Applied and Environmental Microbiology

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Changhui Guan, Jianhua Ju, Bradley R. Borlee, et al. 2007. Signal Mimics Derived from a Metagenomic Analysis of the Gypsy Moth Gut Microbiota . Appl. Environ. Microbiol. 73(11):3669-3676. doi:10.1128/AEM.02617-06.

Applied and Environmental Microbiology, June 2007, p. 3669–3676 Vol. 73, No. 11
0099-2240/07/$08.00+0 doi:10.1128/AEM.02617-06

Signal Mimics Derived from a Metagenomic Analysis of the Gypsy Moth Gut Microbiota▽†

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Received 9 November 2006/Accepted 2 April 2007

Bacterial signaling is an important part of community life, but little is known about the signal transduction pathways of the as-yet-uncultured members of microbial communities. To address this gap, we aimed to identify genes directing the synthesis of signals in uncultured bacteria associated with the midguts of gypsy moth larvae. We constructed a metagenomic library consisting of DNA extracted directly from the midgut microbiota and analyzed it using an intracellular screen designated METREX, which detects inducers of quorum sensing. In this screen, the metagenomic DNA and a biosensor reside in the same cell. The biosensor consists of a quorum-sensing promoter, which requires an acylhomoserine lactone or other small molecule ligand for activation, driving the expression of the reporter gene *gfp*. We identified an active metagenomic clone encoding a monooxygenase homologue that mediates a pathway of indole oxidation that leads to the production of a quorum-sensing inducing compound. The signal from this clone induces the activities of LuxR from *Vibrio fischeri* and CviR from *Chromobacterium violaceum*. This study is the first to identify a new structural class of quorum-sensing inducer from uncultured bacteria.

Microorganisms produce metabolites with diverse chemical features and biological activities and, thus, have been a primary source of antibiotics and other drugs for decades. As the antibiotic resistance crisis has raised concerns about humans' continued ability to manage infectious disease with antibiotics, attention has turned to the development of other types of antimicrobials, such as compounds that inhibit signaling in pathogens (19, 33, 44). Since the vast majority of microorganisms in the environment (>99% in most environments) have not yet been isolated in pure culture (18), environmental microorganisms may present a massive untapped reservoir of metabolites, including signaling compounds. Interest in characterizing the metabolic capacity of the as-yet-uncultured organisms has fueled the development of new culturing methods to coax a greater variety of organisms to grow in culture (21, 51) and the application of culture-independent methods to microbial communities (39, 41, 48).

Metagenomics is a culture-independent strategy involving direct extraction and cloning of DNA from an assemblage of microorganisms, thereby capturing their genetic potential in a surrogate host (18). High-throughput functional screens and sequence-based analysis of metagenomic libraries have led to the identification of novel microbial genes and their products in environmental samples, such as in soil (23), seawater (3), and other environments (24, 37). Metagenomic analysis of insect-associated microorganisms has yielded an understanding of the *Buchnera* genome, an obligate intracellular symbiont of aphids (32), and biosynthetic pathways for secondary metabolites from bacteria associated with beetles (34). Xylanases with unusual primary sequences and novel domains of unknown function have been discovered in metagenomic libraries of microbiota associated with members of the orders *Isoptera* (termites) and *Lepidoptera* (moths) (5).

One goal of our work is to identify metabolites and signal molecules in microorganisms living in communities associated with the lepidopteran midgut. To screen large metagenomic libraries, we developed a high-throughput intracellular screen designated METREX (*met*abolite *r*egulated *ex*pression), in which the metagenomic DNA and a biosensor reside in the same cell. In this case, the biosensor detects compounds that induce the expression of green fluorescent protein (GFP) from a bacterial quorum-sensing promoter that can be detected by fluorescence microscopy or fluorescence-activated cell sorting (46).

Quorum sensing is a system of gene regulation that detects bacterial cell density, enabling bacteria to coordinate diverse biological functions, such as biofilm formation (17, 25), virulence (8, 49), swarming behavior (15, 26), and antibiotic production (12). Quorum sensing is mediated by signal molecules of various specificities that form transcriptional complexes with regulator proteins in the LuxR family. Signal molecules or autoinducers in gram-negative bacteria are typically *N*-acyl-L-homoserine lactones (AHLs) that derive their specificity from the length of the acyl modification (13, 43, 52), yet there are some interesting variations. The opportunistic human pathogen *Pseudomonas aeruginosa*, for example, has two AHL-regulated quorum-sensing systems (*las* and *rhl*) but uses another non-AHL signal molecule, 2-heptyl-3-hydroxy-4(1H)-quinolone, as an additional link between the *las* and *rhl* quorum-

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 13 April 2007.

TABLE 1. Bacterial strains and plasmids used in this work

Strain/plasmid	Characteristics	Source or reference
Biosensor strains and plasmids		
Agrobacterium tumefaciens KYC55(pJZ410, pJZ384, and pJZ372)	KYC55 is an R10 derivative lacking pTiR10; pJZ410, pJZ384, and pJZ372 are a T7 expression system to express TraR	53
Chromobacterium violaceum CV026	CV026 is an acyl-HSL-negative mutant of ATCC 31532, it uses CviR as regulator protein, and it is most sensitive to *N*-hexanoyl-L-HSL	28
Escherichia coli MT102(pJBA132), referred to as reporter strain "JB525"	MT102 is *araD139* (*ara-leu*)*7697* Δ*lac thi hsdR*; pJBA132 is pME6031 carrying *luxR*-P_{luxI}-RBSII-*gfp*(ASV)-T_0-T_1 (Tet^r), is based on components of the *Vibrio fischeri luxIR* quorum-sensing system, and is most sensitive to *N*-(3-oxohexanoyl)-L-HSL	2
Pseudomonas putida F117(pKR-C12)	F117 is an acyl-HSL-negative derivative of IsoF, $PpuI^-$; pKR-C12 is pBBR1MCS-5 carrying P_{lasB}-*gfp*(ASV)-P_{lac}-*lasR* (Gm^r), is based on components of the *Pseudomonas aeruginosa las* quorum-sensing system, and is most sensitive to *N*-(3-oxododecanoyl)-L-HSL and other long-chain acyl-HSL molecules	36
pJBA89	Amp^r; pUC18Not-*luxR*-P_{luxI}-RBSII-*gfp*(ASV)-T_0-T_1, the same quorum-sensing components as in JBA132, but on pUC18Not vector	2
pJBA89m	pJBA89 mutant with partial deletion of *luxR* (140 bp of BstXI/NruI fragment)	This work
pSB401	Contains a fusion of *luxRI´*::*luxCDABE* on a pACYC184 plasmid backbone	47
pCG401	Km^r; pET28b vector harboring an EcoRI fragment of *luxR* from pBS401	This work
E. coli strains and plasmids for cloning		
DH10B	*recA1* and *endA1* cloning strain	Invitrogen, Carlsbad, CA
BL21(DE3)	*E. coli* B F^- *dcm ompT hsdS*(r_B^- m_B^-) *gal* λ(DE3)	Novagen, Madison, WI
pBluescript II	High copy no. cloning/expression vector; Amp^r	Stratagene, La Jolla, CA
pET28b	Protein expression vector; Km^r	Novagen, Madison, WI

sensing systems (29). Another non-AHL autoinducer, namely furanosyl borate diester (AI-2), mediates a gene regulation system in a cell-density-dependent manner, which facilitates interspecies communication among gram-negative bacteria (9). Besides these signals, a number of cyclic dipeptides also mediate quorum sensing in *Pseudomonas* spp. and other gram-negative bacteria (10, 20). The breadth of molecules that can induce quorum-sensing systems may be much greater than is currently known. Some evidence suggests that the expression of quorum-sensing promoters may be stimulated by diverse antibiotics at sublethal concentrations. For example, low concentrations of antibiotics of diverse structures and modes of action induce global changes in gene expression in *Salmonella enterica* serovar Typhimurium, including the expressions of genes regulated by quorum sensing (16, 50). Therefore, quorum-sensing reporter systems may provide a means of detection to identify diverse, biologically active molecules, including antibiotics and new signals.

To begin to assess the diversity of signal molecules that could be produced by the as-yet-uncultured microbial world, we applied the METREX screen to a metagenomic library derived from the microbial consortium associated with the gypsy moth midgut, which harbors a simple community containing typically 7 to 16 species, of which typically about half are culturable (6). Here we report the identification of a metagenomic clone from the gypsy moth midgut community that produces inducers of quorum sensing that are chemically distinct from previously described classes of quorum-sensing inducers.

MATERIALS AND METHODS

Strains, constructs, and PCR primers. The bacterial strains and DNA constructs used in this paper are listed in Table 1. For a list of all PCR primers used in this paper, see Table S1 in the supplemental material.

Gypsy moth rearing and midgut dissection. Gypsy moth egg masses were obtained from culture NJSS (New Jersey Standard Strain) at the United States Department of Agriculture, Animal and Plant Health Inspection Service Laboratory at the Otis Air National Guard Base, Cape Cod, MA. The eggs were hatched, and the larvae were reared as previously described (7). One hundred third-instar larvae (about 2 weeks old) were dissected, and the midguts were incubated in a phosphate buffer (35 mM KH_2PO_4, 17.5 mM Na_2HPO_4, pH 7.0) at 28°C for 5 h. To increase the cell mass to provide sufficient DNA to construct a library, the midgut mix was further incubated for 12 to 48 h in the same buffer in aliquots with the pH readjusted to 5.5, 6.5, 7.5, 8.5, and 9.5. During this time, the bacteria multiplied by 100- to 1,000-fold, and the only source of nutrients was the midgut tissue.

Construction of a metagenomic library. Microbial genomic DNA was isolated using the GenElute bacterial genomic DNA kit (Sigma-Aldrich Corp., St. Louis, MO) according to the manufacturer's protocol. Insect DNA contamination was assessed by PCR using gypsy moth-specific primers: the vitellogenin gene for genomic DNA and the mitochondrial cytochrome *c* oxidase subunit I gene for mitochondrial DNA (see Table S1 in the supplemental material). All restriction and modifying enzymes were purchased from Promega Corporation (Madison, WI). DNA samples from different pH conditions were combined and subjected to partial digestion with Sau3AI, and DNA fragments ranging from 3 to 8 kb were recovered. BamHI-digested pBluescript II KS(+) plasmid (Stratagene, La Jolla, CA) was treated with shrimp alkaline phosphatase and ligated to the Sau3AI fragments. The ligation mix was transformed by electroporation into

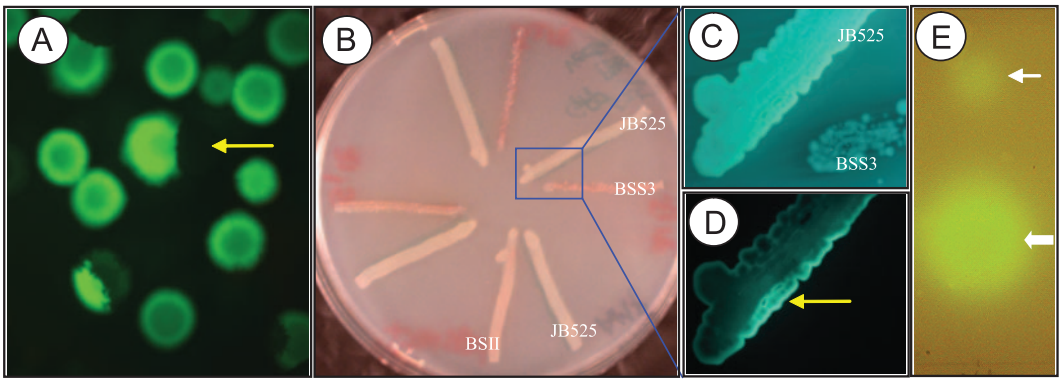


FIG. 1. Metagenomic clone BSS3 induces quorum sensing in the biosensor strain JB525. (A) A positive, pigmented clone (arrow) induced GFP expression of the biosensor plasmid in the neighboring colonies. (B) Transformants containing only the metagenomic clone were streaked side by side with the biosensor; a strain containing the empty vector pBSII was used as the control. (C and D) BSS3 under visible light (C) and with a GFP filter (D). GFP fluorescence in JB525 developed in the area proximal to the BSS3 colony stripe (arrow in panel D). (E) TLC of BSS3 extract overlaid with *E. coli* DH10B(pJBA132) biosensor. Two active spots (arrows) were visible under the fluorescence microscope.

Escherichia coli DH10B-competent cells (40), and the transformants were plated on Luria-Bertani (LB) agar medium with 100 μg/ml ampicillin (Amp). Transformants were pooled and stored at −80°C.

16S rRNA gene library construction and sequencing. The diversity of the midgut microflora was examined by PCR using the isolated metagenomic DNA as a template. The protocol is described elsewhere in detail (6). Briefly, 16S rRNA genes were amplified with 27F and 1492R primers (see Table S1 in the supplemental material) and the products were cloned into the pGEM-T vector (Promega, Madison, WI). Inserts in the transformants were PCR amplified with vector-based primers SP6 and T7 and sequenced with 27F and 1492R, respectively.

Screening the metagenomic library. The complete library pool of metagenomic clones was inoculated in LB amended with Amp (100 μg/ml) liquid medium and incubated at 37°C with shaking for 3 h. Plasmid DNA was isolated using the Miniprep kit (QIAGEN, Inc., Valencia, CA); 0.5 μg of the plasmid DNA was transformed by electroporation into the biosensor *E. coli* MT102 with the pJBA132 AHL biosensor (2). The same cells were transformed with plasmid pBSII (no insert) as a control (Table 1). The plates were incubated at 37°C overnight and further incubated at room temperature (25°C) for six additional hours. Approximately 800,000 transformants were grown (~4,000 CFU/plate) on LB agar containing tetracycline (Tet) and Amp, and the GFP signal in the transformants was detected by examination with a Leica MZ FLIII fluorescence microscope (Leica Microsystems, Inc., Wetzlar, Germany). Micrograph images were obtained using the MagnaFire SP digital imaging system (version 2.1) (Optronics, Goleta, CA). The positive colonies were collected and serially diluted with phosphate-buffered saline, pH 7.2, and the dilution series was plated on LB agar containing Tet and Amp.

Plasmid isolation, retransformation, and DNA sequencing. The plasmids from positive clones were isolated using the Miniprep kit (QIAGEN, Inc., Valencia, CA), and the DNA was transformed into *E. coli* DH10B using Amp to select for the metagenomic clone. Cells were patched on LB plates amended with Tet to confirm that the retransformants (pBSS3) did not contain the biosensor plasmid. The plasmid containing the metagenomic DNA was isolated and sequenced at the University of Wisconsin—Madison DNA Sequencing Facility using a BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA) initially with T3 and T7 primers (see Table S1 in the supplemental material). A primer-walking strategy was used to sequence the rest of the clone.

Biosensor cross-streak assay. The quorum-sensing inducing activities of BSS3 retransformants or subclones were tested by streaking, side by side, the sensor strain and BSS3 clones or subclones on solid agar medium (Fig. 1B). GFP-expressing clones were identified using a Leica fluorescence microscope.

Subcloning of BSS3. Primers were designed for the two open reading frames (ORFs) (*moxY* and *moxZ* [see Table S1 in the supplemental material]), and the PCR products were cloned in frame into the expression vector pET28b (Novagen, Madison, WI). The constructs were transformed into *E. coli* BL21(DE3) (Table 1), and the cultures were induced with isopropyl-β-D-thiogalactopyranoside (IPTG). The subclones were then assayed for activity either by cross-streaking or by the thin-layer chromatography (TLC) overlay method.

***luxR* deletion mutant construction.** A 140-bp fragment delimited by a BstXI site and an NruI site of the 5′ coding region of *luxR* was removed from pJBA89 (2) (Table 1). The resulting fragment was treated with T4 DNA polymerase (Promega Corporation, Madison, WI) to form blunt ends and self-ligated to make the deletion mutant pJBA89m. To confirm that the deletion in pJBA89m did not disrupt the $luxI_p$-*gfp* fusion, a fragment containing the region of *luxR* plus *luxIp* was digested from pSB401 (47) with EcoRI and ligated to pET28b cut with EcoRI to construct plasmid pCG401 (Table 1). The complementation was done by transforming pCG401 into *E. coli* DH10B (40) harboring pJBA89m. A TLC overlay assay using an AHL standard and *q*uorum-*s*ensing signal *m*imic compound 1 (QSM-1) was performed with wild-type pJBA89 and mutant pJBA89m to confirm the activities of the constructs (Table 2).

Culture, extraction, and isolation of BSS3 metabolites. Fresh culture (optical density at 600 nm of 1.0) of *E. coli* DH10B(pBSS3) (200 μl) grown in SOB medium (40) amended with 100 mM KH_2PO_4/K_2HPO_4 saline (pH 7.0) was inoculated into 600 ml of fresh medium with 100 μg/ml Amp in a 2-liter flask. Cultures were incubated at 25°C for about 24 h until the culture turned green. For large-scale isolation, the experiment was scaled up to 20 flasks, or a total volume of 12 liters. The culture was centrifuged at 4,000 rpm for 20 min at 4°C to remove the cells and precipitates. The resulting supernatant was extracted twice with equal volumes of ethyl acetate. The ethyl acetate phase was evaporated under reduced pressure to yield a residue, which induced the biosensor. The residue was applied on a silica gel column (2.5 by 30 cm), and eluted under low pressure with a chloroform-methanol gradient (from 10:0 to 9:1), and collected in 21 fractions, which were spotted on TLC plates and assayed for quorum-sensing activity with the biosensor strain. Fractions 2 to 7 did not exhibit any activity but essentially provided three compounds, namely, indigo (blue), indirubin (pink), and isatin (yellow), in substantial amounts after further purification. The active fractions (fractions 9 to 16) were analyzed by both high-pressure liquid chromatography (HPLC) and TLC, which revealed numerous components. These fractions were finally combined and subjected to repeated silica gel

TABLE 2. LuxR dependence of BSS3 activity[a]

Plasmid 1	Plasmid 2	GFP expression with inducer	
		3-Oxo-C6-HSL (46 nM in medium)	pBSS3 extract (direct spotting on TLC plate)
pJBA89 (*luxR*)*		+	+
pJBA89m (Δ*luxR*)†		−	−
pJBA89m (Δ*luxR*)†	pCG401 (*luxR*)‡	+	+
	pCG401 (*luxR*)‡	−	−

[a] *, pJBA132 contains *gfp* fusion with *luxIp* and a constitutive *luxR*; †, *luxR* in pJBA89m is deleted; ‡, pCG401 contains a constitutive *luxR* on pET28b. −, not expressed; +, expressed.

chromatography using the same solvent conditions, which removed isatin. At this stage, we found that the active fraction was relatively unstable. When stored at 4°C, certain components were converted to isatin, as shown by TLC and HPLC, indicating that the active compound, designated QSM-1, is derived from indoxyl. The combined active fractions were subjected to further purification by HPLC.

For the further purification of bioactive component QSM-1, a small portion of the active fraction was applied to a semipreparative HPLC column and 10 fractions were collected and assayed. The active fraction eluted at 12.5 to 15.0 min. Repeated preparation yielded a fraction that contained several components as demonstrated by analytical HPLC. This fraction was further separated by analytical HPLC, which yielded a peak at 10.5 min that coincided with the elution of active material. However, the small amount of the material was insufficient for nuclear magnetic resonance (NMR) analysis.

HPLC. Crude culture extract fractions collected during purification were monitored by HPLC. HPLC was carried out on a Varian system equipped with ProStar 210 pumps and a photodiode detector. Analytical HPLC was conducted using a Prodigy ODS-2 column (150 by 4.6 mm; 5-μm particle size) and eluted with a linear gradient of 3 to 80% CH_3CN over 20 min, followed by 10 min at 80% CH_3CN at a flow rate of 1.0 ml/min with UV detection at 210 nm. Semipreparative HPLC was conducted using a Microsorb C_{18} column (250 by 10 mm; 5-m particle size) eluted with a linear gradient of 15 to 90% CH_3CN over 25 min at a flow rate of 2.5 ml/min with UV detection at 210 nm.

MS and NMR analyses. Electrospray ionization-mass spectra (MS) were obtained on an Agilent 1100 HPLC-MSD SL quadrupole mass spectrometer equipped with both orthogonal pneumatically assisted electrospray and atmospheric pressure chemical ionization sources. High-resolution MS analyses were acquired on an IonSpec HiResMALDI FT mass spectrometer with a 7 T superconducting magnet. A saturated solution of 2,5-dihydroxybenzoic acid in methanol was used for matrix preparation, and the spectra were peak matched using *m/z* 273.03936 ($[2M - 2H_2O + H]^+$) as a reference peak. ^1H and ^{13}C NMR spectra were recorded at 25°C on Varian Unity Inova 500 instruments operating at 500 MHz for ^1H nuclei and 125 MHz for ^{13}C nuclei. ^1H and ^{13}C NMR chemical shifts were referenced to residual solvent signals: δ_H 2.50 and δ_C 39.51 for dimethyl sulfoxide-d_6.

Characterization of isatin, indigo, and indirubin. Isatin was identified by analyses of its MS, 1D-NMR, and 2D-NMR spectral data. Isatin, electrospray ionization-MS (+): *m/z* 148.0 $(M+H)^+$, 169.9 $(M+Na)^+$; ^1H NMR, δ 11.1 (1H, br.s, 2-OH), 7.50 (1H, br.d, J = 7.5 Hz, H-4), 7.07 (1H, td, J = 7.5, 1.0 Hz, H-5), 7.59 (1H, td, J = 7.5, 1.0 Hz, H-6), 6.91 (1H, br.d, J = 8.0 Hz, H-7); ^{13}C NMR, δ 185.1 (C-3), 160.0 (C-2), 151.4 (C-7a), 139.1 (C-6), 125.4 (C-4), 123.5 (C-5), 118.5 (C-3a). The identities of indigo and indirubin were compared with authentic samples by TLC analyses and were further confirmed by MS. Indirubin, high-resolution matrix-assisted laser desorption ionization-MS (+): *m/z* 263.0828($[M+H]^+$) (calculated 263.0815 for $C_{12}H_{11}O_2N_2$ $[M+H]^+$); indigo, high-resolution matrix-assisted laser desorption ionization MS (+), *m/z* 263.0813 ($[M+H]^+$) (calculated 263.0815 for $C_{12}H_{11}O_2N_2$ $[M+H]^+$).

Chemical and biochemical syntheses of indoxyl. Indoxyl was prepared using either indoxyl acetate treated with sodium hydroxide with heating or indoxyl phosphate (1 mM) with alkaline or acid phosphatase at 37°C (Sigma-Aldrich) under anaerobic conditions. The oxidation of indoxyl was observed when the assay was performed under ambient conditions. The activity of indoxyl was analyzed using the TLC biosensor overlay assay.

TLC and biosensor overlay. The crude extract or purified compound was spotted on the TLC plate (Whatman, Inc., Florham Park, NJ) and developed with a solvent of chloroform-methanol (95:5 vol/vol). The plate was dried briefly and put into a Nunc Immuno OmniTray (Nalge Nunc International, Rochester, NY). SOB soft agar medium (12 ml) (prewarmed at 50°C) containing 10 to 15 μl of overnight culture of biosensor strain was overlaid. The plate was incubated at 37°C for *E. coli* and at 28°C for *Chromobacterium violaceum*, *Agrobacterium tumefaciens*, or *Pseudomonas putida* (Table 1).

Nucleotide sequence accession number. The DNA sequence of BSS3 (*moxY* and *moxZ*) was deposited in GenBank under accession number EF569599.

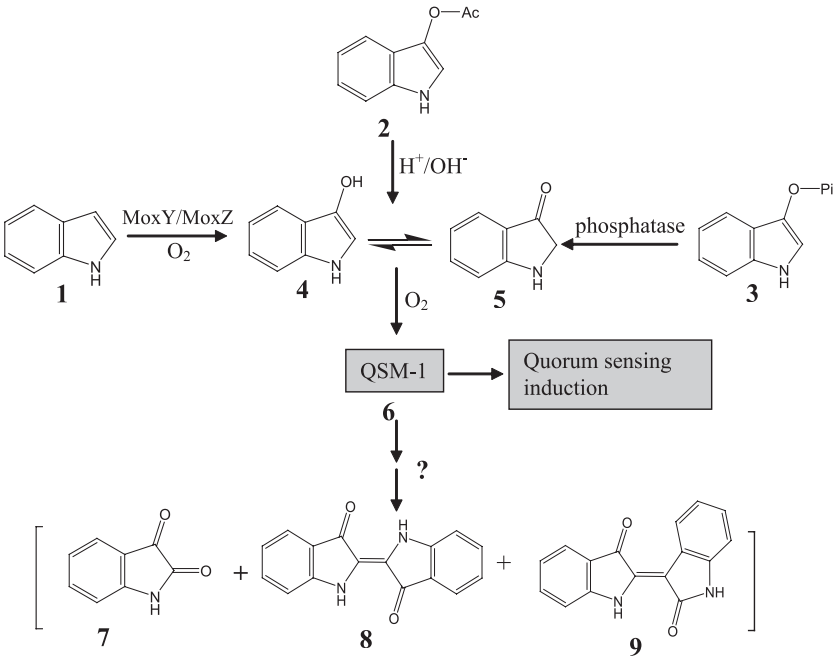
RESULTS

Gypsy moth gut: microbial diversity and construction of a metagenomic library. An analysis of the 16S rRNA genes in the microbial community associated with the gypsy moth midgut revealed *Firmicutes* and *Proteobacteria*, the two phyla we previously found to be most common in the gypsy moth midgut (6). Among these two phyla, the majority of the members aligned with *Staphylococcus* spp. (62%), including *Staphylococcus xylosus* and *Staphylococcus cohnii*, and the *Bacillus* sp. group (26%). The gammaproteobacterial sequences (10%) aligned best with an uncultured gammaproteobacterium clone, CLs90. Members of the *Betaproteobacteria* represented 2% of the clones, and this subphylum was not identified in the gypsy moth midgut previously. Some of the betaproteobacterial sequences affiliated with sequences from taxa that have only recently been cultured (51). We also found a betaproteobacterial sequence that matched an *Achromobacter xylosoxidans* strain that was isolated from a human subject (27). The amplification of genes that act as markers for insect DNA (see Table S1 in the supplemental material) indicated no detectable nuclear DNA in any of the DNA preparations and low levels of mitochondrial DNA in two out of eight samples tested (data not shown). After establishing that the DNA was predominantly bacterial, we constructed a small-insert metagenomic library containing 800,000 clones with inserts that averaged 3.3 kb, representing a total of 2.64 Gb of DNA, which is the equivalent of ~520 average (5-Mb) bacterial genomes.

Intracellular screen for quorum-sensing inducing clones. The intracellular screen of 800,000 metagenomic clones identified 16 clones that induced the expression of GFP in neighboring colonies. The sequences of the inserts of these clones indicated that they either were siblings or had substantially overlapping sequences. One clone, designated BSS3, was chosen for further study. When the BSS3 plasmid was introduced into a strain lacking the biosensor, the BSS3 plasmid behaved in a manner similar to that of the original clone in that the transformants induced the biosensor strain streaked next to them (Fig. 1B, C, and D) but were not fluorescent themselves (Fig. 1A). These results indicate that (i) the plasmid is responsible for the phenotype of the original clone, (ii) the compound responsible for biosensor induction is diffusible in agar-based medium, and (iii) ambient oxidation makes the compound active outside the producing cell.

Characterization of metabolites produced by BSS3. The active compounds were purified through activity-guided fractionation of BSS3 cultures. They were ethyl acetate soluble and the activity was detectable on TLC plates that were overlaid with the biosensor strain in soft agar. In this overlay assay, two spots that coincided with the induction of the quorum-sensing biosensor were observed: a major spot with an R_f of 0.3 (high polarity) and a minor spot with an R_f of 0.9 (low polarity) (Fig. 1E), which were designated QSM-1 and QSM-2, respectively. Since most of the activity was associated with QSM-1, we focused our efforts on this compound.

Clone BSS3 produces a mixture of at least three pigments, isatin (yellow), indirubin (pink), and indigo (blue) (Fig. 2), that are products of indole oxidation. The addition of indole to the culture before it reached stationary phase increased production of the pigments, suggesting that the clone carries genes involved in an indole oxidation pathway (14). Isatin had weak antimicrobial activity: the MIC of isatin in liquid culture was 100 μg/ml for *Escherichia coli*, 200 μg/ml for *Bacillus subtilis*, and 400 μg/ml for *Pseudomonas putida*. Isatin is likely responsible for the slow growth of BSS3 (Fig. 1B). Neither isatin, indirubin, nor indigo induced quorum sensing (data not shown); therefore, we characterized the extracts further to identify the active compound.

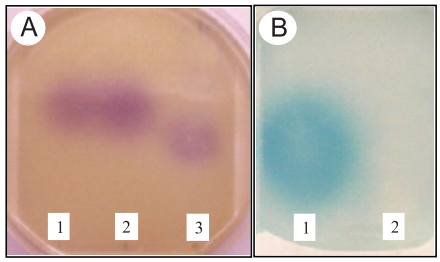


1, indole; **2,** indoxyl acetate; **3,** indoxyl phosphate; **4, 5,** indoxyl; **6,** QSM-1, structure undetermined; **7,** isatin; **8,** indigo; **9,** indirubin

FIG. 2. Proposed MoxY/MoxZ-mediated pathway for synthesis of quorum-sensing inducer.

The purification and characterization of QSM-1 proved difficult because it was unstable. When it was concentrated, QSM-1 spontaneously generated pigments, including isatin, indirubin, and indigo (data not shown; Fig. 2), resulting in a loss of activity. Indoxyl is a precursor for isatin, indirubin, and indigo and is derived from indole oxidation. Indoxyl is known to be unstable and readily converted to indigo, indirubin, isatin, and other compounds (14). After the culture entered stationary phase, BSS3 turned green and then blue and then produced the dark insoluble pigments, indigo and indirubin. The same color shift and pigment formation pattern was observed when indoxyl was prepared using phosphatase from indoxyl phosphate under ambient conditions (data not shown), suggesting that indoxyl might be involved in the synthesis of QSM-1. In this in vitro synthesis, a fraction that eluted from HPLC coincident with QSM-1 induced quorum sensing (data not shown). Pure indoxyl prepared from the same substrate and enzyme under anaerobic conditions differed from QSM-1 in its elution profile on the HPLC and mobility on TLC and had no activity in the overlay assay. However, the exposure of pure indoxyl to open air led to the production of the active compound, suggesting that QSM-1 is a product of indoxyl oxidation (data not shown; Fig. 2).

QSM-1 represents a new class of quorum-sensing inducers. The various regulators in quorum-sensing systems distinguish among closely related AHLs, making the activity of QSM-1 of interest because it represents an entirely new structural group of inducers. We used a series of biosensors to determine the specificity of QSM-1 (Fig. 3). These experiments were conducted with an extract of BSS3 culture fluid, which was the purest preparation of QSM-1 that we found to be stable. The QSM-1 preparation had a unique range of specificity (Fig. 3). *E. coli* containing pJBA132, which is a LuxR-based biosensor used in the initial library screen, was the most sensitive to QSM-1 activity. Another biosensor that was activated by QSM-1 was *Chromobacterium violaceum* CV026, which utilizes



C

Overlay	Biosensor plasmid	Transcriptional regulator	QSM-1 activity
Escherichia coli	JBA132	LuxR	+
Agrobacterium tumefaciens	KYC55	TraR	—
Chromobacterium violaceum CV026		CviR	+
Pseudomonas putida F117	pKR-C12	LasR	—

FIG. 3. Induction of various quorum-sensing biosensors with BSS3 extract. BSS3 culture extract and AHL standards were spotted on the TLC plate and developed with a solvent of chloroform-methanol (95:5 vol/vol). The plate was then overlaid with soft agar medium containing biosensor strains. (A) *Chromobacterium violaceum* CV026: lane 1, C4-HSL (200 ng); lane 2, 3-oxo-C6-HSL (20 ng); lane 3, BSS3 extract (4 μl). (B) *Agrobacterium tumefaciens* KYC55: lane 1, 3-oxo-C6-HSL (0.4 ng); lane 2, BSS3 extract (3 μl). (C) Summary of QSM-1 activity in different biosensors. −, not observed; +, observed.

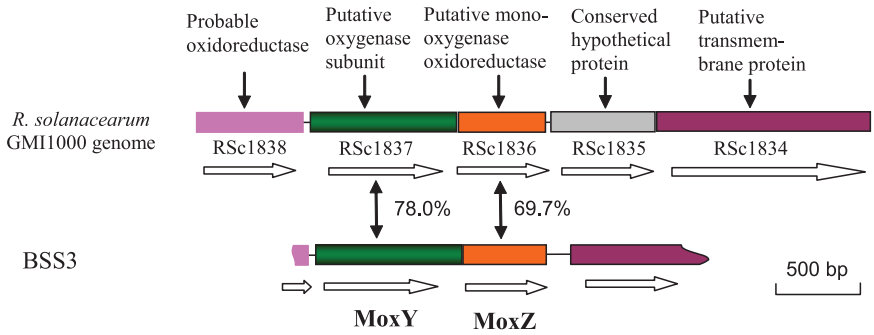


FIG. 4. Genomic organization of BSS3. BSS3 contains two ORFs encoding a homologue of monooxygenase (designated MoxY) and of flavin oxidoreductase (designated MoxZ), respectively. Together with the two truncated ORFs, the four genes were transcribed in the same direction (hollow arrows). The DNA sequences of *moxY* and *moxZ* showed highest similarity to the genomic DNA clusters (labeled under the colored boxes) of Rsc1837 and Rsc1836 of *Ralstonia solanacearum* GMI1000, whose genome sequence has been completed (38). Genes in the upper genomic fragment are labeled with names used in *R. solanacearum* as annotated by the Laboratoire Interactions Plantes Micro-Organismes, Castanet Tolosan, France (http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/ralsto/).

the transcriptional activator CviR, a homologue of LuxR, for quorum-sensing regulation (28) (Fig. 3A). CV026 has been used extensively for the detection of conventional quorum-sensing molecules, including 3-oxo-C6-HSL. BSS3 extract did not induce two other biosensors, *Agrobacterium tumefaciens* KYC55 (53) (Fig. 3B) and *P. putida* F117/pKRC12 (36) (Fig. 3C).

Activity of QSM-1 is LuxR dependent. The biosensor plasmid containing a *luxR* deletion (pJBA89m) failed to express GFP when the cognate AHL (3-oxo-C6-HSL) or BSS3 extract was applied. The introduction of *luxR* restored the responsiveness of pJBA89m to both molecules (Table 2), indicating that the active compound likely interacts directly with the receptor protein (LuxR), thereby mimicking the activity of the AHLs; this interaction remains to be established.

Genes responsible for production of QSM-1. Nucleotide sequence analysis indicates that clone BSS3 contains an insert of 3.2 kb comprising two complete and two truncated ORFs (Fig. 4) that are transcribed in the same direction. Blast analysis indicated that the two complete ORFs, designated *moxY* and *moxZ*, contain significant sequence similarity to a monooxygenase and a flavin oxidoreductase, respectively. The best match was a genomic sequence in *Ralstonia solanacearum* GMI1000 (38), to which MoxY and MoxZ had 78 and 70% identity, respectively (Fig. 4). In addition to the annotated putative oxygenase subunit from *R. solanacearum*, MoxY had similarity to a hypothetical 2-polyprenyl-6-methoxyphenol hydroxylase and related flavin adenine dinucleotide-dependent oxidoreductases in *Burkholderia cepacia* (60%), hypothetical glycine/D-amino acid oxidases in *B. cepacia* (58%), and the well-characterized StyA protein (29%) (4). StyA is a styrene monooxygenase isolated from *P. fluorescens* and *P. putida*, responsible for the transformation of styrene to epoxystyrene (4). The transformation of indole to indigo by StyA has also been reported (31). A monooxygenase (2-hydroxybiphenyl 3-monooxygenase) in *Pseudomonas azelaica* has also been reported to produce indigo, indirubin, and isatin, although it shares little sequence similarity with MoxY (30). These data suggest that *moxY* is a member of a gene family involved in microbial oxidative or hydroxylative pathways. The subcloning and overexpression of *moxY* and *moxZ* indicate that MoxY was necessary and sufficient for the induction of the AHL biosensor and pigment production, and MoxZ enhanced the activity (data not shown). *moxY* and *moxZ* overlap by 1 nucleotide (Fig. 4). The expression of *moxY* and *moxZ* on pBSS3 is dependent on the *lacZ* promoter present on the pBluescript vector (Stratagene, La Jolla, CA); no activity was detected when the insert was cloned in the opposite orientation on the same vector (data not shown).

DISCUSSION

The elucidation of bacterial signal transduction pathways has led to an understanding of microbial ecology that holds interest for both chemists and biologists. Signaling through quorum sensing, for example, provides insight into communication networks that regulate multicellular behavior of single-species populations and communities. Moreover, the chemistry that modulates quorum sensing has revealed novel classes of bioactive molecules and new opportunities to exploit communication as a target for antimicrobial compounds.

Quorum sensing is likely to be a central mechanism of communication within communities, yet few studies have addressed quorum sensing in this context (1, 35). To begin the dissection of communication in a community, we applied a metagenomic analysis to the microbial community that resides in the midguts of gypsy moth larvae. Using METREX, a rapid intracellular screen for metagenomic clones that produce a quorum-sensing inducing compound, we identified one clone (16 siblings from primary screen) among 800,000 that induces a *luxIR*-based biosensor. In this particular study, the active clones were detected intercellularly, demonstrating the versatility of METREX. Chemical analysis indicates that the clone produces an array of molecules, mostly indole-related compounds. The hydrolysis of indoxyl (oxidation product of indole) esters using a chemical or biochemical method, followed by ambient oxidation, results in the production of the active compound QSM-1, which induces quorum sensing. The data indicate that QSM-1 is a derivative of indoxyl, which represents a new class of compound that activates a quorum-sensing promoter.

This study illustrates the power of METREX to identify

rare, active clones in libraries with many members. The complexity of many microbial communities, which manifests in both species richness and uneven species abundance, necessitates the construction of massive libraries to represent all of the genomes of the organisms, making it critical to have selections and rapid screens for functional analysis of metagenomic libraries. METREX presents the opportunity to screen many clones quickly for quorum-sensing inducers and inhibitors. We predict that it will also identify clones producing antibiotics based on the finding that many antibiotics, including members of many different structural and functional groups, when applied at sublethal doses, induce the expression of genes regulated by the quorum-sensing system (16). Here we report the discovery of novel quorum-sensing inducers as well as the antimicrobial molecule, isatin, in the METREX screen. The capacity of METREX to screen a large library without the need for assaying individual colonies or cultures will be even more critical in metagenomic analyses of more complex communities (46).

QSM-1 is an indole compound related to indoxyl, as indicated by the structural elucidation of its derivatives from the culture, chemical, and biochemical syntheses. QSM-1 is not stable under conditions required for structural analysis. During the purification of QSM-1 from BSS3 culture fluid, QSM-1 was converted to pigmented compounds, including isatin, indirubin, and indigo. The synthesis of indoxyl through enzymatic reaction under ambient conditions resulted in the formation of the same pigments. Structural analysis of compounds from the culture fluid did not yield oxindole or dioxindole, two of the indole oxidation products at C-2 that are chemically stable. Commercially obtained oxindole and dioxindole were assayed and did not induce quorum sensing (data not shown). In vitro biochemical assays with purified MoxZ show that this protein is a NADH-flavin oxidoreductase. Purified MoxY can transform indole into indigo only in the presence of MoxZ, suggesting that MoxY/Z is a two-component, flavin-dependent monooxygenase that oxidizes indole into indoxyl through the oxidation of C-3. Further experiments will address the substrate specificity of MoxY/Z (data not shown; Fig. 2).

In the TLC analysis, the R_f of QSM-1 was smaller than the R_fs of isatin and indoxyl, indicating that QSM-1 has a higher polarity. The minor active spot from BSS3 fluid, QSM-2, had a much higher R_f, suggesting that it could be a dimer or a derivative of QSM-1. Based on these data, we propose that QSM-1 is an indole-based, indoxyl derivative, representing a new type of molecule among those that induce quorum sensing. A number of previous findings are consistent with our results, also indicating key roles for indole derivatives. First, many gram-negative species produce indole and it has been proposed to act as an extracellular signal to regulate biofilm formation, which is often regulated by quorum-sensing regulators (11). Furthermore, studies of the microflora of rat intestinal tracts showed that when the pH of the culture medium changed from neutral to alkaline, the production of indole was dramatically induced (22). Interestingly, the gypsy moth midgut, the origin of the insert DNA in BSS3, is highly alkaline, reaching pHs from 9 to 12 (42). Future work will focus on the regulation of indole production and its biological role in the microbial community in this highly alkaline environment. In summary, uniting metagenomic analysis with METREX or other intracellular screens (45) provides a powerful strategy to probe the suite of signal molecules in a microbial community. The identification of the signal and the genes required for its production lays the groundwork for characterizing signal transduction in a microbial community that contains both readily culturable and not-yet-culturable members.

ACKNOWLEDGMENTS

We thank Ronald Binder for AHL standards, Michael Givskov for providing the biosensor strain JB525, Steve Winans for providing the *A. tumefaciens* AHL indicator strain, James Windak for the high-resolution MS analysis, and Jean-Yves Merour and William W. McWhorter, Jr., for critical suggestions on chemical syntheses. We also thank Nichole A. Broderick for assistance with gypsy moth larva rearing and dissection.

This work was supported by Valent Biosciences, the National Science Foundation MCB SGER grant no. 0454823, the Howard Hughes Medical Institute, and the University of Wisconsin—Madison College of Agricultural and Life Sciences (Hatch project 5340).

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