

## ORIGINAL ARTICLE

# *Proteus mirabilis* interkingdom swarming signals attract blow flies

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**Flies transport specific bacteria with their larvae that provide a wider range of nutrients for those bacteria. Our hypothesis was that this symbiotic interaction may depend on interkingdom signaling. We obtained *Proteus mirabilis* from the salivary glands of the blow fly *Lucilia sericata*; this strain swarmed significantly and produced a strong odor that attracts blow flies. To identify the putative interkingdom signals for the bacterium and flies, we reasoned that as swarming is used by this bacterium to cover the food resource and requires bacterial signaling, the same bacterial signals used for swarming may be used to communicate with blow flies. Using transposon mutagenesis, we identified six novel genes for swarming (*ureR*, *fis*, *hybG*, *zapB*, *fadE* and *PROSTU\_03490*), then, confirming our hypothesis, we discovered that fly attractants, lactic acid, phenol, NaOH, KOH and ammonia, restore swarming for cells with the swarming mutations. Hence, compounds produced by the bacterium that attract flies also are utilized for swarming. In addition, bacteria with the swarming mutation *rfaL* attracted fewer blow flies and reduced the number of eggs laid by the flies. Therefore, we have identified several interkingdom signals between *P. mirabilis* and blow flies.**

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## Introduction

Bacteria consumed by immature blow flies feeding on a resource survive larval molting and pupation and are present in emergent adults; hence, resulting flies serve as a dispersal mechanism for the bacteria (Ahmad *et al.*, 2006). Flies and their relatives disperse over 100 pathogens (Greenberg, 1973), many of which are responsible for the estimated 76 million food-borne illnesses occurring annually in the United States. These pathogens include *Escherichia coli* O157:H7, which is responsible for hemorrhagic colitis and hemolytic uremic syndrome (Sanderson *et al.*, 2006) and accounts for 73 000 illnesses and 61 deaths annually (FoodNet, June 2006). Thus, it is imperative to understand how bacterial pathogens are transported by insects.

*Proteus* is a genus of Gram-negative bacteria, many of which cause infections in humans (Liu, 2010), with *Proteus mirabilis* causing 90% of these infections. It commonly inhabits dogs, cows and birds, and can cause nosocomial infections when colonizing human feces in hospital settings. *Lucilia sericata* (Diptera: Calliphoridae) is a common blow fly populating most areas of the world. It is typically one of the first organisms attracted by odors from cadaver decomposition (Clark *et al.*, 2006). *L. sericata* larvae are also the primary species used for maggot therapy (Schmidtchen *et al.*, 2003).

Blow flies are attracted and repelled by various factors, including temperature, light and odors, and flies sense and respond to attractants by receptors on legs, cerci and antennae (Dethier, 1947). Attractants help flies recognize potential mates and kin, oviposition (egg laying) sites and food sources. Repellents usually help protect insects from danger, such as predators. Proteins, fats and oils are the major materials of living organisms. Although these molecules themselves do not produce odor, their decomposition in carrion, feces, urine and animal secretions (that is, sweat, decomposing plant material, fungi and algae) are usually odorous (Dethier,

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1947). Among all the decomposition compounds from fats and proteins, ammonia appears to be the most common single nitrogenous product. It is a major constituent of urine, acting as a primary excretion product as well as a secondary product of urea decomposition. Skatole, indole, mercaptans and sulfides are the most penetrating odors of putrefaction. Another large group of attractants are fatty acids, which are usually fermentation products and decomposition components.

Quorum sensing (QS) is the regulation of gene expression in bacteria as a function of the concentration of secreted small molecules that reflect cell density (Miller and Bassler, 2001). Gram-positive and Gram-negative bacteria both use QS communication to regulate their behavior, including symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation and biofilm formation (Davies *et al.*, 1998; González Barrios *et al.*, 2006). Acylhomoserine lactones in Gram-negative bacteria as well as indole, and autoinducer 2 in both Gram-negative and Gram-positive bacteria (Jayaraman and Wood, 2008; Han *et al.*, 2010) are typical QS signals. QS regulates swarming motility (Daniels *et al.*, 2004).

Swarming is a flagella-driven movement of differentiated hyperflagellated, elongated and multinucleated swarmer cells by which bacteria spread as a biofilm over a surface (Daniels *et al.*, 2004). Glycolipid or lipopeptide biosurfactants work as wetting agents by reducing surface tension. The QS signal acylhomoserine lactone enhances swarming motility in *Serratia liquefaciens* (Daniels *et al.*, 2004), whereas indole diminishes *Pseudomonas aeruginosa* swarming motility (Lee *et al.*, 2009). The quorum-quenching signal brominated furanone (Gram *et al.*, 1996) inhibits *E. coli* swarming motility via inhibiting both acylhomoserine lactone- and autoinducer-2-mediated signaling (Ren *et al.*, 2001).

Bacteria and fruit flies (Diptera: Drosophilidae) share a common cell–cell communication system (Waters and Bassler, 2005). The inner membrane protein AarA of *Providencia stuartii* is required for the release of an extracellular quorum-sensing signal whose structure has not been identified yet (Waters and Bassler, 2005). The homolog of AarA in the fruit fly *Drosophila melanogaster* is a rhomboid protein RHO that controls fly wing vein development and eye organization. Expression of *P. stuartii aarA* in a *D. melanogaster rho* mutant rescued wing vein development, whereas expression of *rho* in a *P. stuartii aarA* mutant complemented the QS signaling defect.

Interkingdom signals can help bacteria recognize the host immune system (Hughes and Sperandio, 2008). For example, the *P. aeruginosa* OprF protein on the cell surface binds to interferon- $\gamma$  from the host, activates the QS system by inducing *rhII* (RhII synthesizes the QS signaling molecule C<sub>4</sub>-homoserine lactone), induces the expression of *lecA* (encodes virulence determinant type I *P. aeruginosa* lectin (PA-I lectin)) and increases the production of

pyocyanin (Wu *et al.*, 2005). *P. aeruginosa* also detects adenosine of injured host cells and activates its PA-1 lectin virulence factor (Patel *et al.*, 2007). Furthermore, indole works as a beneficial signal in intestinal epithelial cells by increasing epithelial-cell tight-junction resistance and attenuating inflammation indicators (Bansal *et al.*, 2010).

The rationale for the work here is that as flies respond to compounds produced by bacteria and as bacteria use signals to swarm, we hypothesized that bacterial strains deficient in swarming signals may also be deficient in interkingdom signaling with flies. After generating *P. mirabilis* transposon mutants that were deficient in swarming, we tested 10 compounds that attract flies (Table 1) for their ability to restore swarming and to restore interkingdom signaling between *P. mirabilis* and *L. sericata*. Using this approach, we identified five new chemicals (fly attractants) that are related to swarming, identified six new swarming pathways related to the synthesis of these compounds and determined part of genetic basis for the interkingdom signaling.

## Materials and methods

### Bacterial growth

*P. mirabilis* from the maggot salivary gland was routinely grown in Luria–Bertani medium at 37 °C. For the plate competition experiments, *E. coli* BW25113 wild-type (Baba *et al.*, 2006) and *P. aeruginosa* PA14 wild-type (Liberati *et al.*, 2006) were utilized.

### Maggot salivary gland extraction

*L. sericata* larvae were grown at room temperature on beef liver in jars until the third instar. Individual larvae with full crops were removed their containers and quickly rinsed in diluted bleach solution (1.25% sodium hypochlorite), followed by two phosphate-buffered saline solutions (0.8% NaCl, 0.02% KCl, 0.144% Na<sub>2</sub>HPO<sub>4</sub> and 0.024% KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) before dissection. The salivary gland was removed and put into a sterile microcentrifuge tube filled with sterile phosphate-buffered saline (20  $\mu$ l per pair of salivary glands to be extracted).

### *P. mirabilis* identification

The harvested glands were mashed and spread on trypticase soy agar plates with 5% sheep blood (TS-blood agar; BVA Scientific, San Antonio, TX, USA). Plates were incubated aerobically for 24 h at 37 °C. Phenotypically distinct colonies were chosen and subcultured repeatedly onto fresh media to attain cultural purity. *P. mirabilis* was initially identified using API Rapid 20E manual identification test strips for *Enterobacteriaceae* (bioMérieux Inc., Marcy l’Etoile, France) using 20 biochemical tests, including glucose acidification, sucrose acidification,  $\beta$ -galactosidase and indole production (Izard *et al.*, 1984).

**Table 1** List of blow fly attractants used to complement the swarming of the *Proteus mirabilis* transposon mutants

Attractant	Insect	Concentration in bacteria	Concentration used to attract flies
Indole	<i>Lucilia Sericata</i> (Dethier, 1947)	<i>Escherichia coli</i> : 0.0703 mg ml <sup>-1</sup> (extracellular) and 0.0234 mg ml <sup>-1</sup> (intracellular) (Domka <i>et al.</i> , 2006).	With 2-mercaptoethanol and sodium sulfide: 10–100 mg ml <sup>-1</sup> , and swormlure-2: 41 mg ml <sup>-1</sup> (Urech <i>et al.</i> , 2004)
Sodium hydroxide	<i>L. cuprina</i> (Dethier, 1947)	NA	Water: 200 mg ml <sup>-1</sup> (Hepburn and Nolte, 1943) <sup>a</sup>
Potassium hydroxide	<i>L. cuprina</i> (Dethier, 1947)	NA	Water: 7.2–240 mg ml <sup>-1</sup> (Hepburn and Nolte, 1943) <sup>a</sup>
Lactic acid	<i>L. sericata</i> , <i>Calliphora erythrocephala</i> (Dethier, 1947)	<i>Lactobacillus acidophilus</i> : 0.07–0.7 mg ml <sup>-1</sup> (Juárez Tomás <i>et al.</i> , 2003)	A total amount of 0.023–0.133 mg h <sup>-1</sup> from human hands is attractive to mosquitoes <i>Aedes aegypti</i> (Smith <i>et al.</i> , 1970) <sup>b</sup>
Ammonia	<i>L. sericata</i> (Hilker and Meiners, 2002)	<i>P. mirabilis</i> : 0.297 mg ml <sup>-1</sup> (Vince <i>et al.</i> , 1973)	Ammonium hydroxide: 5% (Cragg, 1950)
Putrescine	Calliphoridae (Wardle, 1921)	<i>P. mirabilis</i> : 0.003–0.088 mg ml <sup>-1</sup> (Sturgill and Rather, 2004)	Water on filter paper: 0.001–1 mg ml <sup>-1</sup> (Robacker, 2001), 1 µg attracts 70% <i>L. sericata</i> (this study)
p-Cresol	<i>Cochliomyia</i> , <i>Chrysomya</i> (Hilker and Meiners, 2002)	<i>Lactobacillus</i> spp.: 0.8 mg ml <sup>-1</sup> in (Yokoyama and Carlson, 1981)	A total amount of 0.005–0.02 mg ml <sup>-1</sup> used to attract mosquitoes ( <i>Culex quinquefasciatus</i> ) for oviposition (Poonam <i>et al.</i> , 2002) <sup>b</sup>
Benzoic acid	<i>Cochliomyia</i> , <i>Chrysomya</i> (Hilker and Meiners, 2002)	NA	Swormlure-2: 41 mg ml <sup>-1</sup> (Urech <i>et al.</i> , 2004)
Butyric acid	<i>Cochliomyia</i> (Broce, 1980)	Pig cecal bacteria: 0.053–1.057 mg ml <sup>-1</sup> (Kobayashi and Sakata, 2006)	A total amount of 0.02 mg ml <sup>-1</sup> used as a synthetic fly attractant for <i>Fannia femoralis</i> (Mulla <i>et al.</i> , 1984)
Phenol	<i>Cochliomyia</i> , <i>Chrysomya</i> (Hilker and Meiners, 2002)	Marine bacteria: 0.1–1 mg ml <sup>-1</sup> (Updegraff, 1949)	Swormlure-2: 41 mg ml <sup>-1</sup> used in (Urech <i>et al.</i> , 2004)

Abbreviation: NA, not applicable.

<sup>a</sup>The chemical concentrations for sodium hydroxide and potassium hydroxide used for fly attraction in Hepburn and Nolte (1943) were too high for bacterial growth, hence a smaller concentration was used.

<sup>b</sup>Chemical concentrations for mosquito attractions are provided here due to the absence of information for fly attraction.

Sequencing of the gene for 16S ribosomal RNA was also used to confirm the identity of *P. mirabilis*; short-read sequence products of ~300 bp length were produced with forward primer 5'-ACTTAACCCAAC ATCTCACGA-3' and reverse primer 5'-AGGATTAGA TACCCTGGTAGT-3' (Campbell *et al.*, 1995), and long-read sequence products of ~750 bp length were produced using forward primer 5'-ACTCCTACG GGAGGCAGCAG-3' (Moreno *et al.*, 2011) and the same reverse primer. Purified PCR products were sequenced in both directions, with a minimum of 2 × coverage using standard BigDye-terminator Cycle Sequencing (Applied Biosystems Inc., Carlsbad, CA, USA) protocols and submitted to GenBank (JN790943 for *P. mirabilis* and JN790944 for *Providencia* spp.). The species identification of the bacterial strain was determined by comparing the consensus sequence against published 16S recombinant DNA sequences deposited in the GenBank Nucleotide Collection (nr/nt) by utilizing the “blastn” algorithm of the Basic Local Alignment Search Tool (BLAST; NCBI, <http://www.ncbi.nlm.nih.gov>).

#### Transposon mutagenesis and swarming-based screening

Transposon mutagenesis was performed with the EX-Tn5 <DHFR-1>Tnp transposome kit (Epicentre, Madison, WI, USA). After electroporation with 50 µl of competent cells and 1 µl transposome

supplied by the kit, we obtained around 3000 colonies with the Tn5 transposon randomly inserted in the genome. Mueller–Hinton agar plates (Atlas, 2004) were used to select mutants with transposons inserted using 10 µg/ml trimethoprim. The agar concentration was adjusted to 3% to prevent swarming during this step. We then screened 3000 colonies for swarming motility after ~4 h on Luria–Bertani agar plates with 1.5% agar at 37 °C. Fifty mutants with at least threefold decreased swarming were selected and confirmed as swarming-deficient strains using the same conditions.

#### DNA sequencing to identify transposon insertion positions

Genomic DNA was isolated from the swarming mutants via the UltraClean Microbial DNA isolation kit (MO BIO, Carlsbad, CA, USA). For sequencing, arbitrary PCR (Ueda and Wood, 2009) was performed; the first round of arbitrary PCR reaction (PCR1) was performed using 100 ng of genomic DNA and arbitrary primer 1 (5'-GGCCAGGCCTGCAGAT GATGNNNNNNNNNGTAT-3') along with internal specific primer (5'-ACGGATTTCGAAACCTGTAC G-3'). The second arbitrary PCR reaction (PCR2) was performed with the PCR1 product and arbitrary primer 2 (5'-GGCCAGGCCTGCAGATGATG-3') along with external specific primer I (5'-AGGTGGCGGAA ACATTGGATG-3'). The third arbitrary PCR reaction

was performed with the PCR2 product and arbitrary primer 2 (5'-GGCCAGGCCTGCAGATGATG-3') along with external specific primer II (5'-GGCGAAACAT TGGATGCGG-3'). The final PCR product after three sets of arbitrary PCR was purified and sequenced using external specific primer II. NCBI BLAST was used to compare sequences and identify the transposon insertion site.

#### Swarming complementation

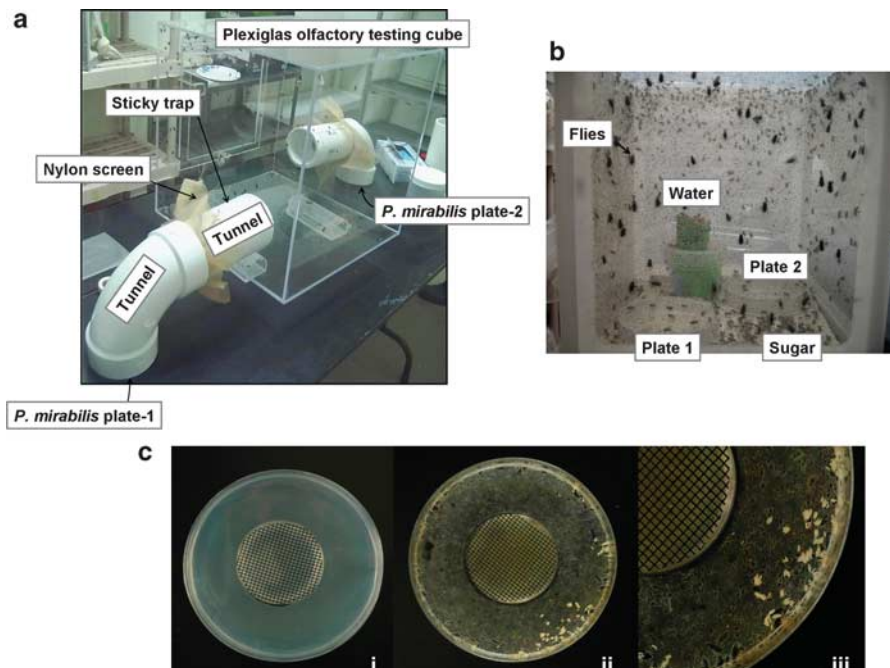
For the swarming complementation test,  $10\ \mu\text{g ml}^{-1}$  and  $250\ \mu\text{g ml}^{-1}$  of each chemical were added to Luria–Bertani agar plates (1.5% agar). The stock solutions of indole and benzoic acid were dissolved in dimethylformamide, and *p*-cresol was dissolved in ethanol. The other chemicals (phenol, butyric acid, lactic acid, NaOH, KOH and putrescine) were dissolved in H<sub>2</sub>O. A volume of  $2\ \mu\text{l}$  of exponential-phase cultures (OD 600 ~1.0) were added to the surface of the agar plates and incubated at 37 °C. For the swarming complementation test with ammonia,  $2\ \mu\text{l}$  and  $15\ \mu\text{l}$  ammonium hydroxide were dropped on the lid of Petri dishes because ammonium hydroxide can easily release ammonia, and the evaporated ammonia can be sensed by bacteria in this way. Swarming halos were measured after 10 h.

#### Fly attraction and oviposition assay

Flies from Davis (CA, USA) (Tarone *et al.*, 2011) were maintained in 30 cm<sup>3</sup> cages (BioQuip Products, Rancho Dominguez, CA, USA) and fed granulated

sugar and water *ad libitum*. Emergent flies were fed blood collected from fresh cow liver for the first 4 days post emergence using cotton. Flies were tested at age 5–7 days, and cages were dried for 24–48 h between experiments after cleaning.

The fly attraction assay was performed at 21 °C as shown in Figure 1a. Two- to three-hundred 7-day-old, blood-fed *L. sericata* blow flies were placed into the clean plexiglas olfactory testing cube we constructed (45 cm<sup>3</sup>) without food or water. Agar plates (17 × 100 mm) with *P. mirabilis* cultures ( $100\ \mu\text{l}$ ,  $10^7\ \text{CFU ml}^{-1}$  bacteria spread onto plates and incubated at 37 °C for 24 h) were put into the ends of each of the two tunnels (white, 10 cm in diameter, by 15 cm length PVC pipe; Charlotte Pipe, Charlotte, NC, USA), which were attached on opposite sides of the cube. The proximal end of the pipe was capped with an inverted funnel, thus allowing flies to enter but making it difficult from them to leave. A nylon screen within the tunnel prevented the flies from reaching the bacterial plates, while allowing odors to pass through. Flies were allowed to roam freely within the testing box for 24 h. Two sticky traps (Bell Laboratories Inc., Chicago, IL, USA) lined the sides at the entrance to the tunnel to catch the flies entering the tunnel that were attracted to odor emitted from the bacterial plate. The flies captured within the tunnels on the sticky traps and the flies remaining within the testing cube were collected after 24 h and counted, and their sex and gravidity was assessed. The plexiglas cage and all components were washed



**Figure 1** Apparatus for fly attraction assay (a) and fly oviposition assay (b), and plate details for the fly oviposition assay (c). (ci) For the fly oviposition assay, small agar plates (35 × 10 mm) were plated with bacteria, covered with a nylon screen and placed in the center of larger (17 × 100) Luria agar plates. (cii) Screen allows volatile emissions, but experimental bacteria are inaccessible to direct contact by flies. (ciii) Eggs deposited on agar after 24 h at 23 °C.

with odorless soap and dried before use in future replicates.

The oviposition assay was performed at 21 °C using the system shown in Figure 1b. One- to two-hundred 7-day-old, blood-fed *L. sericata* adults were placed in a mesh-covered cage with water and sugar provided *ad libitum* (Wal-Mart, Bentonville, AR, USA). Small Luria agar plates (10 × 35 mm; USA Scientific Inc., Ocala, FL, USA) were inoculated with 13 µl of 10<sup>7</sup> CFU ml<sup>-1</sup> bacteria, incubated for 24 h at 37 °C, covered with a plastic screen and sterile placed in the center of Luria agar plates 17 × 100 mm (USA Scientific Inc.; Figure 1c). Two sets of plates (wild type and mutant) were put inside the cage, and the flies were allowed to lay eggs on the Luria agar ring surrounding the centrally placed, small plates with the bacteria. Plates were collected after 24 h and eggs were counted. Cages containing flies were considered replicates and used only once. The heterogeneity G test was used to analyze resulting adult blow fly attraction and oviposition data. This approach allowed us to determine whether expected values of response differed from the overall and pooled ( $G_p$ ) observations, and to determine whether the level of variance and resulting ratio of responses differed across replicates (Sokal and Rohlf, 1995). Furthermore, this approach also was selected over calculating  $\chi^2$ -values, which are not additive and are only approximately correct. Resulting G values are additive and correct, thus reducing the likelihood of type one error (Sokal and Rohlf, 1995).

#### Fly responses to chemicals

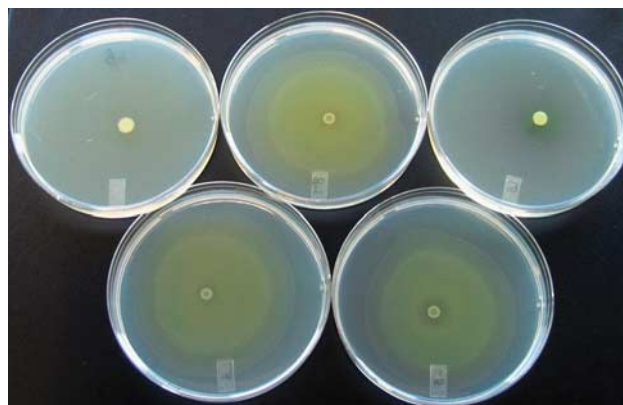
The behavioral response of 7-day-old *L. sericata* adults to either 1 or 10 µg of putrescine paired with a blank control was measured in an olfactometer using methods similar to that of Margolies *et al.* (1997).

## Results

Our hypothesis was that bacteria on decaying resources attract *L. sericata* to the carcass with signals that are related to bacterial swarming. To identify the biochemicals related to this interkingdom signaling, we isolated bacteria from flies, to obtain environmentally-relevant biological samples, then mutated the bacteria and screened for reduced swarming behavior. We determined whether bacterial metabolites that are known to be fly attractants could restore the reduced swarming behavior, thereby linking fly attraction with swarming. Finally, we tested the *rfaL* swarming mutant for differential olfactory and oviposition responses by the flies.

#### *P. mirabilis* isolated from fly salivary glands

By adding *L. sericata* maggot salivary gland extracts to *E. coli* and *P. aeruginosa* cultures, we found that one bacterium from the flies had notable swarming motility that outcompeted that of both *E. coli* and



**Figure 2** *P. mirabilis* outcompetes *E. coli* and *P. aeruginosa* with its swarming motility on Luria-Bertani (LB) plates. Cultures (5 µl at a turbidity of 5 at 600 nm) of each strain were loaded to the top of LB agar plates and incubated at 37 °C for 18 h before taking pictures. Top row from left: *E. coli*, *P. mirabilis*, and *P. aeruginosa*. Bottom row from left: *E. coli* + *P. mirabilis* and *P. aeruginosa* + *P. mirabilis*.

*P. aeruginosa* (Figure 2). This bacterium was identified as *P. mirabilis* (100% match of a 660 bp fragment) using both a biochemical characterization and Sanger sequencing of the bacterial 16S ribosomal gene sequence. Another primary bacterium from the glands was identified as *Providencia* spp. (99% match of a 668 bp fragment). It was expected to find bacteria intimately associated with flies, as *Providencia* spp., *E. coli* O157:H7, *Enterococcus faecalis* (Orla-Jensen) and *Ochrobactrum* spp., have been previously isolated from the screwworm fly *Cochliomyia macellaria* (Ahmad *et al.*, 2006). *P. mirabilis* has also been isolated from maggots of the blow fly *Calliphora vicina* (Erdmann, 1987).

#### Swarming-deficient mutants

After randomly mutating *P. mirabilis*, 3000 colonies were obtained, and 50 swarming-deficient mutants were identified. After confirming the swarming phenotype, 23 mutations were sequenced to identify which genes were related to swarming in *P. mirabilis*. Six groups of genes were identified (Table 2), including genes related to metabolism (*hybG*, *proC*, *pdxA*, *adhE* and *fadE*), regulation (*fis*, *PMI2857* and *yojN*), transcription/regulation (*PMIr001*, *pnp*, *rhlB*, *rpsM*, *rrfG*, *ugd* and *ureR*), cell surface (*rfaL* and *zapB*), flagella (*flgK* and *flhD*) and uncharacterized functions (*PROSTU\_03490*). Four of these genes (*rfaL*, *flhD*, *flgK* and *zapB*) were previously identified to be related to swarming motility; hence, our method was able to recover some known swarming-related mutations for *P. mirabilis*. The *flgK* gene encodes the flagellar hook-associated proteins and is tightly associated with bacterial swarming motility (Fraser *et al.*, 1999). The *flhDC* activator is the central component for regulating swarmer cell differentiation in *P. mirabilis* and other bacteria (Clemmer and Rather, 2007), and *flhDC* mutants are unable to swarm.

**Table 2** Summary of sequencing results for the *Proteus mirabilis* transposon mutants

Mutation	Insert	Organism	Gene function
<i>Metabolism</i>			
<i>hybG</i>	Middle	<i>P. mirabilis</i> HI4320	Hydrogenase nickel incorporation protein
<i>proC</i>	Middle	<i>P. mirabilis</i> HI4320	Pyrroline-5-carboxylate reductase
<i>pdxA</i>	Upstream	<i>P. mirabilis</i> HI4320	4-Hydroxythreonine-4-phosphate dehydrogenase
<i>adhE</i>	Middle	<i>P. mirabilis</i> HI4320	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase
<i>fadE</i>	Middle	<i>P. mirabilis</i> HI4320	Acyl-CoA dehydrogenase
<i>Regulator</i>			
<i>fis</i>	Middle	<i>P. mirabilis</i> HI4320	DNA-binding protein Fis
<i>PMI2857</i>	Middle	<i>P. mirabilis</i> HI4320	Helix-turn-helix XRE-family-like proteins
<i>yojN</i>	Middle	<i>Providencia rettgeri</i> DSM 1131	Putative two-component sensor protein such as YojN
<i>Nucleotide related</i>			
<i>PMIr001</i>	Middle	<i>P. mirabilis</i> HI4320	16S ribosomal RNA
<i>pnp</i>	Middle	<i>P. mirabilis</i> HI4320	Polynucleotide phosphorylase/polyadenylase
<i>rhlB</i>	Middle	<i>P. mirabilis</i> HI4320	ATP-dependent RNA helicase
<i>rpsM</i>	Upstream	<i>P. mirabilis</i> HI4320	30S ribosomal protein S13
<i>rffG</i>	Middle	<i>P. mirabilis</i> HI4320	dTDP- <i>D</i> -glucose-4,6-dehydratase
<i>ugd</i>	Middle	<i>P. mirabilis</i> HI4320	UDP-glucose 6-dehydrogenase
<i>ureR</i>	Middle	<i>P. mirabilis</i> HI4320	Urease operon transcriptional activator
<i>Cell surface related</i>			
<i>rfaL</i>	Middle	<i>P. mirabilis</i> HI4320	O-antigen ligase
<i>zapB</i>	Middle	<i>P. mirabilis</i> HI4320	Cell division protein
<i>Others</i>			
<i>PROSTU_03490</i>	Middle	<i>P. stuartii</i> ATCC 25827	Hypothetical protein
<i>Flagellar</i>			
<i>flgK</i>	Middle	<i>P. mirabilis</i> HI4320	Flagellar hook-associated protein 1
<i>flhD</i>	Upstream	<i>P. mirabilis</i> HI4320	Transcriptional activator FlhD for flagellar

Abbreviation: BLAST, Basic Local Alignment Search Tool.

The transposon insertion site and the relative insertion position are listed (middle indicates that the transposon is inserted in the coding portion of the gene and upstream indicates that the transposon is inserted in the upstream intergenic region). The organism used for BLAST is also listed. *P. mirabilis* HI4320 is the best fit organism for the sequence BLAST. *Providencia* strains were also used for the BLAST search, as these two bacteria were co-isolated from *Lucilia sericata*.

In *P. mirabilis*, the ZapA protein (immunoglobulin A-degrading metalloprotease) works as a virulence factor expressed specifically in swarmer cells, although the *zapA* mutant does not show decreased swarming (Walker *et al.*, 1999). The *zapB* gene that we identified is necessary for ZapA activity (Walker *et al.*, 1999).

#### Complementation of swarming mutations via known fly attractants

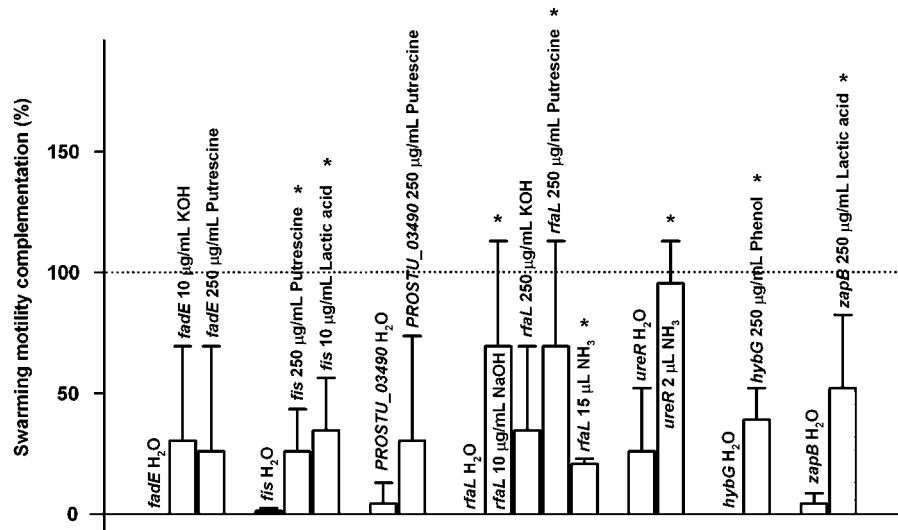
To determine whether any of the swarming mutations are part of interkingdom signaling with blow flies, 10 known blow fly attractants (Table 1) were added to the swarming plates with the swarming mutants to ascertain whether swarming could be restored, that is, whether mutations that disrupted swarming also affected interkingdom signaling between the bacteria and flies. We chose chemical concentrations of 0.01–0.25 mg ml<sup>-1</sup> for our experiments, as these concentrations are close to the physiological concentrations in bacteria and close to the concentrations used to attract flies (Table 1).

The addition of six known fly attractants (putrescine, NaOH, KOH, NH<sub>3</sub>, phenol and lactic

acid) restored the swarming motility of different swarming-deficient mutants (Figure 3). Hence, in addition to attracting flies, these chemicals also function as molecules that control swarming of *P. mirabilis*. With the exception of putrescine (Sturgill and Rather, 2004), these fly attractants have not been previously associated with swarming. We also identified seven (RfaL, UreR, Fis, HybG, ZapB, FadE and PROSTU\_03490) biochemical pathways through which these attractants work by identifying the genes disrupted by the transposon mutagenesis. Notably, the *rfaL* mutant (Figure 3, Supplementary Table 1) had increased motility upon adding NaOH, KOH, putrescine and ammonia, whereas the mutant without chemical addition did not swarm. Moreover, the *ureR* mutant had up to a 3.6-fold increase in swarming motility upon the addition of ammonia compared with the water control. The full motility complementation results are listed in Supplementary Tables 1, 2, and 3.

#### *RfaL* is required for fly attraction and oviposition

Initial tests for fly attraction and oviposition were performed with the *rfaL* mutant versus the wild-type



**Figure 3** Six fly attractants complement the swarming motility of the swarming-deficient mutants. The degree of complementation is determined by comparing the swarming motility of the mutant with the attractant relative to the wild type with H<sub>2</sub>O added. The symbol ‘\*’ indicates statistically significant differences with the control sample (H<sub>2</sub>O added) as determined by a Student’s *t*-test ( $P < 0.05$ ). Error bars indicate the s.d. of at least three independent cultures.

strain, as the *rfaL* mutant has complemented swarming motility with the addition of known signaling molecule putrescine (Figure 3, Supplementary Table 1). Nine trials examining *L. sericata* attraction (total of 2592 flies) and six trials for oviposition (total of 1138 flies) were conducted. When pooling all individuals as one group, *L. sericata* had a significantly ( $G_p = 78.11$ ,  $df = 8$ ,  $P < 0.0001$ ) greater attraction (38% greater) to the wild-type than the *rfaL* mutant. A breakdown based on sex and ovarian status yielded similar results, as significantly greater attraction for flies to the wild-type versus the *rfaL* mutant was determined for males ( $G_p = 11.89$ ,  $df = 8$ ,  $P < 0.0001$ ), gravid ( $G_p = 8.05$ ,  $df = 8$ ,  $P < 0.0001$ ) and nongravid females ( $G_p = 6.51$ ,  $df = 8$ ,  $P < 0.0001$ ). Similar results were determined for the oviposition assay with flies depositing significantly ( $G_p = 473.09$ ,  $df = 5$ ,  $P < 0.0001$ ) more eggs (63% more) on the wild-type versus the *rfaL* mutant. Therefore, RfaL is important for fly attraction and oviposition; it is necessary for swarming, and this swarming deficiency may be complemented by the addition of the known fly attractant putrescine. Hence, putrescine may be an interkingdom signal for this insect.

#### Putrescine attracts *L. sericata*

The compounds indicated as fly attractants in Table 1 have been studied by others for decades (Dethier, 1947; Hilker and Meiners, 2002), for example, Urech *et al.* (2004) showed that 10–100 mg ml<sup>-1</sup> indole can be used together with 2-mercaptoethanol and sodium sulfide as mixtures for attracting *L. cuprina*. In addition, 41 mg ml<sup>-1</sup> indole, 41 mg ml<sup>-1</sup> benzoic acid and 41 mg ml<sup>-1</sup> phenol are components of swormlure-2, which is attractive to *L. cuprina* and *Chrysomya* spp.

In addition, 200 mg ml<sup>-1</sup> NaOH in water and 7.2–240 mg ml<sup>-1</sup> KOH in water are attractive to *L. cuprina*, although the mechanism for how flies sense these nonvolatile chemicals is unknown (Hepburn and Nolte, 1943). Furthermore, 0.001–1 mg ml<sup>-1</sup> putrescine in water was used to attract the Mexican fruit fly *Anastrepha ludens* (Robacker, 2001). Here, we verified the response of *L. sericata* to putrescine, as it was the most important compound for complementing the swarming mutations of *P. mirabilis* and found the fly response level was 70% to 1 µg.

## Discussion

*P. mirabilis* is a Gram-negative urinary tract pathogen for humans (Morgenstein *et al.*, 2010). The prominent feature of this bacterium is its ability to swarm. The swarming behavior involves a complex repeating cycle of differentiation between two cell types, the vegetative (swimmer) and swarmer cells (Janda and Abbott, 2005). The swimmer cells dominate in liquid and change into swarmer cells with longer cell length and more flagella after 3–4 h when they are placed on solid surfaces (Morgenstein *et al.*, 2010). The flagellar rotation is inhibited during this conversion, and an extracellular signal is required to control this multicellular behavior. Proteins related to lipopolysaccharide, flagella, cell wall synthesis, cell division, proteolysis (Belas *et al.*, 1995) and pathogenicity (for example, hemolysin, protease and urease; Liaw *et al.*, 2001) are involved in this conversion. Hence, swarming is related to the pathogenicity of *P. mirabilis* as well as to QS.

In this study, we identified six chemicals (putrescine, NaOH, KOH, NH<sub>3</sub>, phenol and lactic

acid) that are important for restoring swarming in seven mutants (*rfaL*, *ureR*, *fis*, *hybG*, *zapB*, *fadE* and *PROSTU\_03490*). All the chemicals except putrescine have not been associated with swarming previously, and all but the *rfaL* mutation have not been associated with swarming previously; hence, putrescine and the *rfaL* mutation confirm the effectiveness of our approach. Putrescine is an extracellular signal required for swarming in *P. mirabilis* (Sturgill and Rather, 2004). It belongs to the group of polyamines, including putrescine, agmatine and spermidine, and is a constituent of the outer membrane of *P. mirabilis*. Putrescine is the product of SpeB in *P. mirabilis* (Sturgill and Rather, 2004), and mutations in *speA* (encoding arginine decarboxylase) or *speB* (encoding agmatine ureohydrolase) block putrescine production and result in a 2- to 3-h delay in swarmer cell differentiation (Stevenson and Rather, 2006). Adding 25  $\mu$ M exogenous putrescine can completely restore the swarmer cell differentiation of the *speA* mutant (Stevenson and Rather, 2006). Furthermore, putrescine attracts blow flies (Wardle, 1921) and the Mexican fruit fly (Robacker, 2001).

RfaL (WaaL) is the lipopolysaccharide O-antigen ligase. In *P. mirabilis*, the deletion of *rfaL* causes reduced differentiation into swarmer cells primarily due to its repression of the *flhDC* operon (Morgenstein *et al.*, 2010). In *P. aeruginosa*, the RfaL protein is a membrane protein with 11 potential transmembrane segments (Abeyrathne and Lam, 2007). We show for the first time that putrescine can restore the swarming of an *rfaL* mutant. In addition, we show the deletion of *rfaL* decreases fly attraction. Hence, although speculative, it appears RfaL might work as a transporter for putrescine and that putrescine might be an interkingdom signal that is sensed by both blow flies and bacteria. In addition, another O-antigen related gene, *rfaD* (*waaD*), is required for swarming motility in *P. mirabilis* (Belas *et al.*, 1995).

Mechanisms used by arthropods to locate resources vital for their reproduction have been widely investigated (Eisemann and Rice, 1987; Eisemann, 1988; Easton and Feir, 1991; Thomas, 1991; Tessmer *et al.*, 1995). In the case of blow flies, volatile organic compounds released by bacteria are the primary mechanism governing their attraction, acceptance and colonization of such resources (Eisemann and Rice, 1987; Chaudhury *et al.*, 2002, 2010; Morris, 2005). However, no one has demonstrated the role of these volatile compounds in the ecology of the bacteria from which they are released. Our results provide insights into this ecology of insects in that we show that known attractants of flies (putrescine, NaOH, KOH, NH<sub>3</sub>, phenol and lactic acid) restore swarming behavior in of our mutated strains of *P. mirabilis*; hence, we link fly attraction with bacterial swarming. Our discovery is of ecological relevance, as it represents a new facet of trophic interactions between resources and those

entities competing, or collaborating, to consume them. Therefore, swarming molecules could be regulating entire ecosystem processes and interkingdom communication.

Understanding insect signaling is important in fields ranging from agriculture to human health, as arthropods are the source of much benefit and loss. For example, scolytid bark beetles (Coleoptera: Scolytidae) respond to volatile emissions from their fungal food (Paine *et al.*, 1997), and parasitoids of these beetles also respond to these volatiles (Stephen *et al.*, 1993). Moreover, the sites that attract mosquitoes (Diptera: Culicidae) are governed by a range of cues including volatiles emitted by the bacteria residing on our skin (Bernier *et al.*, 2000) as well as from oviposition sites (Ponnusamy *et al.*, 2008). Indole, which is a quorum-sensing molecule (Lee *et al.*, 2007), has also been isolated from human skin and elicits a strong response by mosquitoes (Pelletier *et al.*, 2010). Furthermore, plants respond to salivary excretions released by feeding herbivorous arthropods. In many instances, the salivary excretions harbor microbes, including fungi and bacteria (Felton and Tumlinson, 2008). We contend that in each of these examples, the interkingdom signaling occurring within the associated microbial communities serve as a mechanism by which arthropods are able to locate and assess resources for themselves or their offspring.

Ammonia is another important chemical that is proposed to be an interkingdom signal molecule (Hilker and Meiners, 2002). Ammonia is produced by almost all organisms, it is one of the most characteristic odors in fresh manure (Richardson, 1916), and it can attract *L. sericata* adults (Hilker and Meiners, 2002). Urease catalyzes the hydrolysis of urea into carbon dioxide and ammonia (Nicholson *et al.*, 1993), and in *P. mirabilis*, urease is encoded by the *ure* operon that contains eight genes. UreR is the transcription regulator of *P. mirabilis* urease. The activation of urease leads to an increase of pH and the formation of urinary stones; hence, urease is a virulence factor (Mobley and Belas, 1995). Here, we show for the first time that ammonia can complement the swarming deficiency of the *ureR* mutant. We also propose for the first time that ammonia may be an interkingdom signal that controls both blow fly and bacteria activity.

We also show here that *P. mirabilis* attracts *L. sericata* adults and induces a greater incidence of oviposition, confirming previous observations about bacterial volatiles and their attraction of blow flies (Dethier, 1947). What is novel here is that mutation of one gene in the *P. mirabilis* genome can alter responses of *L. sericata* adults to the wild-type strain significantly. This attraction shift was not complete; however, this lack of universal preference is not a complete surprise. Traits such as orientation and oviposition are very complex behaviors, which are subject to the influences of multiple genetic and environmental factors (Ewing and Manning, 1967;



Sambandan *et al.*, 2008; Miller *et al.*, 2011). Accordingly, environmental differences among experimental replicates (that is, subtle changes in microbial concentrations, fly density or temperature) may have influenced differences among replicates. For example, the 24-h long period of exposure may have saturated the cage for the attractant odors, rendering the flies incapable of choosing between the strains after a certain period of exposure. Future studies will be performed with tighter temporal resolution that may resolve this potential issue.

There is ample evidence that flies harbor a great deal of genetic variation for oviposition (Miller *et al.*, 2011) and olfactory preferences (Rollmann *et al.*, 2010). For example, recent work has shown that some *Drosophila* strains will prefer to lay eggs only on food with yeast on it, whereas others will only lay eggs in substrates where they are absent (Miller *et al.*, 2011). They also prefer oviposition substrates/foods inoculated with certain yeast strains (Vacek *et al.*, 1985; Barker, 1992; Anagnostou *et al.*, 2010), establishing a clear microbial connection to such behaviors in *Drosophila*. Microbial influences in *Drosophila* are not limited to yeast (the major protein source for fruit flies). *Wolbachia* spp. infections can alter attraction to a resource in *Drosophila* (Panteleev *et al.*, 2007) and in mosquitoes (Wiwatanaratnabutr *et al.*, 2010). *Wolbachia* and *Lactobacillus* species have also been shown to affect mating preferences in *Drosophila* (Miller *et al.*, 2010; Sharon *et al.*, 2010), and *Lactobacillus* infection status can influence heritability in mating preferences (Sharon *et al.*, 2010). The insect-mediated attraction observed here may demonstrate particularly high levels of genetic variation, as many insect–microbe interactions show signs of balancing or diversifying selection (Lazzaro and Clark, 2001; Lazzaro *et al.*, 2004; Lazzaro, 2005; Mackay, 2010). Given these known insect–microbe interactions, it is entirely possible that there is genetic variation in *L. sericata* attraction to *P. mirabilis*. Taken as a whole, these observations presented here indicate that the attractant properties of *P. mirabilis* are likely the result of complex interactions between *Proteus* and *Lucilia* genomes.

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