The ISME Journal (2012) 6, 1166–1175

www.nature.com/ismej

ORIGINAL ARTICLE

Verminephrobacter eiseniae type IV pili and flagella are required to colonize earthworm nephridia

Glenn FJ Dulla, Ruth A Go, David A Stahl and Seana K Davidson
Department of Civil and Environmental Engineering, University of Washington, Seattle, WA, USA

The bacterial symbiont *Verminephrobacter eiseniae* colonizes nephridia, the excretory organs, of the lumbricid earthworm *Eisenia fetida*. *E. fetida* transfers *V. eisenia* into the egg capsule albumin during capsule formation and *V. eiseniae* cells migrate into the earthworm nephridia during embryogenesis, where they bind and persist. In order to characterize the mechanistic basis of selective tissue colonization, methods for site-directed mutagenesis and colonization competence were developed and used to evaluate the consequences of individual gene disruptions. Using these newly developed tools, two distinct modes of bacterial motility were shown to be required for *V. eiseniae* colonization of nascent earthworm nephridia. Flagella and type IV pili mutants lacked motility in culture and were not able to colonize embryonic earthworms, indicating that both twitching and flagellar motility are required for entrance into the nephridia.
The ISME Journal (2012) **6,** 1166–1175; doi:10.1038/ismej.2011.183; published online 15 December 2011
Subject Category: microbe–microbe and microbe–host interactions
Keywords: symbiosis; earthworm; flagella; type IV pili; motility

Introduction

Animals and plants form cooperative, beneficial associations with bacteria that range from highly specific and obligate to diverse and facultative. Most animal–bacteria symbioses are initiated in early stages of life as juveniles acquire proper bacteria from either the environment or directly from the parent (Bright and Bulgheresi, 2010). This process presents challenges to a juvenile, as it must simultaneously attract appropriate bacteria while avoiding colonization by potential pathogens. For extracellular symbionts, this process of attracting, collecting and keeping the proper symbiont has been well described in very few systems established for laboratory study including the squid–*Vibrio*, leech–*Aeromonas* and nematode–*Xenorhabdus* associations (Nyholm and Mcfall-Ngai, 2004; Graf, 2005; Goodrich-Blair, 2007). These models are enabled by molecular tools to examine mechanisms governing these stages. Others have elegantly established developmental sequences for early colonization through observations in the environment or lab in the absence of molecular genetic tools (Nussbaumer *et al.*, 2006; Davidson and Stahl, 2008). Without abilities to manipulate and test bacterial genes associated with early colonization processes, mechanisms for this critical phase of microorganism–host partnership remain vague.

Earthworms in the Lumbricidae harbor bacterial symbionts belonging to the *Verminephrobacter* genus in their nephridia, kidney-like osmoregulatory organs (Knop, 1926; Pinel *et al.*, 2008; Lund *et al.*, 2010). Distinct from the gut, nephridia are attached two per segment on either side of the gut. Each nephridium has a ciliated intake (nephrostome) leading to tubules forming three distinct regions. Fluid flows from the coelom through the nephrostome into a set of fine ciliated tubules leading into a second portion with a widened pouch, the ampulla, then to the bladder, and finally through a pore to the exterior (Figure 1a). Bacteria selectively colonize the ampulla. In *Eisenia fetida*, a widely distributed composting worm, three symbionts are known to colonize nephridia, *Verminephrobacter eiseniae*, a *Flexibacter* sp. and a *Microbacteriaceae* member (Davidson *et al.*, 2010). Of these, only *V. eiseniae* can now be routinely maintained in culture independent of the host (Pinel *et al.*, 2008). There is strong evidence for evolutionary longevity of the *Verminephrobacter*–earthworm association (Lund *et al.*, 2010), including a process of vertical transmission, direct passage of symbionts from parent to offspring (Davidson and Stahl, 2006).

During earthworm mating, bacteria are deposited into the nascent egg capsule. Embryos develop in a dense mixed population of bacteria and during embryogenesis, bacteria within egg capsules begin to colonize segments as they mature anterior to posterior (Davidson and Stahl, 2008). The capsule

Correspondence: SK Davidson, Department of Civil and Environmental Engineering, University of Washington, Seattle, WA, 98195-5014, USA.
E-mail: skdavid@u.washington.edu
Received 6 September 2011; revised 31 October 2011; accepted 3 November 2011; published online 15 December 2011

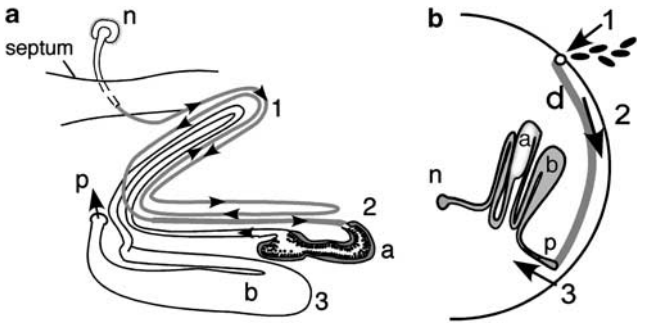

Figure 1 Anatomy of an earthworm nephridium. (**a**) Mature nephridium: 1, first loop, arrow heads indicate fluid flow through ciliated tubules; 2, second loop with ampulla containing bacteria; 3, bladder section before exit to exterior. (**b**) Colonization of nascent nephridium: 1, bacteria accumulate at a pore; 2, cells migrate into the colonization duct; 3, bacteria enter nephridia through a pore into the bladder then ampulla. a, ampulla; b, bladder; d; duct; n, nephrostome; p, pore.

albumen of *E. fetida* contains specific symbiotic strains as well as other bacteria presumably entrained from soil (Davidson *et al*., 2010). Embryos are successfully colonized by specific bacteria from this mix and remain uncolonized by other resident bacteria. Prior microscopy studies have shown that bacterial cells initially accumulate near pores during the development of each segment. They then migrate into ducts that run dorsal to ventral on the lateral sides of each segment. Bacteria reside in ducts for a day or so, then complete migration by traveling into nascent nephridia, accumulating rapidly in the ampulla (Figure 1b). Only specific symbiotic bacteria enter the colonization duct, suggesting either specific attraction to the site or exclusion of incorrect cells (Davidson and Stahl, 2008). These observations suggest directed taxis of motile bacteria through albumen to the pore and into nascent nephridia. Although the physical path of colonization has been well visualized, the molecular mechanisms that facilitate colonization of earthworm embryos remain unknown.

Bacterial movement is essential for initiation of colonization for pathogens and beneficial bacteria in both plants and animals. The majority of well-studied examples are represented by pathogens, and includes the requirement of functional flagella and/or type IV pili (TFP) for pathogen colonization of the animal gut, human lung and plant leaf-surface (Haefele and Lindow, 1987; Nachamkin *et al*., 1993; Wassenaar *et al*., 1993). Beneficial bacterial colonization is less well known and only a few model systems have clearly demonstrated flagellar swimming motility to be essential for initiation of the association (Ames and Bergman, 1981; Malek, 1992; Graf *et al*., 1994). TFP provide adhesion and movement through extension, binding and then retraction of pili. Binding can be charge-dependent, or mediated by highly specific adhesins for binding to select receptors (Mattick, 2002). There are a few examples demonstrating TFP importance for symbiont colonization, but literature is sparse (Stabb and Ruby, 2003; Bohm *et al*., 2007).

Observations of the colonization process and evidence for motility of *V. eiseniae* in culture have led to the hypothesis that both flagella and TFP are involved during colonization of the earthworm embryo. The genome sequence of *V. eiseniae* EF01-2 contains all genes necessary for twitching and flagellar motility guided by chemotaxis (Pinel, 2009). Under the microscope, cells were observed spinning as if tethered on a glass slide, and single polar flagella were evident by transmission electron microscope (Pinel *et al*., 2008). In this paper, we describe application of genetic methods for study of mechanisms used by *V. eiseniae* to colonize embryos of the earthworm *E. fetida*. Specifically, we investigated the necessity of two forms of bacterial motility, flagellar swimming and TFP-mediated twitching, for successful colonization of the host.

Materials and methods

Bacterial strains and culture conditions

Although a genome sequence is available for *V. eiseniae* strain EF01-2, the strain EF05-2r (99.9% 16S rRNA sequence identity to EF01-2) was used for genetic studies because it lacks the extrachromosomal plasmid (p*VE*IS01), has a shorter generation time (Pinel *et al*., 2008) and a spontaneous rifampicin-resistant variant was available (EF05-2r). *V. eiseniae* EF05-2r was maintained on Acidovorax complex media (ACM) at 28 °C (Pinel *et al*., 2008). *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA) and S17-1 (Simon *et al*., 1986) were maintained on Luria–Bertani agar at 37 °C. Kanamycin 30 or 50 μg ml^{-1}, rifampicin 100 μg ml^{-1}, and tetracycline 15 μg ml^{-1} were used as appropriate.

Mutant construction

The *flgK* gene encodes a flagellar hook-associated protein that forms the junction between the hook (FlgE) and filament monomers (FliC) (Berg, 2003). The neomycin phosphate transferase gene, *npt2,* conferring kanamyacin resistance was inserted into the *flgK* gene of *V. eiseniae* EF05-2r. Primers for amplification of 1.3-kb internal portion of the *flgK* gene were designed based on Veis_0560 (gb/CP000542.1/:628810-635405; flgKfrag-F-AVR2: 5′-GCCCTAGGCTGCAAACGACCGGCCACAACATTGCCA-3′; flgKfrag-R-HIND3: 5′-GCAAGCTTGGCCGCCATGACCGGGTTGGC-3′, underlined sequence denotes added restriction-enzyme cut sites) and used to amplify a portion of *flgK* from *V. eiseniae* EF05-2r genomic DNA. The fragment was cloned into a modified Gateway (Invitrogen) vector pENTR/D-Topo-MCS:kan (Shepherd and Lindow, 2009) as an *Avr*II/*Hin*dIII fragment. The resulting construct containing the *flgK* sequence and *npt2* was transferred into pLVC-D suicide vector (Marco *et al*.,

2005) via LR Clonase reaction (Invitrogen) to generate pLVC-D*flgK*, then introduced into donor *E. coli* strain S17-1 by electroporation (Simon *et al.*, 1986). For biparental mating, S17-1 (pLVC-D*flgK*) and EF05-2r were mixed at a 1:10 ratio, plated on ACM and incubated overnight at 28 °C. Mating mixtures were harvested and plated on ACM containing kanamycin (30 μg ml^{-1}) and rifampicin (100 μg ml^{-1}). After 2–3 weeks of incubation at 28 °C, single colonies were restreaked for purity and tested for marker insertion.

In addition, a double crossover insertion was generated to remove coding regions of both the *flgK* and *flgL* genes in EF05-2r. Primers for amplification of 1-kb internal portion of the *flgL* gene were designed based on Veis_0559 (gb/CP000542.1/:628810-635405; flgLFow: 5′-CTCGAGCAGAACTTTCGCGCATTGGTGG-3′; flgLRev: 5′-TCTAGACAGCGACAATCTTTGCACCTGG-3′) and used to amplify a portion of *flgL* from *V. eiseniae* EF05-2r genomic DNA. The fragment was cloned into pENTR/D-Topo-MCS:kan*flgK* as a *Xho*I/*Xba*I fragment. The resulting construct containing *npt2* flanked by *flgK* and *flgL* sequences was transferred into pLVC-D as previously described. pLVC-D*flgKL* was introduced into donor *E. coli* strain S17-1 by electroporation. Biparental mating and selection of mutants were done as previously described.

PilB and PilC are involved in export and assembly of TFP pilin monomers (Watson *et al.*, 1996). Removal of either one or both results in loss of pilus construction (Nunn *et al.*, 1990; Strom and Lory, 1993). Disruption of *pilB* (gb/CP000542.1/:4306572-4311008, Veis_3919) and *pilC* (Veis_3920) of *V. eiseniae* EF05-2r was generated by insertion of the kanamyacin-resistant marker. DNA fragments of ~1 Kb in size were amplified from EF05-2r genomic DNA, using primers pilBfrag-F (5′-CCTAGGATTTACAAGAAGTCCCAGGCCAACCGCA-3′), pilBfrag-R (5′-AAGCTTTACCTGATTCACGCCCGGCAGGTTGATT-3′), pilCfrag-F (5′-CTCGAGAAAGTCTTCGAATGGGAAGGCAAGGACC-3′) and pilCfrag-R (5′-TCTAGACTTCACCAATGGCACACATTTGCAGCACCA-3′). The *pilB* sequence was cloned upstream of *npt2* in pENTR/D-Topo-MCS:kan as a *Avr*II/*Hin*dIII fragment and the *pilC* sequence was subsequently cloned downstream of *npt2* as a *Xho*I/*Xba*I fragment. The resulting construct containing *npt2* flanked by *pilB* and *pilC* fragments was cloned into pLVC-D to create pLVC-D*pilBC*. Conjugation of EF05-2r was performed as above and mutants were screened for marker insertion. A mini-Tn5 transposon conferring kanamycin resistance was introduced into *V. eiseniae* EF05-2r from *E. coli* S17-1 (pRL27) (Larsen *et al.*, 2002) through biparental mating, at a 1:10 ratio, O/N at 28 °C and subsequent selection on ACM with appropriate antibiotics to generate RTn5.1 (Random Tn5 clone1) and RTn5.2. All resulting mutants (VE*flgK*−, VE*flgKL*− and VE*pilBC*−) screened for resistance marker were confirmed using PCR to contain the appropriate insertion.

DNA sequencing

Genomic DNA of *V. eiseniae* EF05-2r was collected with a DNAeasy tissue kit (Qiagen, Germantown, MD, USA). Automated DNA sequencing was preformed at the University of Washington High Throughput DNA Sequencing Facility via illumina sequencing. The DNA sequence was assembled and open reading frames were assigned with a combination of the Glimmer3 algorithm (Delcher *et al.*, 2007), GeneSifter (Geospiza, Seattle, WA, USA) and BLAST (Altschul *et al.*, 1990). Accession numbers of DNA sequences: *pilBCDcoaE* (JN900250), *flgIJKL* (JN900251).

Motility assays

Cells were grown on ACM plates, harvested and washed in 10 mM KPO_4 buffer, then 10^7 cells were stab inoculated into 0.3% agar ACM plates, incubated overnight at 28 °C and examined for cell migration through agar. Twitching motility was observed by stab inoculation into the bottom of 1.5% phytagel ACM plates and examined for movement along the petri dish bottom-surface. Strains were also harvested from plates, suspended in ACM broth ($OD_{600} \approx 1.0$) and observed in suspended static culture at room temperature for adherence behavior, aggregation and binding to the culture tubes.

Electron microscopy

Cells were grown on ACM plates, collected and suspended in 1.25% glutaraldehyde, 0.1 M sodium phosphate buffer, pH 7.3, overnight at 4 °C, washed in buffer, and post-fixed with 2% osmium tetroxide, in 0.05 M phosphate buffer 1.5 h, rinsed and stored in 0.1 M phosphate buffer, pH 7.3. Samples for transmission electron microscope were mounted on 150 mesh rhodium/copper grids, stained with uranyl acetate and lead citrate and examined using a JEM 1200EX II TEM (JEOL Ltd, Tokyo, Japan).

Elimination and reestablishment of V. eiseniae *from earthworm egg capsules and nephridia*

Adult and juvenile *E. fetida* were maintained as previously described (Davidson and Stahl, 2006; Davidson *et al.*, 2010). Egg capsules were collected and maintained on moistened filter paper in petri dishes. Bacterial symbionts were eliminated by treatment of egg capsules with antibiotics as previously described (Davidson and Stahl, 2006). In addition to 150 μg ml^{-1} kanamycin for elimination of *V. eiseniae*, 150 μg ml^{-1} erythromycin was used to eliminate both *V. eiseniae* and *Flexibacter* species. Hatchlings from each treatment were assayed for nephridial bacteria by fluorescence *in situ* hybridization (FISH; see next section) ($n = 10$–15). Cured worms were maintained until sexual maturity and offspring monitored for nephridial bacteria.

Capsules from cured adults were used to test colonization competence of mutants.

Cured capsules were inoculated with *V. eiseniae* EF05-2r by injection to reestablish nephridial bacteria. Eggs were first surface sterilized with 50% ethanol for 15 s, rinsed with sterile water three times and then allowed to dry until a small dimple appeared in the shell to accommodate additional fluid. Bacterial suspensions, approximately 1–3 μls, were injected into capsules with a 50 g needle and capsules were maintained in petri dishes at room temperature (~20 °C). Eggs were partially submerged in diH20 to maintain humidity and hydration of eggs, and 30 μg ml^{-1} kanamycin was supplemented in suspension water to maintain selective pressure on mutants. At least 10 capsules were used per treatment, and at least 6 worms assayed from each treatment by FISH 2 days after hatching. Colonization of hatchlings was observed from 10 segments, each typically containing 2 nephridia, in the anterior, middle and posterior areas of hatchlings.

Optimal bacterial cell number needed to achieve full colonization was established by injecting a series of *V. eiseniae* EF05-2r cell solutions, ranging from 8×10^3 to 3.6×10^8 cells (~0.002–100-fold average normal number), into capsules (0–1-day-old). The optimal time for injection for full colonization was tested by injecting the effective number of cells into capsules containing embryos staged at 0–3 days, 5–7 days and 10+ days of development. Cured capsules lacking both *V. eiseniae* and *Flexibacter* species or with *Flexibacter* sp. present were inoculated to test interference by precolonization of the nephridia by *Flexibacter* sp.

FISH for detection of nephridial colonization
Fixation and FISH were performed on intact hatchlings as described previously using stringent conditions established for each probe (Schramm *et al.*, 2003; Davidson and Stahl, 2008). The following probes were used: LSB 145—*V. eiseniae* (Schweitzer *et al.*, 2001; Schramm *et al.*, 2003), EUB 338—bacterial domain (Amann, 1995) and Flexi 145—genus *Flexibacter* (Davidson *et al.*, 2010). Specimens were mounted in Vectashield (Vector Labs, Inc., Burlingame, CA, USA) and fluorescence detected using excitation at the appropriate wavelength with a Zeiss LSM Pascal laser scanning confocal microscope (Carl Zeiss, Jena, Germany).

Results

Motility and aggregation of V. eiseniae
Flagellar motility. *V. eiseniae* possesses genes needed for flagella production. The gene cluster *flgIJKL* is arranged in an apparent operon in *V. eiseniae* EF01-2. Sequence analysis of *V. eiseniae* EF05-2r *flgIJKL* showed identical synteny and 99% sequence similarity. FlgK and FlgL function as flagellar hook-associated proteins that form the junction between the hook (FlgE) and filament monomers (FliC) (Berg, 2003). After 24 h of incubation, a band of *V. eiseniae* EF05-2r cells had clearly migrated away from the point of inoculation in soft agar plates (Figure 2a). Interruption of the *flgKL* coding sequences of EF05-2r was confirmed by PCR, after transformation via conjugation. The flagella mutants, VE*flgK*– (Figure 2a) and VE*flgKL*–(data not shown), failed to move away from the point of inoculation in semisolid media.

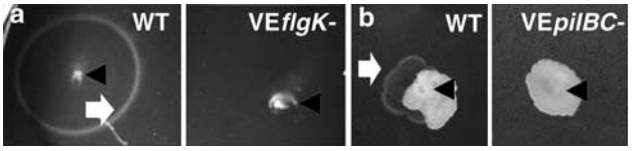

Figure 2 Motility tests of *V. eiseniae* wild-type and mutants. (**a**) Soft agar plate inoculated with *V. eiseniae* EF05-2r (left) and VE*flgK*– (right); (**b**) Phytagel plate stab inoculated with EF05-2r (left) and VE*pilBC*– (*right*). Arrowheads, point of inoculation; arrows, migrating front of cells. Note: label markings were digitally removed from A.

TFP-mediated motility. *V. eiseniae* also possesses genes required for TFP synthesis. The gene cluster *pilBCDcoaE* is arranged in an apparent operon in *V. eiseniae* EF01-2. Sequence analysis of *V. eiseniae* EF05-2r *pilBCDcoaE* showed identical synteny and 99% sequence similarity. The *pilB* and *pilC* genes encode components of the TFP machinery that export and polymerize PilA, pilin monomers. PilD cleaves PilA subunits prior to TFP assembly (Strom *et al.*, 1993; Mattick, 2002). The function of *coaE* in TFP construction is undetermined. After stab inoculation, *V. eiseniae* EF05-2r cells were observed to move along the interface of the plastic bottom and the phytagel media (Figure 2b), a behavior characteristic of twitching motility. Transmission electron microscope analysis of cells from static cultures revealed rod-like structures extending as much as 4 μM away from the polar ends of wild-type (Figure 3a) VE*flgK*– and VE*flgKL*– cells moved between the plastic–phytagel interface and possessed rod-like structures identical to wild type (data not shown). Replacement of portions of coding regions of both *pilB* and *pilC* in EF05-2r, confirmed by PCR as above, eliminated twitching motility (Figure 2b) and cell surface pilus structures in VE*pilBC*– (Figure 3b).

Aggregation in liquid media. In static culture, the wild-type *V. eiseniae* EF05-2r cells aggregated and adhered weakly to the surface of plastic falcon tubes (Figure 4a). The flagellar mutants, VE*flgK*– (Figure 4b) and VE*flgKL*– (data not shown), adhered to the plastic surface as well. Although association with the tube wall was not stable, as gentle agitation easily displaced the aggregates, cell clumping was visible by light microscopy. Disruption of *pilB* and *pilC* resulted in loss of the aggregation phenotype,

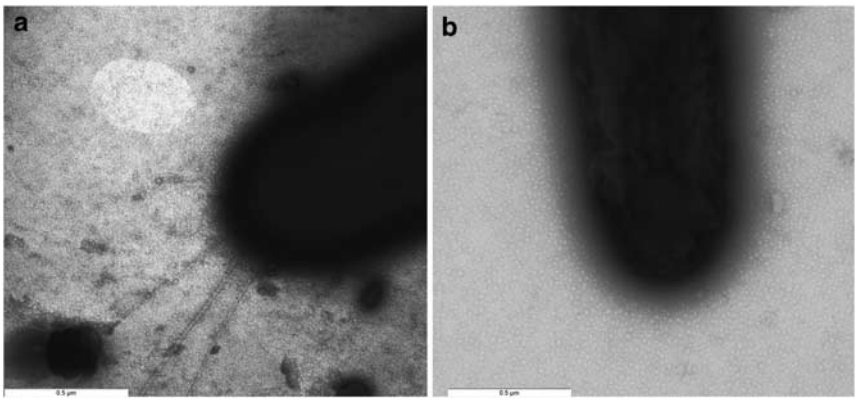

Figure 3 Transmission electron images of *V. eiseniae* TFP. Transmission election micrographs of *V. eiseniae* EF05-2r (**a**) and VE*pilBC*– (**b**). Scale bar, 0.5 μm.

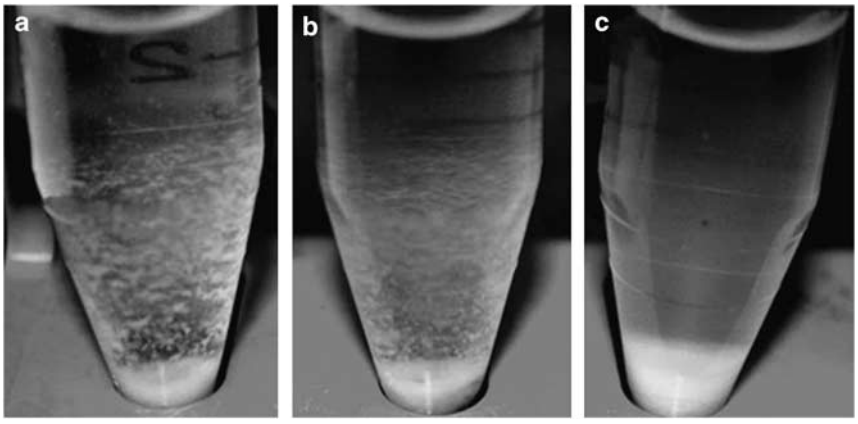

Figure 4 Aggregation and attachment of *V. eiseniae*. *V. eiseniae* EF05-2r (**a**), VE*flgK*– (**b**), and VE*pilBC*– (**c**) in static culture within falcon tubes.

and cells failed to form clumps or bind to the sides of the tubes. VE*pilBC*– cells often remained dispersed in solution, and eventually settled to the bottom of the tube (Figure 4c).

Curing and reestablishment of V. eiseniae in earthworm nephridia. Nephridia of untreated hatchling worms are colonized by both *V. eiseniae* and *Flexibacter* sp. in the ampulla and only *V. eiseniae* in the bladder (Figure 5a). Treatments of capsules with 150 μg ml^{-1} kanamycin resulted in *V. eiseniae*-free hatchlings that retained *Flexibacter* sp. (Figure 5b). Treatment with 150 μg ml^{-1} erythromycin eliminated all nephridial symbionts, confirmed by FISH LSCM (Figure 5c) with EUB-338, Flexi 145 and LSB 145 probes, which resulted in no signal. Hatchlings from both treatments grew to sexual maturity.

Capsules (0–1-day old), each containing approximately 5 μl of albumin, have an estimated 9x10^5 bacterial cells per μl of albumin, with approximately 3.2×10^6 *V. eiseniae* cells per capsule (Davidson and Stahl, 2008). In capsules lacking both *V. eiseniae* and *Flexibacter* sp., 8×10^5 and 8×10^6 EF05-2r cells (0.25 and 2.5 times the estimated normal population, respectively) were required for full colonization of hatchling nephridia ($n = 10$ hatchlings, 60 nephridia per hatchling observed). Inconsistent colonization resulted from injection of 8×10^4 cells, and no colonization was observed with 8×10^3 EF05-2r cells. Embryos failed to develop following injection of cell numbers >100-fold the normal *V. eiseniae* population.

Injections of cultivated EF05-2r into egg capsules lacking both *Flexibacter* sp. and *V. eiseniae* resulted in full colonization regardless of the stage of embryos at time injection ($n = 6$, 60 nephridia per *E. fetida* hatchling at each stage) (Table 1). In contrast, inoculations of EF05-2r into capsules containing *Flexibacter* sp. resulted in variable *V. eiseniae* colonization associated with developmental stage (Table 1). The excess (10 ×) number of injected *V. eiseniae* cells relative to naturally occurring numbers did not alter normal co-colonization of *Flexibacter* sp. symbiont regardless of embryonic stage at inoculation. Worms associated with only EF05-2r or EF05-2r and *Flexibacter* sp. were raised to sexual maturity and no developmental defects were observed in lab culture.

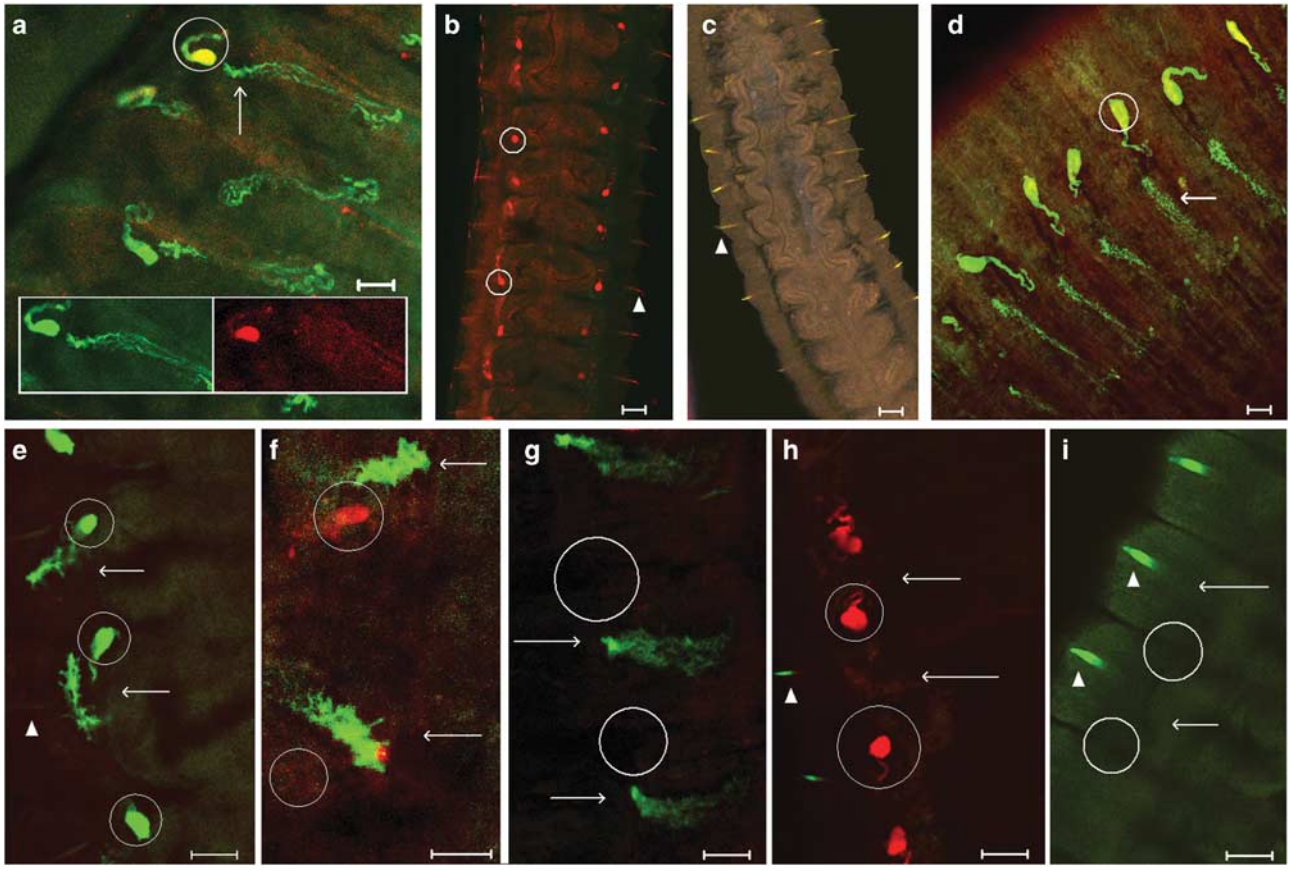

Figure 5 FISH LSCM images of nephridia of *E. fetida* hatchlings. Hatchlings from (**a**) untreated capsule, (**b**) 150 μg ml^{-1} kanamycin treatment and (**c**) 150 μg ml^{-1} eurythromycin treatment. (**d**) Hatchlings from capsules lacking *V. eiseniae* inoculated with EF05-2r and (**e**) capsules lacking both symbionts inoculated with EF05-2r. (**f**) Hatchlings from *V. eiseniae*-free egg capsules inoculated with VE*flgK*– and (**g**) symbiont-cured egg capsules inoculated with VE*flgK*–. (**h**) Hatchlings from *V. eiseniae*-free egg capsules inoculated with VE*pilBC*– and (**i**) symbiont-cured egg capsules inoculated with VE*pilBC*–. Green, *V. eiseniae*; Red, *Flexibacter* sp. Scale bars, 50 μm; arrows, bladder; arrowheads, autofluorescent locomotive setae structures; circles, ampulla.

Table 1 Nephridial colonization timecourse of *V. eiseniae*[a]

Bacteria present in egg capsule	*Embryo age at inoculation*		
	0–3 days	*5–8 days*	*10+ days*
Kanamycin-treated colony (*V. eiseniae*–, *Flexibacter sp.*+)	99 (± 2)	91 (± 6)	65 (± 13)
Erythromycin-treated colony (*V. eiseniae*–, *Flexibacter sp.*–)	100	100	100

[a]Average percentage (± s.e.) of hatchling nephridia normally colonized with EF05-2r, $n = 6$ hatchlings, 60 nephridia observed per worm.

Assessment of mutant colonization competence. Capsules (0–3 days) inoculated with 5×10^7 EF05-2r cells yielded fully colonized hatchlings with *V. eiseniae* cells in the ampulla (circled) and bladder (arrows) of the nephridia, with either *Flexibacter sp.* present or absent (Figures 5d and e). Consistent with normal colonization, cells only transiently resided in the bladder. At maturity, bacteria were found only in the ampulla. Hatchlings from capsules injected with VE*flgK*– cells rarely showed *Verminephrobacter* cells in the ampulla and primarily only in the bladder, regardless of the presence or absence of *Flexibacter* sp. These cells failed to move beyond the bladder (Figures 5f and g). Juveniles examined 2–3 weeks post hatching did not contain VE*flgK*– cells in either bladder or ampulla. *Flexibacter* sp. colonization of the ampulla appeared normal. Hatchlings from capsules injected with *VEflgKL*– exhibited the same results.

The TFP mutant failed to colonize the earthworm nephridia regardless of the presence or absence of the *Flexibacter* sp. symbiont. Inoculation of 5×10^7 VE*pilBC*– cells into capsules (0–3 days) lacking *V. eiseniae*, or both *V. eiseniae* and *Flexibacter* sp., failed to show *V. eiseniae* cells in any region of the nephridia when examined by FISH LSCM (Figures 5h and i). *Flexibacter* sp. appeared in the ampulla as in controls. Repeated colonization experiments with

Table 2 Nephridial colonization by *V. eiseniae* in hatchlings

	Flexibacter *sp. present*[b]	*Colonization of nephridia*[a]	
		Bladder	*Ampulla*
EF05-2r	+	+	+
	−	+	+
VE*flgK*−	+	+	−
	−	+	−
VE*flgK*−.c2	+	+	−
	−	+	−
VE*flgKL*−	+	+	−
	−	+	−
VE*pilBC*−	+	−	−
	−	−	−
VE*pilBC*−.c2	+	−	−
	−	−	−
Rtn5.1	+	+	+
	−	+	+
Rtn5.2	+	+	+
	−	+	+

[a]*V. eiseniae* innoculated into 0–3 day-old egg capsules, presence or absence in nephridial segment detected by FISH, $n \geqslant 6$ hatchlings.
[b]Presence or absence of *Flexibacter* sp. in egg capsule prior to inocculation and detected in ampulla by FISH, $n \geqslant 6$ hatchlings.

identical, yet independently generated, mutations of the flagella and TFP mutants displayed the same colonization deficiencies (Table 2, $n = 6$ hatchlings). Random Tn5 insertion mutants of *V. eiseniae* were used as controls to determine secondary effects of the *npt2* insertion or presence of antibiotics throughout the colonization period. *V. eiseniae* strains RTn5.1 and RTn5.2 containing a random mini-Tn5 cassette displayed normal colonization in similar inoculations of egg capsules, appearing in both the ampulla and the bladder of hatchlings (Table 2, $n = 10$ hatchlings).

Discussion

This work provides a significant advancement in development of a new model system for understanding mechanisms of non-pathogenic bacteria–host interactions. The results describe the first mechanisms identified for early stages of establishment of a symbiont in an embryonic host stage. Methods for targeted gene disruption were demonstrated for the first time for host species-specific symbiont *V. eiseniae* EF05-2r and used to show the requirement of two distinct mechanisms of motility for successful colonization of the host. Using the *V. eiseniae* EF01-2 genome to guide gene selection, both flagellar and TFP systems were successfully disrupted, as shown by the loss of flagellar motility with *flgK* and *flgL* mutations, and loss of twitching with *pilB* and *pilC* disrupted. These two modes of motility are necessary for *V. eiseniae* to complete the journey from albumin into the colonization duct, then into the bladder and finally the ampulla of nascent nephridia. Without flagella, but TFP intact,

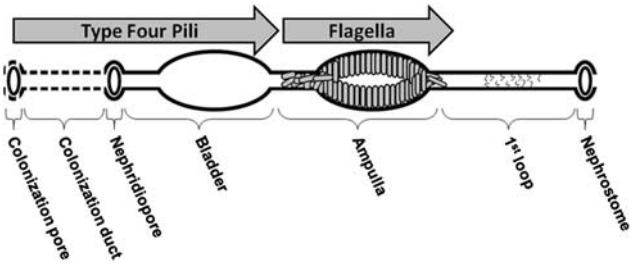

Figure 6 Colonization pathway of *V. eiseniae* in protonephridia. *V. eiseniae* use TFP to reach, enter and migrate to the bladder, then require flagella to reach the ampulla.

cells make it as far as the bladder, indicating that TFP are used to migrate into the duct, and bladder, but that flagella are required to make it the rest of the way. The failure of TFP mutants to enter the duct, even with flagellar motility intact, indicates that TFP are essential for successful migration into and/or through the colonization pore and duct (Figure 6). Use of redundant mutant strains producing identical phenotypes in culture and colonization experiments supports our conclusions and lessens the possibility of secondary mutations obfuscating colonization defects observed, despite the lack of a mutant complementation method.

These observations suggest two important features of this colonization process. First, surface features of the embryo and the colonization duct are critical for initial migration mediated by TFP-binding. These may be specific receptors that act to select bacteria with the proper adhesins. If chemotaxis is involved in directing the cells, it is by directed twitching motility. Second, swimming is required to get past the bladder and stay in the ampulla, suggesting an outward current produced by ciliated tubules in the nephridia is active at this stage and may serve as a barrier to bacterial cells that cannot swim. In addition to swimming, flagella can serve as an attachment point during colonization. Flagellar cap protein FliD of *Pseudomonas aeurugenosa* and *Clostridium difficile* has been shown to adhere to epitopes present in host tissues (Arora *et al*., 1998; Tasteyre *et al*., 2001), and flagellin monomer FliC of enteropathogenic and enterohemorrhagic *E. coli* has a strong binding affinity for mucin of host mucosal surfaces (Erdem *et al*., 2007). The polar orientation of *V. eiseniae* cells within the ampulla (Pinel *et al*., 2008) indicates possible binding interactions with polar flagella or TFP. If flagella are required to bind the ampulla wall, flagellar mutants would be cleared from the system and collected in the bladder before being eliminated.

The process of migration terminates with binding to the ampulla surface. The ampulla is not a blind end pouch, but tubules enter and exit the ampulla. The receptors here, or the chemical signals, lead to binding mediated by TFP, flagella and/or cell surface receptors, and a cessation of motility. Although both pili and fimbriae are among

the better-characterized structures of microbial attachment (Mattick, 2002), fimbriae are not likely involved because neither fimbriae genes in the genome nor structures in micrographs were detected in *Verminephrobacter*. Pili are well described in pathogen–host interactions, including regulation of immune responses through binding to cell surface complement regulators, and persistence at colonization sites through binding (Kallstrom *et al.*, 2001). However, there are few characterized beneficial bacteria TFP-mediated host interactions, but these examples support a critical role for TFP in early colonization. Similar to *E. fetida* (Davidson *et al.*, 2010), hatchling squid acquire *Vibrio* spp. symbionts from a mixed microbial community, and *pilA* mutation in *Vibrio fisheri* generated a competitive disadvantage when challenged with wild type for colonization of light organ (Stabb and Ruby, 2003). For endophytic N_2-fixing *Azoarcus* sp. BH72, TFP are necessary to initiate colonization of rice plants (Bohm *et al.*, 2007). Although dense *Verminephrobacter* populations in the albumen (Davidson and Stahl, 2008) and movement of worm embryos throughout this suspension suggest the embryo surfaces would be in constant contact with bacteria, the TFP or flagella may enable the symbiont to maneuver through the albumen to the embryo surface and duct entrance. The nature of the binding specificity of *V. eiseniae* to surfaces of the earthworm embryo remains to be explored.

Important inter-specific bacterial interactions were noted during the development of this model system. Associations with other bacterial species have been documented in well-established model animal-symbiont systems. *Aeromonas veronii* associates with *Rikenella*-like bacteria in microcolonies that colonize the digestive tract of medicinal leech *Hiurdo verbena* (Kikuchi and Graf, 2007). *V. fisheri* outcompetes related nonsymbiotic species during colonization of squid hatchling mucus secretion in aggregates outside infection sites (Nyholm and McFall-Ngai, 2003). Although aggregates of *Flexibacter* sp. and *V. eiseniae* cells are observed in the albumen, associate at the entrance pore and are found in the colonization duct together (Davidson and Stahl, 2008; Davidson *et al.*, 2010), observations from selective curing experiments indicate that *V. eiseniae* and *Flexibacter* sp. symbionts are not dependent on each other for colonization and persistence. Single member colonization does not rule out the possibility that both are working in concert in nephridia of mature worms under normal field conditions. Introduction of *V. eiseniae* later in development demonstrated that the presence of *Flexibacter* sp. can interfere with *V. eiseniae* colonization possibly by competitive exclusion. *Flexibacter* sp. has been visualized to be bound to ampulla cells as was *V. eiseniae* (Davidson *et al.*, 2010). It is possible that *Flexibacter* sp. migration into nascent nephridia prior to *V. eiseniae* occupies binding sites too densely for co-colonization by late arrivals. Specific development events, such as change in host chemical cueing or duct architecture, may also limit colonization. Crypt cell morphology of the squid light organ is altered, increased microvillar density and cell swelling, post inoculation with *Vibrio* symbionts (Montgomery and McFall-Ngai, 1994; Lamarcq and McFall-Ngai, 1998), presumably increasing interactions to establish symbiosis. These observations indicate that synchronization of bacterial colonization in the embryo is important for full colonization. Preemptive colonization of biocontrol agent *Pseudomonas fluorecens* A506 reduces colonization of the *Erwinia amylovera* fire blight pathogen on pear nectaries (Wilson and Lindow, 1993). Previous observations showed that *Flexibacter* sp. may arrive earlier than *V. eiseniae* on occasion, but under normal egg capsule circumstances, *V. eiseniae* likely enters soon after to colonize the ampulla (Davidson *et al.*, 2010).

Essential tools for analysis of mechanisms governing *V. eiseniae* interactions with the earthworm *E. fetida* during colonization were established in this study. This new model system adds to the limited number of symbiotic associations being studied that have one or both members amenable to genetic manipulation (Ruby, 2008). With this project, we have begun to study specific mechanisms of this association fulfilling some of molecular Koch's postulates (Falkow, 1988) for in-depth analysis of molecular mechanisms. This is a novel model based on molecular mechanisms of selective bacterial colonization during embryogenesis. Although much has been gleaned looking at the end point colonization of the nephridia in hatchling worms, further analysis throughout embryonic development is needed to refine mechanisms of the observed colonization deficiencies.

Acknowledgements

This work was supported by the National Science Foundation Grants IOB 0951119 (to SKD) and IOB 0345049 (to DAS and SKD). Funding for GD was provided by an awarded NSF Minority Postdoctoral Fellowship (DBI 0805653). RG was funded by the REU/NSF program under amendment IOB 0619945 to NSF IOB 0345049.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990). Basic local alignment search tool. *J Mol Biol* **215**: 403–410.

Amann RI. (1995). Fluorescently labeled, ribosomal-rna-targeted oligonucleotide probes in the study of microbial ecology. *Mol Ecol* **4**: 543–553.

Ames P, Bergman K. (1981). Competitive advantage provided by bacterial motility in the formation of nodules by rhizobium-meliloti. *J Bacteriol* **148**: 728–729.

Arora SK, Ritchings BW, Almira EC, Lory S, Ramphal R. (1998). The Pseudomonas aeruginosa flagellar cap protein, FliD, is responsible for mucin adhesion. *Infect Immun* **66**: 1000–1007.

Berg HC. (2003). The rotary motor of bacterial flagella. *Ann Rev Biochem* **72**: 19–54.

Bohm M, Hurek T, Reinhold-Hurek B. (2007). Twitching motility is essential for endophytic rice colonization by the N-2-fixing endophyte Azoarcus sp strain BH72. *Mol Plant Microbe Interact* **20**: 526–533.

Bright M, Bulgheresi S. (2010). A complex journey: transmission of microbial symbionts. *Nat Rev Microbiol* **8**: 218–230.

Davidson SK, Stahl DA. (2006). Transmission of nephridial bacteria of the earthworm Eisenia fetida. *Appl Environ Microbiol* **72**: 769–775.

Davidson SK, Stahl DA. (2008). Selective recruitment of bacteria during embryogenesis of an earthworm. *ISME J* **2**: 510–518.

Davidson SK, Powell RJ, Stahl DA. (2010). Transmission of a bacterial consortium in Eisenia fetida egg capsules. *Environ Microbiol* **12**: 2277–2288.

Delcher AL, Bratke KA, Powers EC, Salzberg SL. (2007). Identifying bacterial genes and endosymbiont DNA with Glimmer. *Bioinformatics (Oxford)* **23**: 673–679.

Erdem AL, Avelino F, Xicohtencatl-Cortes J, Giron JA. (2007). Host protein binding and adhesive properties of H6 and H7 flagella of attaching and effacing Escherichia coli. *J Bacteriol* **189**: 7426–7435.

Falkow S. (1988). Molecular kochs postulates applied to microbial pathogenicity. *Rev Infect Dis* **10**: S274–S276.

Goodrich-Blair H. (2007). They've got a ticket to ride: Xenorhabdus nematophila–Steinernema carpocapsae symbiosis. *Curr Opin Microbiol* **10**: 225–230.

Graf J. (2005). Molecular requirements for the colonization of Hirudo medicinalis by Aeromonas veronii.. In: Overmann J (ed), *Molecular mechanisms of symbiosis. Series: Progress in Molecular and Subcellular Biology*. Springer-Verlag: Berlin.

Graf J, Dunlap PV, Ruby EG. (1994). Effect of transposon-induced motility mutations on colonization of the host light organ by vibrio-fischeri. *J Bacteriol* **176**: 6986–6991.

Haefele DM, Lindow SE. (1987). Flagellar motility confers epiphytic fitness advantages upon pseudomonas-syringae. *Appl Environ Microbiol* **53**: 2528–2533.

Kallstrom H, Gill DB, Albiger B, Liszewski MK, Atkinson JP, Jonsson AB. (2001). Attachment of Neisseria gonorrhoeae to the cellular pilus receptor CD46: identification of domains important for bacterial adherence. *Cell Microbiol* **3**: 133–143.

Kikuchi Y, Graf J. (2007). Spatial and temporal population dynamics of a naturally occurring two-species microbial community inside the digestive tract of the medicinal leech. *Appl Environ Microbiol* **73**: 1984–1991.

Knop J. (1926). Bakterien und bakteroiden bei Oligochaeten. *Z Morphol Ökol Tiere* **6**: 588–624.

Lamarcq LH, McFall-Ngai MJ. (1998). Induction of a gradual, reversible morphogenesis of its host's epithelial brush border by vibrio fischeri. *Infect Immun* **66**: 777–785.

Larsen RA, Wilson MM, Guss AM, Metcalf WW. (2002). Genetic analysis of pigment biosynthesis in Xanthobacter autotrophicus Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. *Arch Microbiol* **178**: 193–201.

Lund MB, Davidson SK, Holmstrup M, James S, Kjeldsen KU, Stahl DA *et al.* (2010). Diversity and host specificity of the Verminephrobacter-earthworm symbiosis. *Environ Microbiol* **12**: 2142–2151.

Malek W. (1992). The role of motility in the efficiency of nodulation by rhizobium-meliloti. *Arch Microbiol* **158**: 26–28.

Marco ML, Legac J, Lindow SE. (2005). Pseudomonas syringae genes induced during colonization of leaf surfaces. *Environ Microbiol* **7**: 1379–1391.

Mattick JS. (2002). Type IV pili and twitching motility. *Annu Rev Microbiol* **56**: 289–314.

Montgomery MK, McFall-Ngai M. (1994). Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid Euprymna scolopes. *Development (Cambridge)* **120**: 1719–1729.

Nachamkin I, Yang XH, Stern NJ. (1993). Role of Campylobacter-Jejuni flagella as colonization factors for 3-day-old chicks—analysis with flagellar mutants. *Appl Environ Microbiol* **59**: 1269–1273.

Nunn D, Bergman S, Lory S. (1990). Products of 3 accessory genes, Pilb, Pilc, and Pild, are required for biogenesis of pseudomonas-aeruginosa Pili. *J Bacteriol* **172**: 2911–2919.

Nussbaumer AD, Fisher CR, Bright M. (2006). Horizontal endosymbiont transmission in hydrothermal vent tubeworms. *Nature* **441**: 345–348.

Nyholm SV, McFall-Ngai MJ. (2003). Dominance of Vibrio fischeri in secreted mucus outside the light organ of Euprymna scolopes: The first site of symbiont specificity. *Appl Environ Microbiol* **69**: 3932–3937.

Nyholm SV, Mcfall-Ngai MJ. (2004). The winnowing: establishing the squid-Vibrio symbiosis. *Nat Rev Microbiol* **2**: 632–642.

Pinel N. (2009). Physiological and genomic insights into the biology of verminephrobacter eiseniae, a bacterial symbiont of the earthworm eisenia foetida. Ph.D. thesis, University of Washington, Seattle.

Pinel N, Davidson SK, Stahl DA. (2008). Verminephrobacter eiseniae gen. nov., sp nov., a nephridial symbiont of the earthworm Eisenia foetida (Savigny). *Int J Syst Evol Microbiol* **58**: 2147–2157.

Ruby EG. (2008). Symbiotic conversations are revealed under genetic interrogation. *Nat Rev Microbiol* **6**: 752–762.

Schramm A, Davidson SK, Dodsworth JA, Drake HL, Stahl DA, Dubilier N. (2003). Acidovorax-like symbionts in the nephridia of earthworms. *Environ Microbiol* **5**: 804–809.

Schweitzer B, Huber I, Amann R, Ludwig W, Simon M. (2001). Alpha- and beta-proteobacteria control the consumption and release of amino acids on lake snow aggregates. *Appl Environ Microbiol* **67**: 632–645.

Shepherd RW, Lindow SE. (2009). Two dissimilar N-acyl-homoserine lactone acylases of pseudomonas syringae influence colony and biofilm morphology. *Appl Environ Microbiol* **75**: 45–53.

Simon R, Oconnell M, Labes M, Puhler A. (1986). Plasmid vectors for the genetic-analysis and manipulation of rhizobia and other gram-negative bacteria. *Methods Enzymol* **118**: 640–659.

Stabb EV, Ruby EG. (2003). Contribution of pilA to competitive colonization of the squid Euprymna scolopes by Vibrio fischeri. *Appl Environ Microbiol* **69**: 820–826.

Strom MS, Lory S. (1993). Structure-function and biogenesis of the Type-Iv Pili. *Annu Rev Microbiol* **47**: 565–596.

Strom MS, Nunn DN, Lory S. (1993). A single bifunctional enzyme, PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family. *Proc Nat Acad Sci USA* **90**: 2404–2408.

Tasteyre A, Barc MC, Collignon A, Boureau H, Karjalainen T. (2001). Role of FliC and FliD flagellar proteins of Clostridium difficile in adherence and gut colonization. *Infect Immun* **69**: 7937–7940.

Wassenaar TM, Vanderzeijst BAM, Ayling R, Newell DG. (1993). Colonization of chicks by motility mutants of Campylobacter-Jejuni demonstrates the importance of flagellin-a expression. *J Gen Microbiol* **139**: 1171–1175.

Watson AA, Alm RA, Mattick JS. (1996). Identification of a gene, pilF, required for type 4 fimbrial biogenesis and twitching motility in Pseudomonas aeruginosa. *Gene* **180**: 49–56.

Wilson M, Lindow SE. (1993). Interactions between the biological control agent Pseudomonas fluorescens A506 and Erwinia amylovora in pear blossoms. *Phytopathology* **83**: 117–123.