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Antibiotic-producing bacteria isolated from the giant sand scorpion, *Smeringurus mesaensis* (Scorpiones: Vaejovidae)

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Abstract. Antibiotic resistance is a global health crisis. Our current arsenal of antibiotics—drugs meant to kill bacteria and stop their population growth—is becoming less effective at treating bacterial infections as resistant bacteria emerge, fueling the dire need to discover new antibiotics. Most antibiotics in use today have been discovered from bacteria. To increase the chances of finding potentially novel antibiotic molecules, we studied the relatively unexplored microbial environment of scorpion tissues, using the giant sand scorpion, *Smeringurus mesaensis* (Stahnke, 1957). Bacterial symbionts were isolated and cultured from the mesosoma and metasoma, and isolates were tested in a functional assay for production of antibiotics. Under the culture conditions utilized, most scorpion-derived bacteria were from the phyla of Firmicutes, Proteobacteria and Actinobacteria. Fifty-five percent of the clonal isolates tested produced antibiotics, with most being *Bacillus* species. None of the bacterial conditioned media were cytotoxic to mammalian cells. This study suggests scorpion tissues may provide a rich source of antimicrobial molecules to help combat the antibiotic resistance crisis.

Keywords: Arachnid, microbiome, culture

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Symbiotic bacteria are known to play multiple different roles in their hosts. In arthropods, these functions include digestion (Noda et al. 2009), vitamin uptake and synthesis (Salem et al. 2014), immune system maturation (Weiss et al. 2011), reproduction (Duron et al. 2008), and toxin degradation (Mason et al. 2014). Symbionts can also play a protective role. For example, symbiotic bacteria can suppress the growth of other bacteria (Goto et al. 2006), protect against parasitic fungi (Kaltenpoth et al. 2005; Łukasik et al. 2013), parasitoids (Vorburger et al. 2010; Xie et al. 2014), and viruses (Teixeira et al. 2008; Glaser & Meola 2010).

Scorpions represent an ancient arachnid order, with a fossil record extending to the Siluran Period, with the oldest dating to 433–438 Ma (Dunlop & Selden 2013). Found on every continent except Antarctica, scorpions are a resilient lineage that can survive under extreme conditions of heat, drought, and starvation. Little is known about the factors that contribute to their success; however, microorganisms are likely to play a key role. While endosymbionts are known to drive arthropod evolution, the links between scorpions and bacterial symbionts are understudied (Goodacre & Martin 2012). A few studies have detected bacteria in scorpions, with predicted roles regarding metabolic functions and toxin degradation, as well as possible roles in parthenogenesis (Suesdek-Rocha 2006; Bolanos et al. 2016). Further, the importance of endogenous bacteria in scorpions is underscored by evidence of their vertical transmission from parent to offspring and cospeciation between host and endosymbiont (Elmnasri et al. 2018; Bolanos et al. 2019). Most studies addressing scorpion endosymbionts use molecular methods (Shimwell et al. 2023; Bolanos et al. 2016; Elmnasri et al. 2018, and others), which bypass the isolation and culture of bacteria from their host. However, testing cultured clonal isolates can reveal important discoveries. For example, bacteria isolated from the gut and feces of *Heterometrus spinifer* (Ehrenberg, 1828) produced molecules that deterred the growth of various types of cancer cells (Soopramanien et al. 2020). Whether cultured bacteria from other scorpion species and tissues additionally produce clinically relevant compounds, such as antibiotics, is unknown.

The need to discover new antibacterial compounds is critical. According to the World Health Organization (2021), we are presently entangled in a global health crisis of antibiotic resistance – the increasing inability of our current antibiotics to kill common bacterial infections. Every year in the U.S. antibiotic-resistant infections affect more than 2.8 million people causing 35,000 deaths, and many more deaths occur due to related complications (Centers for Disease Control and Prevention 2019). Fueling the shortage of effective antibiotics is the sharp decline in antibiotic discovery, from about 16 new antibacterial agents from 1983–1987 compared to only 2 from 2008–2012 (Infectious Disease Society of America 2011). Antimicrobial molecules can be found in unusual locations, for example, tissues from organisms living in unsanitary conditions (cockroaches, snakes, crocodiles) (Lee et al. 2012; Siddiqui et al. 2022) or molecules found in or based on scorpion venom (Cao et al. 2012; Rincón-Cortés et al. 2022). Since most antibiotics in use today were derived from bacteria (Clardy et al. 2009), and knowing that scorpions are an ancient lineage that likely co-evolved with their endosymbiont bacteria (Bolanos et al. 2019), we aimed to determine whether bacteria cultured from scorpion tissues could produce antibiotics—molecules that would deter the growth of other bacteria. The giant sand scorpion, *Smeringurus mesaensis,* (Stahnke, 1957), was chosen for this study because of their abundance in the region and large size, allowing for ease of manipulation. Using culture-dependent methods, we isolated bacteria from the metasoma and mesosoma of *S. mesaensis*, identified the bacterial isolates with DNA sequencing and used antibiotic-production assays to test clonal isolates for molecules capable of inhibiting the growth of bacteria.

METHODS

Scorpion collection and dissection.—*S. mesaensis* specimens were collected from two locations, the Sonoran Desert ($n = 5$) and Borrego Springs, CA ($n = 8$), during the spring of 2017. Specimens were identified based on morphology, habitat, geographic location, and sequencing of DNA barcodes from individuals from the

sample populations. For arachnid-related studies such as this, no ethics approval is required. The scorpions were held in captivity for 3–5 weeks without feeding. The exoskeleton was cleaned with three wash/rinse cycles of 70% ethanol/sterile water (Bolanos et al. 2016). Scorpions were euthanized by insertion of a needle through the carapace and into the ganglia. Mesosomal and metasomal tissues were harvested using a dissection microscope and sterile tools. Mesosomal segments 1 and 7and metasomal segments 1 and 5 were excised using sterilized equipment to prevent contamination from the surrounding tissues. After removal of the tissues from the exoskeleton, the tissues were triturated and incubated at 37°C for three hours with shaking in 3 ml of nutrient broth (1 g Lab-Lemco powder, 2 g yeast extract, 5 g peptone, 5 g NaCl per liter; pH7.4). To test for bacterial growth, 100 µl aliquots of the nutrient broth from the incubated tissues were plated onto two types of agar plates: Trypticase soy agar (TSA; 15 g pancreatic digest of casein, 5 g papaic digest of soybean, 5 g NaCl, 15 g agar per liter; pH7.3) and LB agar, Miller (10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar per liter). Cycloheximide diluted in dimethyl sulfoxide at a final concentration of 25 µg/ml was added to deter fungal growth. The plates were grown at ambient room temperature, ~ 20°C, until colony growth appeared, typically after 5–9 days. Clonal isolates were frozen and subsequently used for DNA extraction and to test for antibiotic production. No bacterial growth was detected from the negative controls that included nutrient broth without scorpion tissue.

Freezing of bacteria.—Bacteria were frozen either viably at −80°C in growth medium with 25% glycerol or as pellets at −20°C for DNA extractions.

DNA extraction from bacterial isolates.—DNA was extracted using a DNeasy Blood and Tissue (Qiagen) kit using the manufacturers protocol. The DNA was eluted from the column using 100 µL of Qiagen AE buffer.

PCR amplification of 16S rRNA.—We used three different sets of 16S rRNA primers to test for bacteria. All three sets of primers produced similar PCR and DNA sequencing results. The primers used were: 27F 5′-AGAGTTTGATCMTGGCTCAG-3′, 1492R 5′-GGTTACCTTGTTACGACTT-3′; 27FHT 5′-AGRGTTTGATYM TGGCTCAG-3′, 1492RHT 5′-GGYTACCTTGTTACGACTT-3′; 63F 5′-CAGGCCTAACACATGCAAGTC-3′, 1387R 5′-GGGCG GWGTGTACAAGGC-3′. Final concentrations for 25 µl PCR reactions were 10–50 ng/µl DNA template, 0.5 µM of each primer, 200 µM dNTPs, 1.2 units of Taq DNA Polymerase, 20 mM Tri-HCl (pH8.4), 50 mM KCl and 1.5 mM $MgCl_2$. The reaction conditions were 96°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for one minute, and a subsequent final extension at 72°C for 10 minutes. PCR reactions included *E. coli* DNA as a positive control and no template negative controls. Electrophoresis of PCR products (~1.4 kb 16S rRNA gene) was performed on a 1% agarose gel with SYBR-Safe (1:10,000, Invitrogen). Gels were visualized with GelDoc (BioRad) software.

Sequencing and bacterial identification.—PCR products of the correct size were cleaned with ExoSAP-IT (Applied Biosystems) using the manufacturer's protocol. Samples were sequenced at the DNA Resource Core of Dana-Farber/Harvard Cancer Center. Sequencing reactions were performed on an ABI3730xl DNA analyzer. Each sample was sequenced in the forward and reverse directions. Contigs were assembled in Geneious 7.1.9 and edited manually. The resulting consensus sequences were used for bacterial identification of FASTA sequences using BLASTN and the 16S ribosomal RNA sequence database with Megablast to optimize for highly similar sequences. Annotated FASTA sequences for the mesosoma and the metasoma isolates can be found Supplemental File S1 (online at https://doi.org/10.1636/JoA-S-22-039.s1); their numbering is the same as in Supplemental Table S1 (online at https://doi.org/10.1636/JoA-S-22-039.s4). The percentage of bacteria from each phylum was calculated as: (the number of bacterial colonies isolated from a phylum/total number of colonies isolated) x 100%.

Phylogenetic analysis.—To explore relationships among bacterial taxa, we generated a phylogenetic tree using methods similar to Shimwell et al. (2023). We used all 16S rRNA sequences generated in this study combined with sequences from the literature that had been isolated using molecular methods rather than culture (Bolanos et al. 2016, 2019; Elmnasri et al. 2018; Shimwell et al. 2023). The sequences were from various scorpion tissues (gut/digestive tract, gonad, telson) and species: *Hadrurus arizonensis* Ewing, 1928 (Hadruridae), *S. mesaensis*, *Androctonus australis* (Linnaeus, 1758) (Buthidae), *Centruroides limpidus* (Karsch, 1879) (Buthidae), *Vaejovis smithi* Pocock, 1902 (Vaejovidae) and others. Sequences were aligned according to secondary structure with SSU-ALIGN v0.1.1 (Nawrocki 2009). Using the program, we masked columns in which nucleotides were assigned with low confidence. The alignments were used to generate a Maximum Likelihood (ML) phylogeny with iQtree version 1.6.6 (Nguyen et al. 2015), implementing ModelFinder (Kalyaanamoorthy et al. 2017) to determine best-fit substitution models (we used TIM3+I+G4, as suggested by the software), and ultrafast bootstrap resampling to gauge nodal support (Minh et al. 2013). The consensus phylogeny was visualized in FigTree v. 1.4.4 (online at http://tree.bio.ed.ac.uk/software/) and annotated in Adobe Illustrator.

Antibiotic production tests.—Clonal isolates were recovered from −80°C storage and grown at 37°C in LB broth, Lennox (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter; pH7.0) overnight with shaking. The clonal suspensions were patched onto LB and TSA plates and allowed to grow for 1–3 days until well-established. To test for antibiotic production, different bacterial strains were overlaid and we tested for the ability of the underlying scorpion-derived bacteria to deter the growth of the bacteria in the overlay. Overlays consisted of a diluted top agar made with nutrient broth plus 7.5 g agar (per liter). Overlays were inoculated with 50 µL of overnight bacterial culture from the tester strain prior to spreading ~ 7 ml atop the plate with the scorpion-derived bacterial patches. The overlays were made with the following bacterial tester strains: *Escherichia coli* (Migula) Castellani and Chalmers, (ATCC 11775), *Acinetobacter baylyi* (ATCC 33305), *Pseudomonas putida*, and *Klebsiella aerogenes* (both Presque Isle Cultures). These strains were chosen because they represent safe relatives of ESKAPE pathogens – the leading cause of nosocomial infections, most of which are multidrug-resistant (Santajit & Indrawattana 2016). Overlaid cultures were grown at 37°C for two days, at which time they were scored for the presence or absence of zones of growth inhibition. Zones of inhibition would suggest that the underlying bacteria were producing antibiotics, and thus deterring the regional growth of the overlaid tester bacteria. All bacterial isolates obtained from scorpion tissues were tested for antibiotic production on two separate occasions; evidence of a zone of inhibition in one or two out of the two assays was considered positive for antibiotic production.

Preparing bacterial conditioned medium.—DMEM (Dulbecco's modified Eagle's medium) was inoculated with single colonies of scorpion-derived bacteria and grown with shaking at 200 rpm for

24 hours at 37°C. DMEM without bacteria provided a negative control. *E. coli* (ATCC 11775) and *B. subtilis* (Presque Isle Cultures) served as non-pathogenic, non-scorpion bacterial controls to indicate background levels of cell death in cytotoxicity assays. The cultures were spun at 2,000 g for 10 minutes at 4°C to pellet the cells, prior to sterile filtration using 0.22 µm syringe filters. The Bradford assay (BioRad) was used to quantitate the protein in each conditioned medium. The resulting conditioned media were stored at −80°C until needed.

Cell culture and cytotoxicity assays.—Mouse BV2 cells (microglia; Accegen Biotechnology) and human 293T cells (embryonic kidney; ATCC CRL-3216) were maintained at 37°C with 5% CO_2 in DMEM media containing 10% FBS (fetal bovine serum). To assess cytotoxicity of scorpion bacteria conditioned media, cells were plated in a 24-well plate at a concentration of 10,000 cells per well and incubated for 1 hour to allow the cells to adhere to the plate. Conditioned media (10 µg/ml of protein) was added to the cells and incubated for 24 hours at 37°C with 5% CO_2. All cells in each well were then labeled using Hoechst 33342 (2 mg/mL, Fisher Scientific), while dead cells were labeled using propidium iodide (2.5 mg/mL, Fisher Scientific). Fluorescent images were obtained using a CKX41 inverted microscope (Olympus) and a SPOT idea camera with Spot 5.2 Software (SPOT). Images were taken using the same exposure settings. Area of fluorescent staining in each image was quantified using a fixed threshold in ImageJ 1.53t (NIH) (Schneider et al. 2012). Cell viability was determined by dividing the total area of propidium iodide staining (dead cells) by the total area of Hoechst staining (all cells). Prism 5 (GraphPad) was used to create the graphs and perform statistical analyses.

Statistical analysis.—Chi-squared tests were used to determine whether there were significant differences between mesosoma and metasoma for bacteria that were common to each tissue, and to compare the antibiotic production from bacteria isolated from the mesosoma and metasoma. For the cytotoxicity assays, all treatments were compared to the negative control using a one-way ANOVA with Dunnett's multiple comparisons test. Values are presented as the mean and standard error of the mean.

RESULTS

Isolation and culture of bacteria from scorpion tissue.—To better understand the microbiome of scorpions, we isolated and cultured bacteria from different tissues of the giant sand scorpion, *S. mesaensis* (Fig. 1A). Initial efforts attempted to grow bacteria from tissue pieces from the mesosoma and metasoma cultured directly on Lysogeny Broth (LB) or Tryptic Soy (TS) agar plates, however, this method did not produce any bacterial growth ($n = 4$ scorpions). Successful cultures were achieved after the scorpion tissues were triturated and incubated in liquid culture prior to plating (Fig. 1B). No colony growth was detected on the negative controls that contained medium incubated without the addition of scorpion tissues (Fig. 1C). Cultures of the negative controls and scorpion tissues were performed at the same time. Of nine scorpions, four yielded three or fewer bacterial colonies; the remaining five produced 5 to 47 bacterial colonies (Fig. 1D). In total, we grew 102 colonies, 52 from the mesosoma and 50 from the metasoma (Fig. 1E). Approximately half of all colonies were grown on either LB or TS agar, indicating no obvious preference for culture medium.

DNA sequencing identifies the bacteria isolated from scorpions.—To identify the strains of bacteria isolated from scorpion tissues, DNA from thawed and regrown bacterial clones underwent PCR to detect the 16S rRNA gene. After freeze/thaw, we recovered 87/102 clones (85%) and sent 80 isolates representing 8 scorpions for sequencing. The identified sequences represented 12 different bacterial strains from 3 phyla (Table 1). The most highly represented phylum was Firmicutes (87.5%), followed by Proteobacteria (10%) and Actinobacteria (2.5%). Of the bacterial genera, *Enterococcus* (61.2%), *Bacillus* (23.6%) and *Stenotrophomonas* (7.5%) were the most highly represented (Table 1). The diversity of bacteria within any single scorpion was difficult to gauge, given that 4/8 scorpions produced only 1–3 colonies. However, of the two most highly represented genera, *Enterococcus* and *Bacillus*, *Enterococcus* was detected in 3 scorpions, and one strain of *Bacillus* was detected in 4 scorpions, while an additional 6 strains of *Bacillus* were each found in one scorpion (Table 1).

Having determined the overall diversity of the bacterial isolates, we asked if the types and abundance of bacteria differed between the mesosoma ($n = 44$) and the metasoma ($n = 36$). There was a common set of bacteria, found in both mesosomal and metasomal tissue, that included (in order of abundance) *Enterococcus*, *Bacillus* and *Stenotrophomonas* (Figs. 2A, B). However, the abundance of these bacteria differed between tissues. The mesosoma compared to the metasoma yielded a significantly different number of *Enterococcus* (33 vs. 16, $\chi^2 = 7.8$, $P < 0.005$) and *Bacillus* (6 vs. 13, $\chi^2 = 5.5$, $P < 0.01$), and a similar number of *Stenotrophomonas* (3 vs. 3, $\chi^2 = 0.06$, $P > 0.7$; Fig. 2A, B). The remaining bacteria were unique to each tissue, with the mesosoma containing 2% each of *Streptomyces* and *Kocuria* (Fig. 2A), whereas the metasoma contained 5.5% each of *Variovorax* and *Staphlococcus* (Fig. 2B). These results suggest that the mesosoma and metasoma contain both a common set and unique set of bacteria, with varying abundance.

Phylogenetic analysis identifies related scorpion bacterial sequences.—A phylogeny (see Supplemental Files S2, S3, online at https://doi.org/10.1636/JoA-S-22-039.s2 and https://doi.org/10.1636/JoA-S-22-039.s3) was constructed to determine the relatedness among the sequences isolated in this study compared to studies that surveyed scorpion gut, gonad and telson tissue using molecular methods (Bolanos et al. 2016, 2019; Elmnasri et al. 2018; Shimwell et al. 2023). The comparison samples included a variety of scorpion species including *A. australis* (Elmnasri et al. 2018), *C. limpidus, V. smithi* (Bolanos et al. 2016), *S. mesaensis*, and *H. arizonensis* (Shimwell et al. 2023), and others. There were 3 large clades whose taxa included *Enterococcus faecalis*, *Bacillus* species, and *Stenotrophomonas maltophilia*. The single clade of *E. faecalis* consisted of 49 sequences plus a single sequence from the gonad of *A. australis* (Elmnasri et al. 2018). The *Bacillus* clade included 12 sequences, all from this study, of which surprisingly 8 were from *Bacillus mojavensis*. The *S. maltophilia* clade included 6 sequences from this study plus 3 sequences derived from tissues of *C. limpidus* and *V. smithi* (Bolanos et al., 2016). There were 4 pairs of sequences representing *Bacillus* species (2 pair), *Variovorax* species, and *Staphlococcus epidermis*. Five samples did not group with other sequences, indicating low similarity to the collection of sequences in our phylogenetic analysis. These results indicate that many of our cultured scorpion bacteria form their own clades, while some clades included additional sequences that were isolated from scorpions in separate studies.

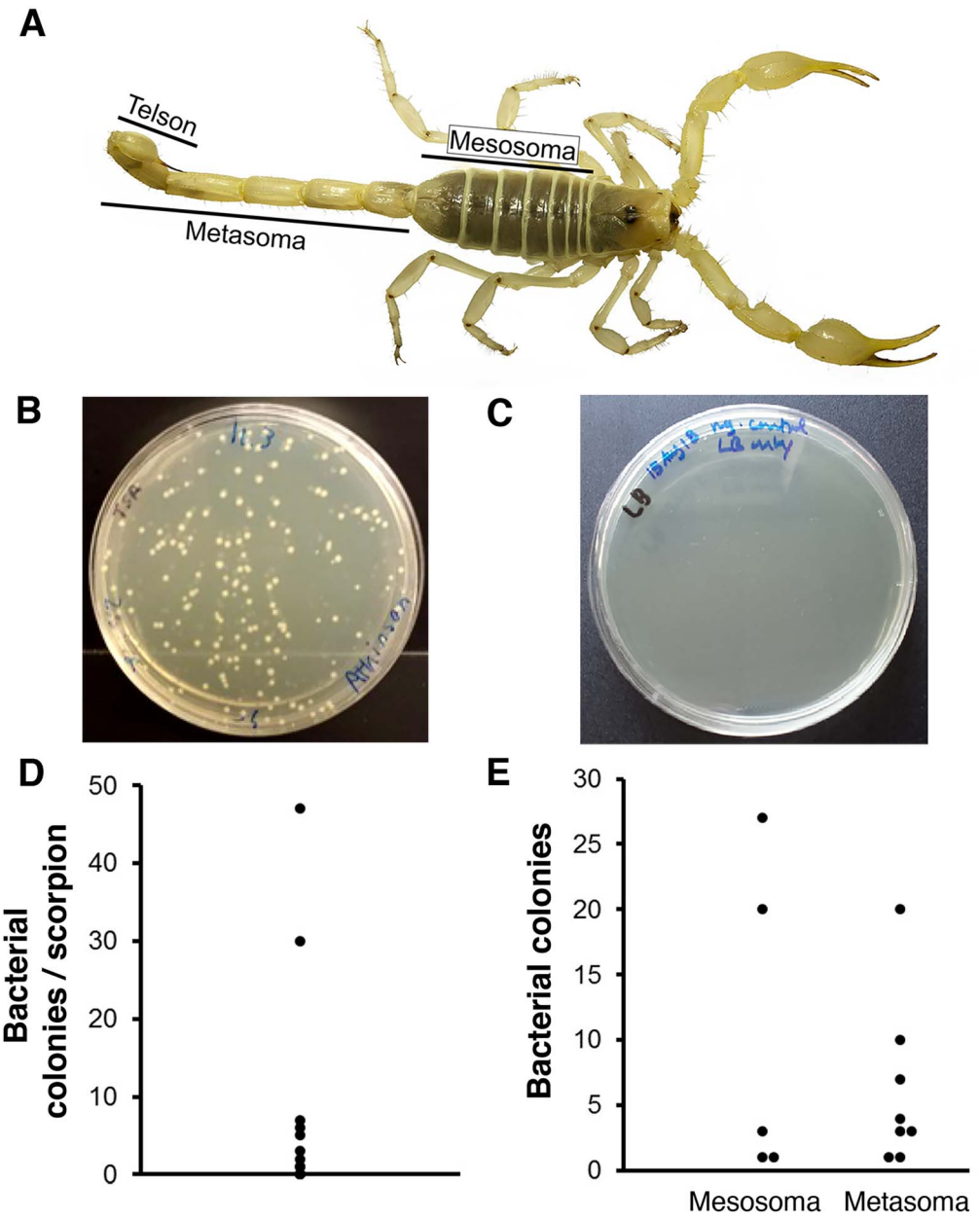

Figure 1.—Culture of bacteria from *S. mesaensis* tissues. (A) Mesosoma and metasoma tissues were isolated and cultured from the giant sand scorpion, *S. mesaensis*. Colonies grew from scorpion tissues (B), but not from media alone, the negative control (C). The plate in (B) represents a subculture from a single bacterial clone. The number of bacterial colonies grown from (D) each scorpion ($n = 102$ colonies) and (E) each tissue (mesosoma $n = 52$, metasoma $n = 50$ colonies). Each dot in (D, E) represents a single scorpion.

We used the phylogenetic tree as a basis to find closely related sequences for comparison of percent identity using BLAST. Table 2 shows the taxa, clone number and query sequences from this study (as seen in Supplemental File S2), in addition to the subject sequence accession numbers, percent identities (both from Genbank), and citations for the related studies. From our cultured data, 19 sequences had matching scorpion bacterial sequences with percent identities of >97%, a cutoff level suggestive of similar species. We found that 6 of our *Stenotrophomonas* clones matched KM978218, KM978283, KM978322 (98.29–99.57%), 2 *Staphlococcus* clones matched KM978310 (97.69–98.66%) (Bolanos et al. 2016); 4 *Enterococcus* clones matched MG733111 (97.9–98.13%) (Elmnasri et al. 2018); 4 *Bacillus* clones matched OP050295, OP050308 (98.44–100%), 1 *Bacillus* clone matched OP050052 (97.22%), 1 *Kocuria* clone matched OP050310 (97.1%) (Shimwell et al. 2023). These results indicate similarity between approximately 25% of the sequences isolated in this study to sequences found in three previous microbiome studies that covered a wider range of scorpion species.

Testing for antibiotic production from bacterial isolates.—Having successfully cultured bacteria from *S. mesaensis* tissues, we used antibiotic production assays and scoring for zones of inhibition to determine whether the scorpion-derived bacteria could deter the growth of other bacteria (Fig. 3A). The tests used isolates from 8

Table 1.—Summary of bacterial strains cultured from *S. mesaensis* tissues. The percentage of bacteria identified was calculated by taking the [(number of colonies from a bacterial strain/total bacterial colonies sequenced) x 100%].

Phyla	Bacterial strain	# bacteria identified (total = 80)	% of all bacteria identified	# scorpions with strain ($n = 8$)
Firmicutes	*Enterococcus faecalis*	49	61.2%	3
(87.5%; $n = 70$)	*Bacillus mojavensis*	8	10.0%	4
	Bacillus subtilis	4	5.0%	1
	Bacillus licheniformis	2	2.5%	1
	Bacillus paramycoides	2	2.5%	1
	Bacillus cereus	1	1.2%	1
	Bacillus foraminis	1	1.2%	1
	Bacillus spp.	1	1.2%	1
	Staphylococcus epidermidis	2	2.5%	2
Proteobacteria	*Stenotrophomonas maltophilia*	6	7.5%	1
(10%; $n = 8$)	*Variovorax* spp.	2	2.5%	1
Actinobacteria	*Streptomyces* spp.	1	1.2%	1
(2.5%; $n = 2$)	*Kocuria rhizophila*	1	1.2%	1

scorpions and included 10 different bacterial strains. We found that 11/20 (55%) bacterial clones tested were capable of producing antibiotics. Most antibiotic production came from the *Bacillus* genus (10/11 positive clones), with one antibiotic-producing clone from *Variovorax* (Table 3, Fig. 3A). Six isolates inhibited 1–2 tester strains, the remaining 5/11 inhibited 3 to 4 of the tester strains (Table 3), indicating a broad spectrum of inhibition by the scorpion bacteria. When tested against the four bacterial tester strains, no

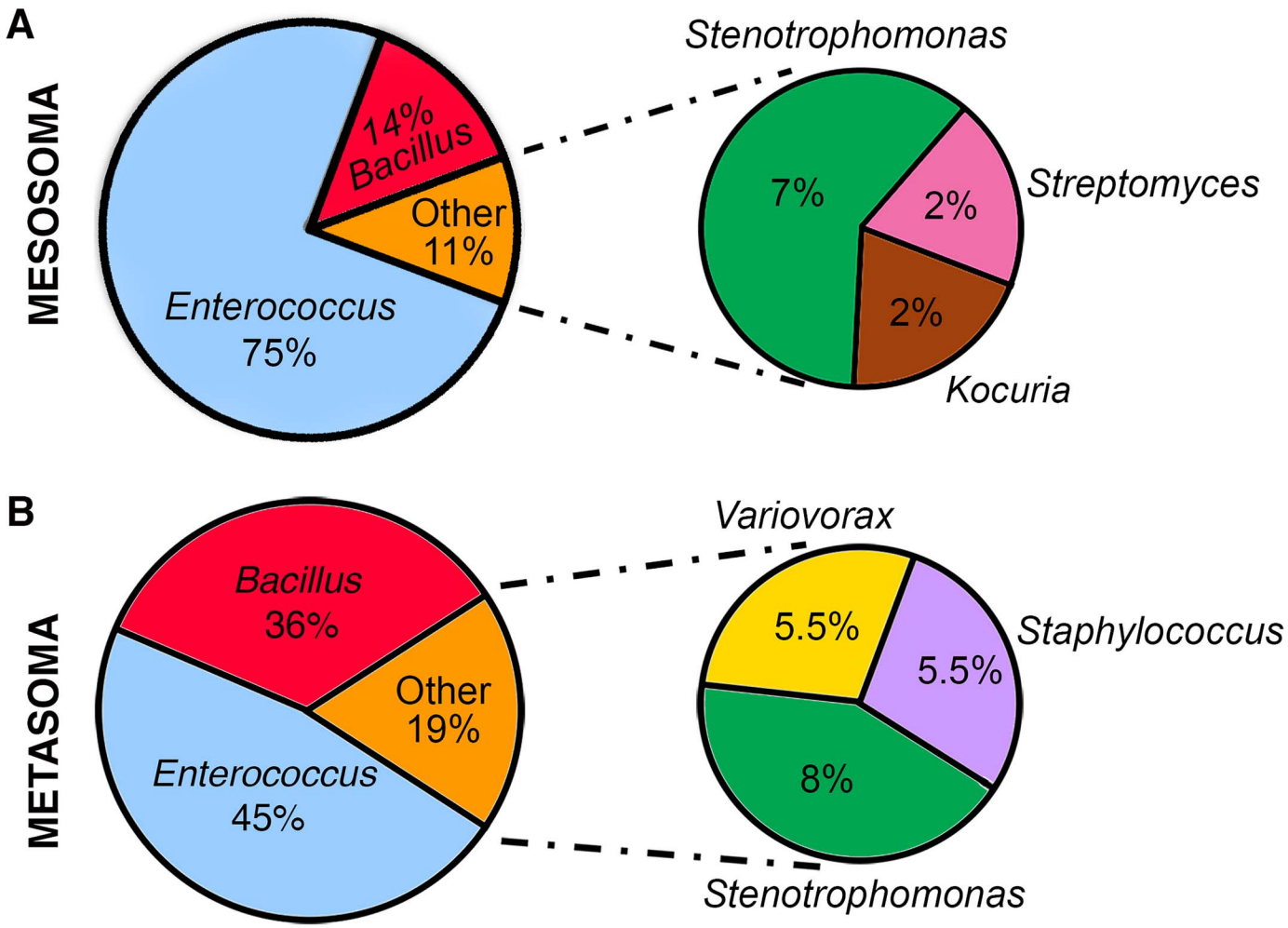

Figure 2.—The abundance and types of bacteria cultured from the scorpion mesosoma and metasoma. (A) Mesosoma ($n = 44$ clones) and (B) Metasoma ($n = 36$ clones). DNA sequencing combined with BLAST was used for bacterial identification.

Table 2.—Comparison of percent identity of bacterial sequences from this study with related scorpion bacterial sequences. The percent identity of closely related sequences as determined by phylogenetic analysis were compared using BLAST. Only sequences with greater than 97% identity are shown. Clone # - sequences from this study; Query sequence - sequences from this study as labeled in the phylogenetic tree (Supplemental File S2); Accession # - identifies related sequences from Genbank; Reference - indicates the study from which the accession numbers originated.

Taxa	Clone #	Query sequence	Accession #	% ID	Reference
Bacillus	2	#2_21M_Bacillus_subtilis_99.9	OP050295	100	Shimwell, 2023
Bacillus	36	#36_49Mt_Bacillus_licheniformis_99.5	OP050295	100	Shimwell, 2023
Bacillus	2	#2_21M_Bacillus_subtilis_99.9	OP050308	99.74	Shimwell, 2023
Stenotrophomonas	3	#3_21M_D_Stenotrophomonas_maltophilia_99.3	KM978218	99.57	Bolanos, 2016
Stenotrophomonas	50	#50_23Tx_Stenotrophomona_maltophilia_99.2	KM978322	99.57	Bolanos, 2016
Stenotrophomonas	50	#50_23Tx_Stenotrophomona_maltophilia_99.2	KM978218	99.57	Bolanos, 2016
Stenotrophomonas	3	#3_21M_D_Stenotrophomonas_maltophilia_99.3	KM978322	99.5	Bolanos, 2016
Stenotrophomonas	1	#1_21Mx_Stenotrophomonas_maltophilia_98.9	KM978322	99.24	Bolanos, 2016
Stenotrophomonas	1	#1_21Mx_Stenotrophomonas_maltophilia_98.9	KM978218	99.24	Bolanos, 2016
Stenotrophomonas	51	#51_28952-7_24T_Stenotrophomonas_maltophilia_99.3	KM978322	99.22	Bolanos, 2016
Stenotrophomonas	51	#51_28952-7_24T_Stenotrophomonas_maltophilia_99.3	KM978283	99.22	Bolanos, 2016
Stenotrophomonas	51	#51_28952-7_24T_Stenotrophomonas_maltophilia_99.3	KM978218	99.22	Bolanos, 2016
Stenotrophomonas	3	#3_21M_D_Stenotrophomonas_maltophilia_99.3	KM978283	99.21	Bolanos, 2016
Stenotrophomonas	50	#50_23Tx_Stenotrophomona_maltophilia_99.2	KM978283	99.14	Bolanos, 2016
Bacillus	74	#74_72Tt_Bacillus_mojavensis_99.7	OP050308	98.99	Shimwell, 2023
Bacillus	75	#75_101T_Bacillus_mojavensis_99.8	OP050308	98.99	Shimwell, 2023
Bacillus	36	#36_49Mt_Bacillus_licheniformis_99.5	OP050308	98.96	Shimwell, 2023
Stenotrophomonas	1	#1_21Mx_Stenotrophomonas_maltophilia_98.9	KM978283	98.8	Bolanos, 2016
Staphlococcus	71	71_28952-2_53T_Staphlococcus_epidermis_99.0	KM978310	98.66	Bolanos, 2016
Bacillus	74	#74_72Tt_Bacillus_mojavensis_99.7	OP050295	98.46	Shimwell, 2023
Bacillus	75	#75_101T_Bacillus_mojavensis_99.8	OP050295	98.44	Shimwell, 2023
Stenotrophomonas	6	#6_28952-13_23M_Stenotrophomonas_maltophilia_96.8	KM978322	98.3	Bolanos, 2016
Stenotrophomonas	6	#6_28952-13_23M_Stenotrophomonas_maltophilia_96.8	KM978283	98.3	Bolanos, 2016
Stenotrophomonas	6	#6_28952-13_23M_Stenotrophomonas_maltophilia_96.8	KM978218	98.3	Bolanos, 2016
Stenotrophomonas	47	#47_28952-1_22TT_Stenotrophomonas_maltophilia_98.3	KM978322	98.29	Bolanos, 2016
Stenotrophomonas	47	#47_28952-1_22TT_Stenotrophomonas_maltophilia_98.3	KM978218	98.29	Bolanos, 2016
Stenotrophomonas	47	#47_28952-1_22TT_Stenotrophomonas_maltophilia_98.3	KM978283	98.2	Bolanos, 2016
Enterococcus	17	#17_41M_Enterococcus_faecalis_100	MG733111	98.13	Elmnasri, 2018
Enterococcus	21	#21_43M_ Enterococcus_faecalis_100	MG733111	98.13	Elmnasri, 2018
Enterococcus	27	#27_45M_ Enterococcus_faecalis_100	MG733111	98.09	Elmnasri, 2018
Enterococcus	23	#23_28952-17_43ML_ Enterococcus_faecalis_99.6	MG733111	97.9	Elmnasri, 2018
Staphlococcus	48	48_28952-12_22TL_Staphlococcus_epidermis_98.1	KM978310	97.69	Bolanos, 2016
Bacillus	79	#79_112T_Bacillus_paramycoides_99.1	OP050052	97.25	Shimwell, 2023
Variovorax	46	#46_21Tx_Variovorax_spp_99.6	OP050009	97.22	Shimwell, 2023
Kocuria	5	#5_28952-8_22M_Kocuria_rhizophila_99.7	OP050310	97.1	Shimwell, 2023

antibiotic production was detected from *E. faecalis*, *Kocuria rhizophila*, *S. maltophilia*, or *Streptomyces* (n = 1–4 isolates tested per strain). *Bacillus foraminis* was not tested for antibiotic production. The antibiotic-producing bacteria were more highly detected from the metasoma compared to the mesosoma. Seventy-five percent of the metasoma bacteria (9/12 tested) compared to 25% (2/8 tested) of the mesosoma bacteria produced antibiotics ($\chi^2 = 4.8$, $P < 0.02$). These results suggest a skewed anatomical distribution of antibiotic-producing bacteria in the scorpions under the conditions used in this study.

Conditioned media from scorpion bacteria does not affect the viability of mammalian cells.—We used conditioned media prepared from antibiotic-producing scorpion bacteria to determine the effects on cell viability (Figs. 4A–C). Cell viability was assessed by staining for dead cells with propidium iodide (Fig. 4C, red) compared to total cells stained with Hoechst (Fig. 4C, blue). When compared to the negative controls, none of the 11 conditioned media tested showed significant cytotoxicity towards either mouse BV2 microglia (0–4.8% cell death; Fig. 4A) or human 293T embryonic kidney cells (0.2–8.6% cell death; Fig. 4B). The negative controls included media conditioned either without bacteria or conditioned by non-pathogenic bacteria that were commercially purchased (*E. coli*: 4.2% and 0.6% cell death for BV2 and 293T cells, respectively, and *B. subtilis*: 4.7% and 3.9% cell death for BV2 and 293T cells, respectively; Fig. 4A, B). In contrast, significant cell death was detected in the positive control, staurosporine-treated cells—55.6% for BV2 cells ($P < 0.0001$) and 53.8% for 293T cells ($P < 0.0001$). Additionally, the staurosporine treatment showed an altered rounded cell morphology indicative of cell death, whereas scorpion conditioned media and the negative controls looked flat and adherent, demonstrating a normal phenotype for each cell line (Fig. 4C). These results indicate that the scorpion bacteria conditioned media does not affect the viability of mammalian cells.

DISCUSSION

The global crisis of antibiotic resistance with its potential for loss of life and vast socioeconomic impact, dictates the urgent need for the discovery of new antibiotics. Most antibiotics in use today have been discovered in bacteria. To increase the chances

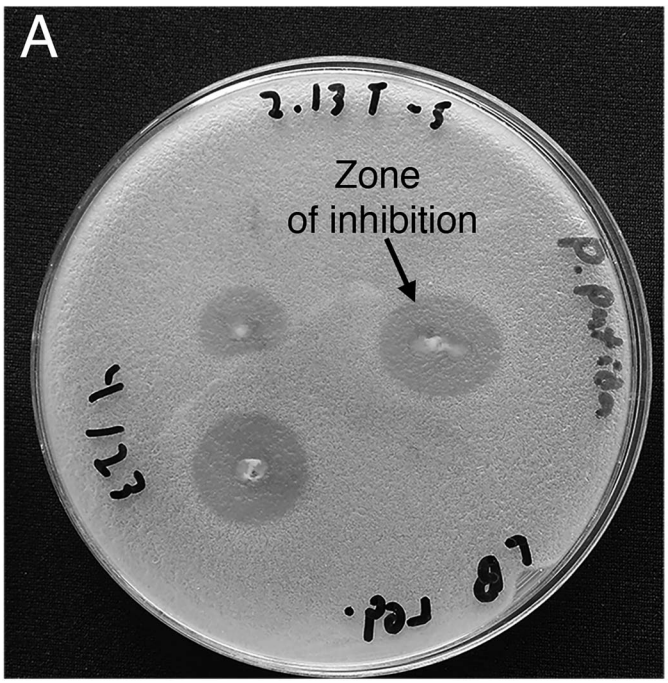

Figure 3.—Example of an antibiotic production assay and zones of inhibition. (A) Scorpion-derived bacteria, *Variovorax* spp., from the metasoma that produce antibiotics, are surrounded by a clearing, termed a zone of inhibition (arrow), where the bacterial tester strain, *Pseudomonas putida,* cannot grow due to its sensitivity to the antibiotic produced. Except for the zones of inhibition surrounding the three patched regions of the scorpion-derived bacteria, growth of the tester strain is seen over most of the plate as an opaque lawn.

of finding potentially novel antibiotic molecules, we chose to study the relatively unexplored environment of scorpion tissues. By tissue region, we found a common set of bacteria, combined with bacteria unique to each body part. Antibiotic-producers were significantly more prominent in the metasoma than mesosoma and most were of the *Bacillus* genus. Our study shows for the first time, the ability of scorpion-derived bacteria to produce antimicrobial compounds.

Because bacteria can be found everywhere, the potential for contamination is of great concern and associated with every study involving bacteria. We had several lines of evidence to support the fitness of our aseptic technique. For example, our first cultures with scorpion tissue pieces lacked bacterial growth; our cultures initiated with triturated scorpion tissue grew bacteria whereas the negative controls did not; many of our sequences had high percentage matches to sequences from other scorpion microbiome studies (Table 2) (Bolanos et al. 2016; Elmnasri et al. 2018; Shimwell et al. 2023), and these data were supported by our phylogenetic analysis (Supplemental Files S2, S3). These results are inconsistent with contamination, but rather support that our bacterial sequences arise from the culture of scorpion tissues.

Since most bacteria remain uncultured, the bulk of knowledge regarding bacterial diversity has been revealed using molecular profiling (Steen et al. 2019). It is estimated that less than 2% of existing bacteria can be cultured in a laboratory setting, as the conditions for their growth are simply unknown (Steen et al. 2019). One limitation of culture-dependent methods is their narrow representation of bacterial taxa. However, functional assays that test for the production of antibiotics or other useful compounds typically require the isolation and culture of clonal bacterial isolates. Our overall aim was not to evaluate the vast diversity of the scorpion bacterial community, but rather to determine if the bacteria that we successfully cultured differed by anatomical region and if they were capable of antibiotic production. Since culture conditions can introduce bias towards the growth of some microbes while excluding others (Overmann et al. 2017), our experiments tested the scorpion tissues side-by-side using the same culture conditions to minimize any bias. Changes to the types and abundance of bacteria cultured from scorpions could arise by using different growth conditions, for example by changing the medium, temperature, time of culture, etc. (Pold et al. 2016). Under the culture conditions utilized, the most highly represented phyla were Firmicutes, Proteobacteria, and Actinobacteria. Our results are consistent with the literature given that these phyla are amongst those taxa most likely to be successfully cultured,

Table 3.—Antibiotic production by bacteria cultured from *S. mesaensis* tissues. Each bacterial clone represents an individual and unique isolate; 11 of 20 clones tested showed antibiotic production. Only antibiotic producers are shown. A "+" indicates that the sample produced a zone of inhibition; "-" indicates no inhibited growth zone of the tester strain. The scorpion number indicates individual scorpions, the sequence number identifies the sequence as found in Supplemental Table S1.

			Bacterial Tester Strains			
			Gram negative relatives		Gram positive relatives	
Scorpion #, sequence #	Anatomical region of isolate	Species of bacterial clone	*A. baylii*	*P. putida*	*K. aerogenes*	*S. epidermidis*
2, 45	metasoma	*Variovorax* spp.	+	+	+	-
7, 73	metasoma	*B. cereus*	+	+	-	+
11, 77	metasoma	*B. paramycoides*	+	+	-	-
11, 76	metasoma	*Bacillus* spp.	+	+	-	-
2, 53	metasoma	*B. subtilis*	+	-	+	-
10, 75	metasoma	*B. mojavensis*	-	+	-	-
7, 74	metasoma	*B. mojavensis*	+	+	+	+
7, 72	metasoma	*B. mojavensis*	+	+	-	-
13, 80	metasoma	*B. mojavensis*	+	+	+	+
4, 36	mesosoma	*B. licheniformis*	-	+	-	-
7, 44	mesosoma	*B. mojavensis*	+	+	-	+

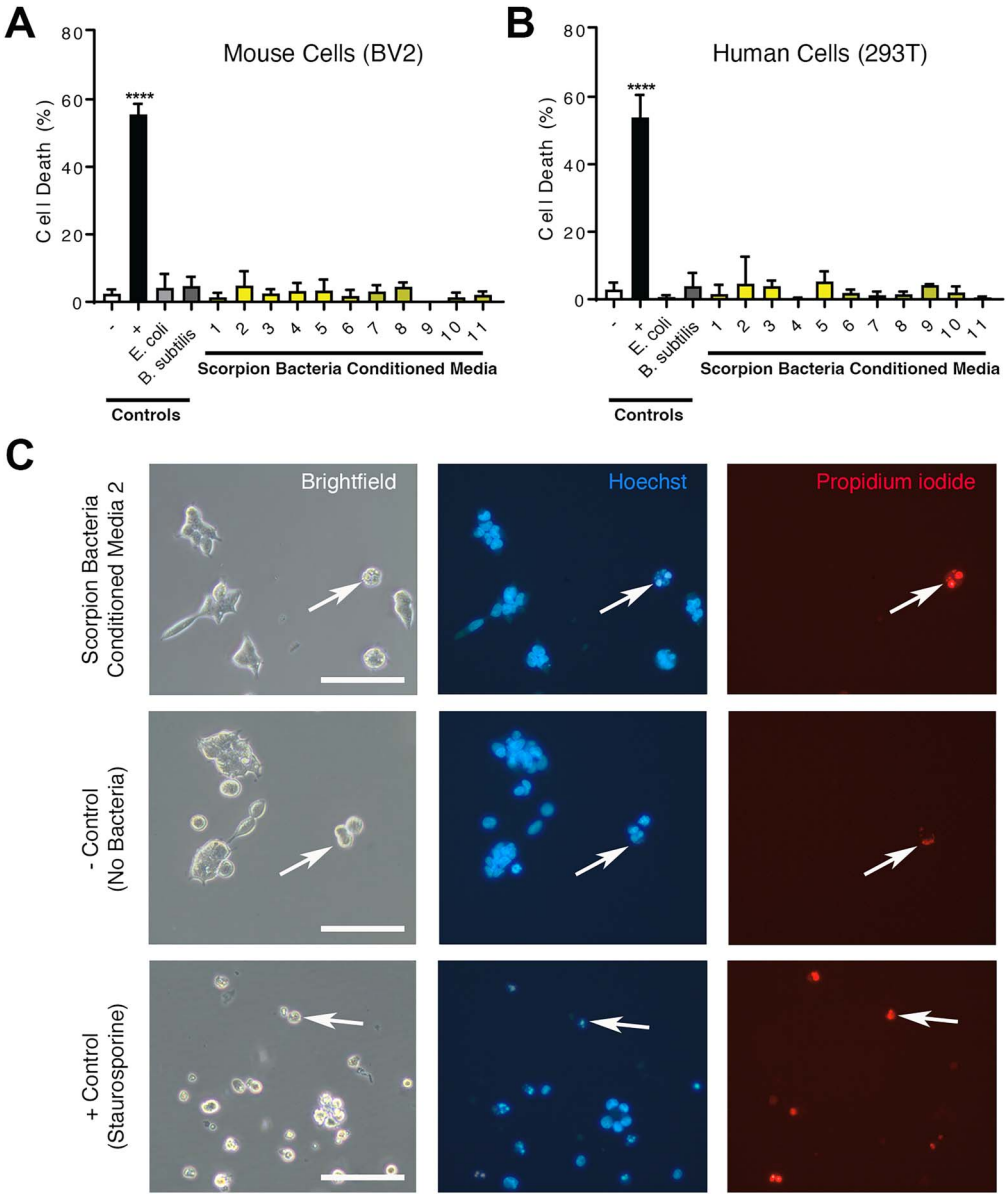

Figure 4.—Conditioned media from scorpion bacteria does not affect the viability of mammalian cells. (A) Mouse BV2 cells and (B) human 293T cells were subjected to conditioned media from scorpion bacteria or commercially available bacteria (*E. coli*, *B. subtilis*) for 24 hours. A cohort of bacteria were isolated using LB broth (Samples 1–5), while another cohort was isolated using TSA broth (Samples 6–11). The percentage of cells that were dead following treatment with conditioned media was then assessed microscopically by dividing the relative number of dead cells (as stained by propidium iodide) by the relative number of all cells (as stained by Hoechst). Staurosporine was used as a positive control for cell death (+) while conditioned media without bacteria was used as a negative control (-). Controls are represented by white, gray and black bars, conditioned media samples are represented by yellow shaded bars. (C) Representative images are shown for 293T cells treated with conditioned media (with or without bacteria) or staurosporine. Arrows indicate examples of dead cells. Scale bar, 100 μm. Results were compared using a one-way ANOVA with a Dunnett's multiple comparisons test where all treatments were compared to the negative control treatment. Values are mean ± SEM ($n = 3$ per group). ****$P < 0.0001$.

either from the environment or from other organisms (Schloss & Handelsman 2004). Most of the bacterial taxa that we identified via culture have been previously detected in scorpion tissues using molecular methods by us (Shimwell et al. 2023) and others (Bolanos et al. 2016; Elmnasri et al. 2018). Indeed 19 of our sequences showed >97% percent identity with sequences from these three prior studies that represented various bacterial taxa and scorpion species (Table 2). Some bacterial isolates have also been cultured from either scorpion guts or feces (Soopramanien et al. 2020), or from cockroach tissue (Akbar et al. 2018).

In their hosts, symbionts play many different roles, however, the links between scorpion and bacterial symbionts is relatively unknown. The importance of scorpion endosymbionts is highlighted by their transmission from parent to offspring and cospeciation between host and symbiont (Elmnasri et al. 2018; Bolanos et al. 2019). In this study we refer to symbionts in the broadest context to simply mean bacteria isolated from our scorpion samples. The isolated 16S rRNA gene sequences showed high identities (>97%) to known bacteria in Genbank, indicating that the bacteria isolated using the current culture conditions were not necessarily unique to

scorpions. These results suggest bacterial acquisition through ingestion of prey. This contrasts with previous studies using molecular rather than culture-based methods (Bolanos et al. 2016, 2019; Elmnasri et al. 2018; Shimwell et al. 2023), that showed unique bacterial sequences from scorpions, based on BLAST results, identities below 97%, long branches and clustering of sequences from scorpions in phylogenetic trees. As aforementioned, changing the culture conditions may result in the discovery of novel culturable bacteria from scorpion tissues.

Natural products provide a promising approach for the discovery of new compounds to combat many medical conditions including antimicrobial resistance, as they may reveal unique structures and functions that can provide new therapies and a rational approach to produce synthetic therapeutic molecules. The evolution of scorpions and their molecules over hundreds of millions of years suggests that they could be a specialized source of natural therapeutic compounds. Drug discovery inspired by natural compounds from scorpion tissues typically focuses on their venom, and includes molecular shuttles designed to carry drugs across the otherwise impenetrable blood brain barrier (Diaz-Perlas et al. 2018), a fluorescent imaging agent that binds specifically to cancerous pediatric brain cells and other solid tumor cancers (Soroceanu et al. 1998; Lyons et al. 2002), and a venom-derived peptide to deliver anti-inflammatory steroids specifically to arthritic joints, rather than systemically, to reverse cartilage inflammation (Cook Sangar et al. 2020). Each of these therapeutics is based on the molecular characteristics of chlorotoxin, a peptide derived from *Leiurus quinquestriatus* (Ehrenberg, 1828) (Buthidae); reviewed in Cohen et al. 2018). Further, scorpion venom includes peptides that can inhibit the growth of multidrug-resistant bacteria (Hernandez-Aponte et al. 2011), in addition to pathogenic fungi, viruses and parasites (reviewed in Rincón-Cortés et al. 2022).

Beyond their venom, scorpions likely house symbionts that produce molecules of interest. For example, microbes represent a vital resource for potentially useful antibiotics and other therapeutic natural products (Challinor & Bode 2015). Here we cultured bacteria from scorpion tissues; using functional assays we found many to have antimicrobial characteristics against both Gram-positive and Gram-negative bacteria. The metasoma compared to the mesosoma contained more antibiotic producers, which may be linked to the ecological role of antibiotics in nature (see below). Conditioned media from the scorpion-derived bacteria were not toxic to mammalian cells (Fig. 4) indicating the potential for clinical use in humans. Most antibiotic producers were of the *Bacillus* genus. *Bacillus* have a wide distribution including the gut microbiota of arthropods, can be readily grown in culture, and are well known for the production of antimicrobial compounds (Nicholson 2002). The identity of the antimicrobial compounds detected in this study are unknown, but likely represent small peptides or metabolites, as was found for molecules produced from the gut bacteria of cockroaches (Akbar et al. 2018). Whether the genetic divergence between the scorpion-derived *Bacillus* compared to other *Bacillus* isolates found in Genbank is enough to yield unique antibiotic compounds that have not been discovered elsewhere remains to be determined. Interestingly, scorpion-derived bacteria have yielded additional natural products with vast clinical potential, including molecules with anti-cancer properties (Soopramanien et al. 2020). It would be interesting in future studies to isolate specific bacterial metabolites detected in this study, to identify and test them for antimicrobial activity and additional biochemical properties of clinical relevance.

While we detected antibiotic production in a lab environment, this unnatural setting does not imply the production or function of antibiotics in nature. Although antibiotic effects in microbial environments are not well understood, a common viewpoint is that antibiotics deter the growth of other bacteria due to competition for resources. This perspective views antibiotics as chemical weapons and in the present study would predict more limited resources in the metasoma, where most antibiotic producers were found, than in the mesosoma. Alternatively, since antibiotics can act as signaling molecules when present at much lower levels (as low as 1%) than those required for growth inhibition of other organisms (Goh et al. 2002; Yim et al. 2007; Clardy et al. 2009), the resulting change in gene expression may select for community members that could live cooperatively and thrive in this privileged microenvironment. Hence antibiotics would facilitate a community-level screening effect via cell-to-cell communication (Davies 2006; Linares et al. 2006; Fajardo & Martínez 2008). This mechanism could operate in both the metasoma and mesosoma tissues.

To the best of our knowledge, this study testing bacteria isolated from scorpion tissues for antibacterial activity is novel and serves as a launch point for future studies. The high rate of success in isolating antibiotic-producing bacteria from scorpions (55% of clones tested), together with the non-toxicity of bacterial metabolites on mammalian cells, indicates that scorpions may be a useful study system for the discovery of new antibiotic compounds for clinical use in humans, especially using varied culture conditions.

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SUPPLEMENTAL MATERIALS

Supplemental File S1.— Annotated FASTA sequences for bacteria isolated from the mesosoma and metasoma of *Smeringurus mesaensis* (Scorpiones: Vaejovidae), online at https://doi.org/10.1636/JoA-S-22-039.s1

Supplemental File S2.—Figure legend for Supplemental File 3, online at https://doi.org/10.1636/JoA-S-22-039.s2

Supplemental File S3.— Phylogenetic tree diagram including sequences obtained in this and other studies of scorpion bacterial symbionts, online at https://doi.org/10.1636/JoA-S-22-039.s3

Supplemental Table S1.— Scorpion number, body part, and identity of the closest bacterial relative for each of 80 sequences obtained from bacteria cultured from scorpion tissues, online at https://doi.org/10.1636/JoA-S-22-039.s4

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