good's coverage was calculated and ranged from 0.67 to 0.92. There were 210 OTUs generated following editing and clustering of the sequences (Table S1, Supporting Information). Most of the isolates (62%) had only one variant of the KSα gene, while 25% had two variants, 10% had three variants, 0.7% had four variants and 2% had five variants (Fig. 1A).

When compared to KSα genes that encoded for known small molecules (excluding the BLAST hits that were unknown PKS II genes), there were 15 (17%) of the Arizona sequences and 30 (25%) of the New Mexico sequences that shared <85% sequence similarity (Fig. 2A). This indicates that up to 45, or 21%, of the experimental sequences may produce uncharacterized polyketide products. In a comparison of the experimental sequences to themselves, only 15 sequences (7%) were unique OTUs, sharing <85% similarity to other sequences in the data set (Fig. 2B). Three KS α sequences were present across multiple isolates with six instances each. The first sequence came from four caves and shared 84% identity to urdamycin. The second came from three caves and shared 85% identity with a spore pigment. The third came from two caves and shared 86% identity to another spore pigment. In total, bat-acquired bacterial cultures demonstrated the genetic potential to produce 30 distinct characterized polyketide compounds arising from PKS II biosynthetic pathways. These are known to exhibit antibiotic (kanamycin, Ito *et al.* 1970; granaticins, Chang *et al.* 1975; medermycin, Takano *et al.* 1976; trioxacarcin, Tomita *et al.* 1981; urdamycin, Drautz *et al.* 1986; auricin, Novakova *et al.* 2002; oxytetracycline, Pickens and Tang 2010; merochlorin, Sakoulas *et al.* 2012, Ryu *et al.* 2019), antitumor (granaticins, Chang *et al.* 1975; trioxacarcin, Tomita *et al.* 1981; urdamycin, Drautz *et al.* 1986; cosmomycin, Garrido *et al.* 2006; daunorubicin, Löwenberg *et al.* 2009; nivetetracyclates, Chen *et al.* 2013; grincamycin, Lai *et al.* 2018), antiviral (trioxacarcin, Tomita *et al.* 1981; rubromycin, Goldman *et al.* 1990) and antiparasitic (nemadectin, Doscher *et al.* 1989) activities. Additionally, there were 45 sequences identified to produce spore pigments (Table S1, Supporting Information). However,

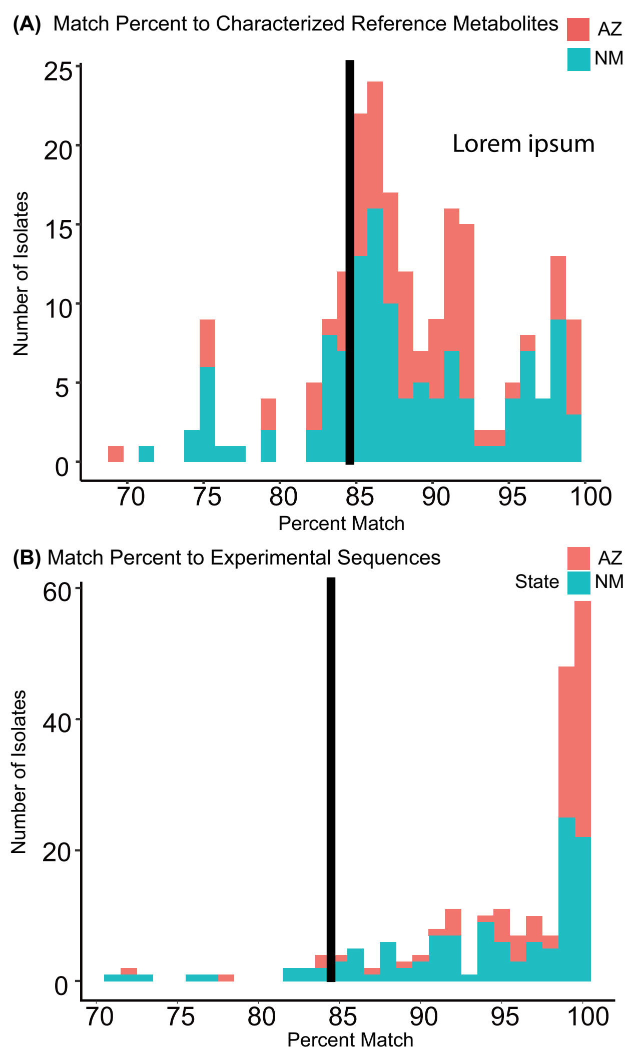

Figure 2. Comparison of KSα sequences to determine novelty. The bold black line represents 85% homology. **(A)** Experimental sequences compared with curated reference sequences. **(B)** Sequences compared with themselves.

only 165 of the 210 KSα sequences were identifiable with greater than 85% homology using antiSMASH 5.1.2 (Blin *et al.* 2019), leaving 45 (22%) with undetermined identities and functions (Table S1, Supporting Information).

The vast majority, 196 (93%), of KSα sequences recovered were produced by isolates identified as *Streptomyces*, and 39 of those sequences are uncharacterized (sharing <85% homology to characterized metabolites) (Table S1, Supporting Information). KSα sequences were also produced by other Actinobacteria genera: *Streptosporangium* (4), *Arthrobacter* (2), *Amycolatopsis* (1), *Nocardiopsis* (3) and *Nocardia* (1), as well as one alpha-proteobacteria bacteria, *Brevundimonas* (3) (Table S1, Supporting Information).

Bat microbiome-derived KSα sequences fall into clades that are known to encode for structurally diverse aromatic polyketide small molecules, as well as several clades with functionally uncharacterized sequences (Fig. 3A, orange triangles). Sequences were captured from the bacterial microbiome of every bat species investigated although sequences do not appear to group by state, bacterial species or bat species-specific clades and are instead distributed throughout the tree (Fig. 3). Notably, there are clades containing isolates from multiple bats species across various locations. One such clade contains closely related bacterial KSα sequences (>99.4% similarity) from three different bat species (*T. brasiliensis*, *C. townsendii* and *M. californicus*) in both Arizona and New Mexico (Fig. 3B). Other KSα sequences may be more specialized such as a clade of five sequences from only *C. townsendii* in New Mexico or another clade of seven sequences dominantly from *P. hesperus* and one *M. californicus* bat in Arizona (Fig. 3C).

Fourteen bat bacterial genomes were sequenced and assembled to explore total BGC diversity and novelty. Genome assemblies consisted of 140 to 2,122 contigs and N50 values from 21,392 to 217,020 (Table S2, Supporting Information). Genome sizes range from 8.3 to 13.6 Mb and GC content from 70% to 73% (Table S2, Supporting Information). With the addition of two genomes acquired from GenBank, the genomes from 16 Actinobacteria isolated on bats were evaluated for the total biosynthetic capacity using antiSMASH 5.1.2. Thirty to 65 BGCs were documented per genome of which the dominant gene cluster types were terpenes, type-1 PKS (T1PKS), siderophores and nonribosomal peptides (NRPS) (Fig. 4A).

Of the 16 genomes analyzed, 10 possessed KSα sequences for a total of 18 sequences (Table S2, Supporting Information). The relationship between the % homology of experimental KSα sequences to the antiSMASH reference database and the overall BGC novelty was explored with a linear regression (Fig. 4B; R^2 = 0.302). For any genome with more than one KSα sequence, the average of the % homology was used. We used a Bayesian linear regression (brms package in R; Bürkner 2021) with weakly informed priors to determine the relationship between KSα and total BGC. We elected to use 50% uncertainty intervals for computational stability versus 95% uncertainty intervals. Given the data and model chosen, the general trend reveals total BGC novelty increases as the KSα homology decreases (L50% = −1.23, U50% = −0.59), although we acknowledge the small sample size of this data set. A *Streptosporangium* isolate AC469_CC789 has the highest total BGC novelty, with 39 of the 42 BGCs (93%) sharing <85% homology to known clusters and an average BGC homology of 21% (Fig. 4C; Table S2, Supporting Information). Both KSα sequences, CC789_MYVE_contig A (Fig. 3C) and CC789_MYVE_contig B (Fig. 3A), were novel as well. Conversely, isolate AC555_RSS877 produced three KSα sequences that were all well characterized (>85% homology). The total biosynthetic capacity analysis confirms the second lowest novelty, with 27 of 36 BGCs (75%) sharing <85% similarity to known clusters (Fig. 4C). Our data set suggests that one could have anticipated that AC469_CC789 possesses high bioactive novelty and AC555_RSS877 is lower in bioactive novelty using the KSα sequences to screen for global biosynthetic capacity (Fig. 4B) and that this trend is worth further exploration. Overall, bat bacterial genomes have a high amount of novelty, with 69–93% of their BGCs having <85% homology to the antiSMASH database (Fig. 4C). An overestimation of BGCs may be possible due to fragmented sequences.

Discussion

Although a diverse group of microbes produce secondary metabolites, a survey of 100 of the most important secondary metabolites showed 68% are produced by *Streptomyces* species, 15% by other actinomycetes, 5% by other bacteria and 12% by fungi (Katz and Baltz 2016). The bat microbiome offers an untapped niche with a high amount of Actinobacteria diversity and novelty, particularly within the genus *Streptomyces* (Hamm *et al.* 2017, Park *et al.* 2021). In this study, 196 (93%) of the KSα sequences were isolated from *Streptomyces* species, and they encompassed 29 char-

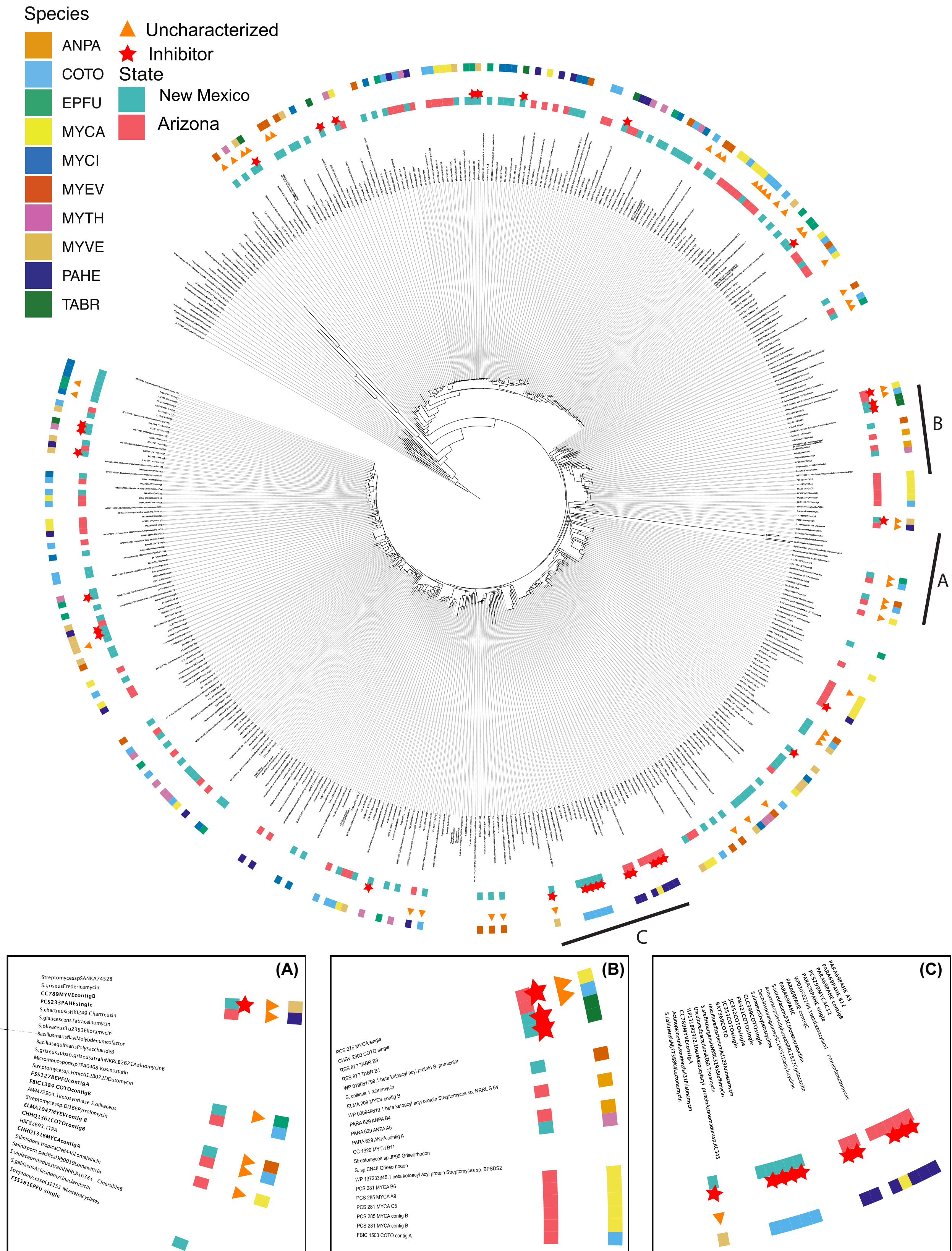

Figure 3. Maximum likelihood phylogenetic tree of KSα sequences. State from which the isolate originated is indicated by inner ring. Isolates shown to be inhibitors of *P. destructans* are indicated with a red star and uncharacterized sequences are indicated with an orange triangle. Outer ring indicates bat species: ANPA, *Antrozous pallidus*; COTO, *Corynorhinus townsendii*; EPFU, *Eptesicus fuscus*; MYCA, *Myotis californicus*; MYCI, *Myotis ciliolabrum*; MYEV, *Myotis evotis*; MYTH, *Myotis thysanodes*; MYVE, *Myotis velifer*; PAHE, *Parastrellus hesperus*; TABR, *Tadarida brasiliensis*. **(A)** A clade of novel KSα sequences from multiple bat species across various locations. **(B)** KSα sequences from recently described novel species, *Streptomyces corynorhini*, and other *P. destructans* inhibiting isolates. **(C)** Closely related KSα sequences from multiple bat species across various locations that can inhibit *P. destructans*.

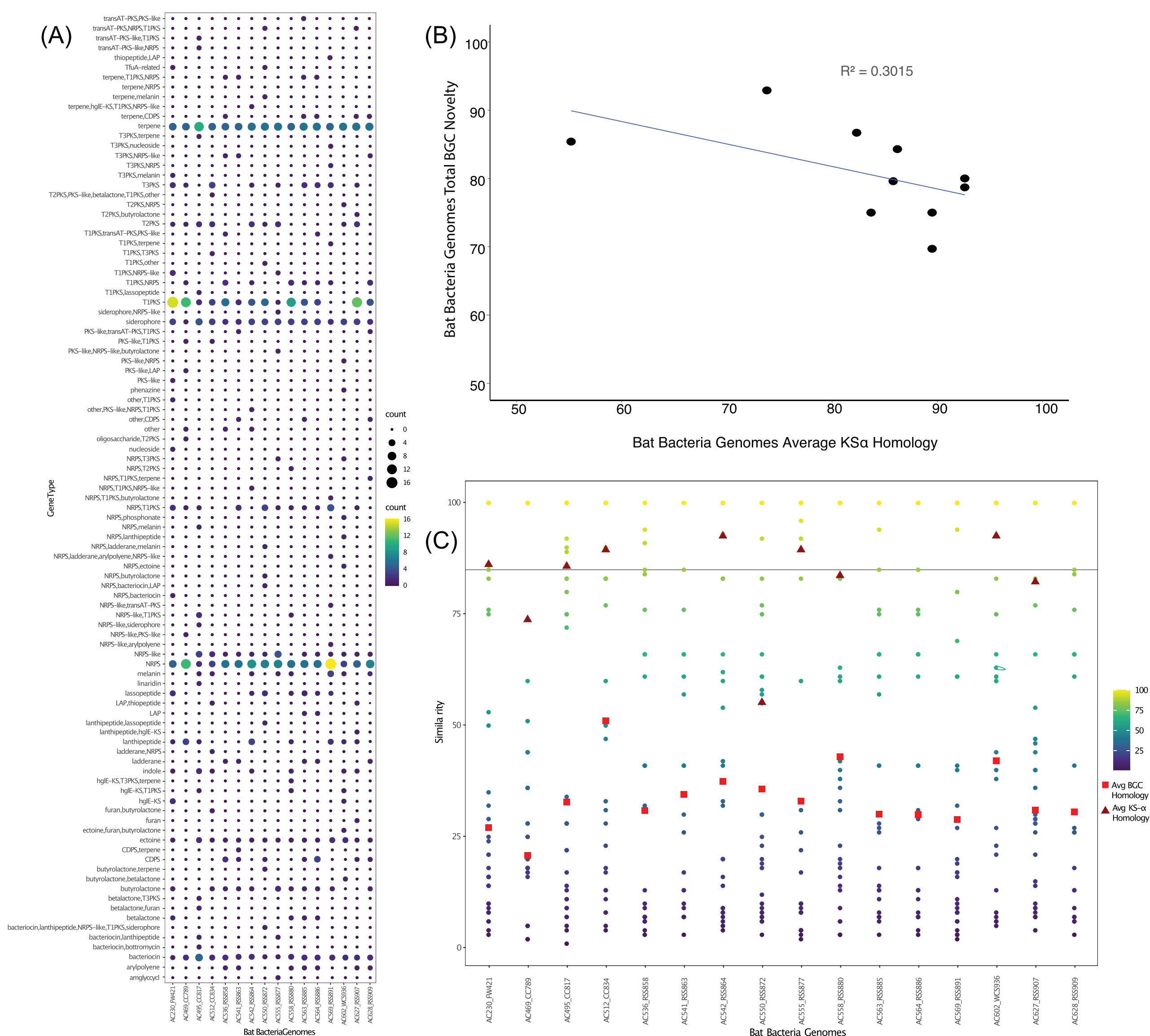

Figure 4. Bat microflora genomic analyses. **(A)** Number of gene cluster types per bat bacterial genome. Dot size and color indicate the number of genes in each sample's genome. **(B)** Correlation between KS*α* homology and total BGC novelty in bat bacterial genomes. **(C)** Bat microflora genome comparison with known BGCs. Each dot represents one BGC and its percentage similarity to known gene clusters in antiSMASH 5.1.2. The average BGC homology to known clusters is denoted with a square for each isolate, as well as the average KS*α* homology denoted with a triangle. An absence of a triangle for a sample implies there were no KS*α* sequences identified. The horizontal black line represents the 85% homology to known clusters' cutoff to determine novelty.

acterized metabolites. Additionally, 39 of the sequences shared <85% identity to characterized compounds indicating they possibly arise from biosynthetic systems encoding yet uncharacterized metabolites. The highly variable phylogenetic distribution of these sequences should not be surprising as, even among closely related *Streptomyces* genomes, there is great diversity in secondary metabolite profiles due to gene loss and horizontal gene transfer events prevalent within the genus (Egan *et al.* 2001, Doroghazi and Buckley 2010, Andam *et al.* 2016, McDonald and Currie 2017). A few studies have highlighted major differences in metabolomics even at the strain level (Seipke *et al.* 2015, Antony-Babu *et al.* 2017, Belknap *et al.* 2020). Furthermore, combinations of major BGC classes integrated into hybrid BGCs can generate a remarkable BGC diversity and compounds the reasons why species of *Streptomyces* continue to be an attractive reservoir for novel compounds.

Twenty of the isolates producing 31 KS*α* sequences have previously tested positive for antifungal activity against *P. destructans* in an *in vitro* assay (Fig. 3, red stars) as described in Hamm *et al.* (2017). Although we recognize that each strain has between 15 and 60 BGCs that could be responsible for the antifungal activity, we believe the bioactivity of the isolates coupled with the taxonomic novelty is noteworthy. While PKS II pathways do not produce the most common classes of antifungals, some compounds (actinomadurone, lysolipin, xantholipin and citreamicin) have been reported to display an array of biological activities including antifungal capabilities (Chu *et al.* 1997, Lopez *et al.* 2010, Zhang *et al.* 2012, Bunyapaiboonsri *et al.* 2017, Annang *et al.* 2018, Liu *et al.* 2019). Here, 21% of the KS*α* sequences could not be identified at <85% homology; thus, the function remains unknown. The recent discovery of the PKS II-derived turbinmicin, a marine microbiome antifungal that targets urgent-threat drug-resistant fungi, rein-

forces the possibility of novel antifungal discovery (Zhang *et al.* 2020).

Several genome-wide analyses and data mining have been used to predict and prioritize bioactive metabolite discovery (Rudolf *et al.* 2016, Adamek *et al.* 2017, Thomford *et al.* 2018, Belknap *et al.* 2020), but genome sequencing still exists as a time and financial restraint. The typical genetic KSα/KSβ/ACP architecture, with few exceptions, synthesizes aromatic polyketides (Wawrik *et al.* 2005, Hertweck *et al.* 2007), supporting the reliability of KSα for PKS II biosynthesis predictions. Here, in all instances where KSα was detected through genome annotation, the PCR screening also detected KSα. However, in two cases the PCR screening did not capture every variant identified by antiSMASH. Specialized niches with a high amount of actinobacterial diversity, like the bat microbiome, could benefit from a simple KSα PCR screen to determine homology to characterized sequences that in turn may suggest overall BGC novelty (Fig. 4B). In practice, PCR screening for biosynthetic domain richness patterns of NRPS and PKS systems have been fruitful (Lemetre *et al.* 2017, Benaud *et al.* 2019). That being said, a major caveat of this study is the reliance on the composition of the BGC database used for comparison and the ability for it to correctly identify BGCs. An additional stipulation is the cutoff of homology to known BGC clusters chosen by researchers. Domain similarity cutoffs of 70% have been previously reported in large-scale genomics and metabolomics studies to identify a wider net of biosynthetic capacity (Doroghazi *et al.* 2014, Parkinson *et al.* 2018). While looser cutoffs provide a broader perspective on related families of natural products, tighter cutoffs are more appropriate for grouping BGCs that produce identical compounds (Navarro-Muñoz *et al.* 2020). In this study, with a cutoff of >90% homology, there were only 61 of the 210 (29%) KSα sequences identified versus a cutoff of >85% homology, which had 165 of the 210 (79%) identified in the database (Fig. 2A). Interestingly, KSα sequences in this study were more closely related to other experimental sequences than the reference database (Fig. 2B), a similar finding to Wawrik *et al.* (2005).

The exploration of the external microbiome and discovery of bioactive small molecules is of particular importance due to the emergence of several animal fungal diseases that invade the skin of their hosts, causing morbidity or mortality, such as WNS in bats (Fisher *et al.* 2020). We recognize that environmental conditions of hibernacula may be strong predictors of species impacts (Langwig *et al.* 2016); however, additional mechanisms (e.g. host immunity, host behavior and microbial defense) may play a role in disease susceptibility. Bat microbiota have shown potential to provide host protection against *P. destructans* (Hoyt *et al.* 2015, Hamm *et al.* 2017, Lemieux-Labonté *et al.* 2020), but bacterial augmentation has yet to be commonplace in management of wildlife disease. Foresight from the research of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans*, the invasive fungal pathogens causing massive amphibian die-off, highlights the potential role of skin microbiota in patterns of resistance and susceptibility and potential management practices that might help conserve host populations (Harris *et al.* 2009, Bletz *et al.* 2013, 2017, Woodhams *et al.* 2014, Bataille *et al.* 2016). This study and recent bat microbiome research begin to tease apart the complex interactions of the bat microbiota (Ange-Stark *et al.* 2019; Lemieux-Labonté *et al.* 2020).

Herein, we document the diversity and novelty of KSα sequences isolated from culturable bacteria on bat skin and fur surfaces in the southwestern United States. The results suggest that the bat microbiome might be a fruitful environment for investigating bioactive small molecules, and further that KSα gene homology could serve as a proxy for its total biosynthetic capacity. In doing so, this could allow for a simple PCR screening for isolates' KSα genes, enabling more rapid strain prioritization when applied to other specialized niches. Understanding the bat microbiome in particular could be informative for management and risk assessment of bat populations in terms of infectious diseases such as WNS.

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Supplementary data

Supplementary data are available at *FEMSMC* online.

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