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Arabinose Induces Pellicle Formation by *Vibrio fischeri*

Karen L. Visick, Kevin P. Quirke, Sheila M. McEwen

Department of Microbiology and Immunology, Loyola University Medical Center, Maywood, Illinois, USA

Biofilms are multicellular communities of bacteria attached to a surface and embedded in a protective matrix. In many cases, the signals that induce biofilm formation are unknown. Here, we report that biofilm formation by the marine bacterium *Vibrio fischeri* can be induced by the addition of arabinose to LBS (Luria-Bertani-salt), a tryptone-based medium. Growth of cells in the presence of 0.2% arabinose, but not other sugars, induced the production of a pellicle at the air/liquid interfaces of static cultures. *V. fischeri* failed to grow on arabinose as the sole carbon source, suggesting that pellicle production did not occur as a result of increased growth, but experiments using the acid/base indicator phenol red suggested that *V. fischeri* may partially metabolize arabinose. Pellicle production was independent of the *syp* polysaccharide locus but was altered upon disruption of the *bcs* cellulose locus. Through a screen for mutants defective for pellicle production, we found that loss of motility disrupted the formation of the arabinose-induced pellicle. Among the ~20 mutants that retained motility were strains with insertions in a putative *msh* pilus locus and a strain with a defect in *yidK*, which is involved in galactose catabolism. Mutants with the *msh* gene disrupted grew poorly in the presence of arabinose, while the *yidK* mutant appeared to be "blind" to the presence of arabinose. Finally, arabinose impaired symbiotic colonization by *V. fischeri*. This work thus identifies a novel signal and new pathways involved in control of biofilm formation by *V. fischeri*.

Bacteria are highly adaptable: they recognize, integrate, and ultimately respond to numerous cues in their environments. The presence of nutrients, for example, could indicate a favorable environment and thus promote induction of genes involved in growth and/or attachment. Alternatively, a lack of nutrients could promote motility and a search for a better environment.

One type of nutrient readily sensed by cells is the presence of an appropriate carbon source. The surfaces of animals often contain carbohydrates, which bacteria use for attachment, signaling, and/or growth. For example, *Bacteroides thetaiotaomicron* recognizes L-fucose from fucosylated glycans present on the surfaces of intestinal cells. The availability of this molecule causes *B. thetaiotaomicron* to induce a fucose utilization pathway, resulting in the ability of these cells to grow and induce the continuing production by the host of the fucosylated glycans (1).

Another response of bacteria to carbon sources in the environment is the production of a biofilm, a community of bacteria typically attached to a surface and embedded in a self-produced matrix (2–4). For example, in *Pseudomonas aeruginosa*, pyruvate and pyruvate metabolism are required for microcolony formation, an early step in the production of biofilms (5). In *Vibrio cholerae*, either mannose or glucose can induce biofilm formation (6). Biofilms provide protection from environmental stresses, such as antibiotics and other antimicrobial agents. It is now well accepted that bacteria present in biofilms are responsible for the majority of infectious diseases. However, much remains to be learned about the conditions that signal for or induce biofilm formation.

More is known about components necessary for the production of biofilms. For example, flagella have been identified in numerous organisms as playing a role in the early stages of biofilm development (7–10). Pili, such as mannose-sensitive hemagglutinin (Msh), promote attachment by various bacteria (11–13). The biofilm matrix is typically comprised of macromolecules such as polysaccharides and proteins. Although many bacteria produce a unique polysaccharide, one polysaccharide present in the matrix of biofilms produced by numerous microbes is cellulose (14–17). Finally, proteins secreted into the matrix often play structural roles (18, 19).

One useful model for studying the control over biofilm formation is the marine bacterium *Vibrio fischeri*. Under typical laboratory conditions, *V. fischeri* forms biofilms poorly. However, genetic conditions have been established that induce strong biofilm phenotypes, including strong pellicles (biofilms at the air/liquid interfaces of static cultures) and rugose (wrinkled) colonies (20). In laboratory culture, the formation of these biofilms depends on the symbiosis polysaccharide (*syp*) locus. The *syp* locus is also required for biofilms that *V. fischeri* forms in the context of its symbiotic host, the squid *Euprymna scolopes* (21–24). However, the natural signals that induce the *syp* locus and biofilm formation are as yet unknown. *V. fischeri* also contains a cellulose polysaccharide locus, *bcs*, which is involved in biofilm formation under certain conditions (25, 26). It is thought that cellulose plays a role in cell surface attachment, but the role of the cellulose locus has been only minimally investigated, and its regulatory control remains poorly understood.

Here, we report the novel finding that *V. fischeri* can respond to the addition of L-arabinose with the production of a pellicle at the air/liquid interfaces of static liquid cultures. The bacteria do not

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Address correspondence to Karen L. Visick, kvisick@lumc.edu.

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TABLE 1 *V. fischeri* strains used in this study

Strain	Genotype or characteristic	Reference
DM127	*flrA*::*kan*	27
DM138	*flaC*::*kan*	28
DM143	*flaA*::*kan*	28
ES114	Wild-type	29
KV1172	Δ*cheR* (frameshift)	30
KV1356	Δ*galK*	31
KV1787	Δ*sypG*	32
KV3299	Δ*sypE*	33
KV4389	attTn*7*::Erm^r	23
KV5005	Δ*rpoN*	This study
KV5098	Δ*sypN*	34
KV5192	Δ*sypC*	34
KV5365	*flg*::Tn*5*	This study
KV5366	Δ*bcsA*	This study
KV5633	*ptsI*::Tn*5*	This study
KV5836	Δ*motA*	This study
KV5837	Δ*yidK*	This study
KV5849	Δ*galM*	This study
KV5939	*bcsE*::Tn*5*	This study
KV5940	*bcsE*::Tn*5*	This study
KV5941	IG (*VF_A0885-bcsE*)::Tn*5*[a]	This study
KV6004	Δ*mshA*	This study

[a] IG, intergenic.

use L-arabinose as a carbon source, which suggests that arabinose instead serves as a signal to induce pellicle production. Production of this pellicle did not require the *syp* polysaccharide locus, but investigation into the genetic requirements for arabinose-induced pellicle production revealed the involvement of the cellulose locus as well as motility genes and a number of previously uncharacterized genes. Finally, exposure to arabinose was detrimental to symbiotic colonization. This work thus uncovers a novel pathway for induction of biofilm formation in *V. fischeri.*

MATERIALS AND METHODS

Strains and media. Strains used in this study are listed in Table 1. For most of the work, *Vibrio fischeri* strain ES114 (29) and its derivatives were used in these studies. For cloning, *Escherichia coli* strains Tam1 (Active Motif, Carlsbad, CA), CC118 λ*pir* (35), DH5α, DH5α λ*pir*, β3914 (36), and π3813 (36) were used. For routine culturing, *V. fischeri* cells were grown in LBS (Luria-Bertani-salt) (37, 38), which contains 1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, 2% (wt/vol) sodium chloride, and 50 mM Tris-HCl, pH 7.5. In some experiments, we used SWT (seawater tryptone) (39), which contains 0.5% (wt/vol) tryptone, 0.3% (wt/vol) yeast extract, 35 mM $MgSO_4 \cdot 7H_2O$, 7 mM $CaCl_2 \cdot 2H_2O$, 210 mM NaCl, and 7.0 mM KCl. To evaluate the use of arabinose as a carbon source, we used a modified version of HMM (HEPES minimal medium) (40) that contained the following ingredients: 0.1% (wt/vol) ammonium chloride, 0.0058% K_2HPO_4, 150 mM NaCl, 25 mM $MgSO_4 \cdot 7H_2O$, 5 mM $CaCl_2$, 5 mM KCl, 10 μM ferrous ammonium sulfate, 100 mM HEPES (pH 7.5), 0.03% Casamino Acids, and either 0.2% glucose or 0.2% arabinose or lacking an added carbon source. We performed similar experiments to assess galactose as a carbon source, using 0.2% galactose. For motility assays, TB-SW (41) was used. To evaluate different carbon sources for their ability to induce pellicle formation, sugars or carbon sources were added to LBS at final concentrations of 0.2% or 0.4%; for experiments with some sugars, additional Tris was added to compensate for a pH effect. *E. coli* strains were grown in Luria-Bertani (LB) (42) or brain heart infusion medium. Agar was added to a final concentration of 1.5% for solid media. The following antibiotics were added to *V. fischeri* media, where necessary, at the indicated concentrations: chloramphenicol (Cm), 1 to 2.5 μg ml^{-1}, and erythromycin (Em), 5 μg ml^{-1}. The following antibiotics were added to *E. coli* media, where necessary, at the indicated concentrations: Cm, 25 μg ml^{-1}; Em, 150 μg ml^{-1}; kanamycin (Kn), 50 μg ml^{-1}; or ampicillin (Ap), 100 μg ml^{-1}.

Plasmids and molecular biology techniques. Plasmids and primers used in this study are shown in Tables S1 and S2 in the supplemental material. Standard molecular biology techniques were used to generate various plasmids used in this study. Restriction enzymes were purchased from New England BioLabs or Fermentas. DNA was introduced into *V. fischeri* using triparental conjugations (31, 43). The use of the pVO8-based BglII library of *E. coli* ES114 chromosomal DNA was previously described (44).

We used the method of LeRoux et al. (36) to generate unmarked, in-frame deletions. We generated DNA fragments, ~500 to 600 bp in length, upstream and downstream of the gene of interest, using KOD HiFi DNA polymerase (Novagen) with *E. coli* ES114 as a template and appropriate primers indicated in Table S2 in the supplemental material. The primers were designed such that the very 5′ and 3′ ends of the gene of interest were retained and fused in-frame to a short, nonnative sequence (lowercase in Table S2). These upstream and downstream fragments were then fused in overlap extension PCRs (45) such that the gene of interest was largely replaced by the nonnative sequences. Each resulting PCR product was then cloned into pJET1.2/blunt (Fermentas) and subcloned into the suicide plasmid pKV363 (46). The deletion constructs were then introduced into *V. fischeri* strain ES114, and following the mutagenesis procedure, the resulting recombinants were confirmed using PCRs with *Taq* polymerase (Promega) and primers outside each deletion.

Arabinose-induced pellicle formation. To observe arabinose-induced pellicle formation, we grew *V. fischeri* cells in LBS medium with shaking overnight and then diluted the cells 1:200 in 2 ml of fresh LBS medium containing 0.2% arabinose. The diluted cultures were placed into 24-well plates (Greiner Bio-One) and incubated at room temperature (about 23 or 24°C) for 24 h. The resulting growth was imaged using a Zeiss Stemi-C dissecting microscope with an attached camera. To make it easier to visualize the presence of a pellicle, each sample was perturbed with a pipette tip prior to being imaged.

Phenol red assays. Cultures were grown at 28°C overnight with shaking in SWT containing 0.05% phenol red and 0.2% arabinose or as controls, SWT containing 0.2% glucose or with no added sugar. The next day, cultures were scored as being the same color as the no-added-sugar control (red) or the glucose control (yellow) or a distinct color (orange).

Transposon mutagenesis screen and determining the site of insertion. Transposon (Tn) mutagenesis was performed using the mini-Tn*5* from pEVS170 (47), which was introduced via conjugation into *V. fischeri* ES114 cells. This Tn*5* derivative contains an erythromycin resistance gene, oriR6K (an origin of replication not functional in *V. fischeri*), and *oriT* (for transfer via conjugation) within the ends of the transposon (47), which can be used to facilitate cloning of DNA sequences flanking the Tn. Tn mutants were selected on LBS plates containing Em, and colonies that arose were selected and transferred to 96-well plates containing LBS with 0.2% arabinose. Mutants that failed to form a pellicle were purified and retested to confirm their phenotypes. The Tn and flanking sequences were cloned from mutants that were defective in forming a pellicle but that retained normal motility. Chromosomal DNA was isolated and digested with a restriction enzyme, and then the resulting DNA fragments were self-ligated and transformed into *E. coli* cells as described previously (47). The location of the Tn was determined by sequencing the DNA flanking the Tn insertion with primer 908 (see Table S2 in the supplemental material).

Motility assays. Cells were grown with shaking in SWT overnight, and then 10-μl aliquots were used to inoculate TB-SW soft agar plates. Cells that failed to migrate beyond the point of inoculation within 6 to 8 h were scored as nonmotile.

β-Galactosidase assay. Plasmid pKV143, which contains the *lacZ* gene under the control of the arabinose-inducible pBAD promoter, was

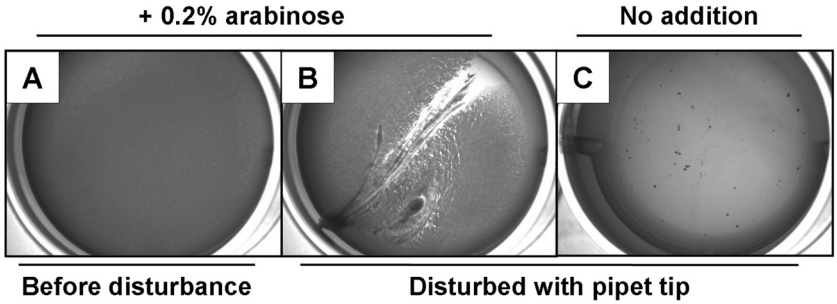

FIG 1 Wild-type *V. fischeri* forms a pellicle in response to arabinose. (A to C) Cultures of wild-type *V. fischeri* (ES114) were grown statically in the presence (A and B) or absence (C) of 0.2% arabinose for 24 h. A pipette tip was then used to disturb the surface of the culture. (B) The presence of a pellicle is apparent following the disturbance.

introduced into *V. fischeri* ES114 and specific mutant strains. Cells were grown with shaking in LBS containing Cm overnight at 28°C and diluted 1:200 in 2 ml LBS containing Cm and 0.2% arabinose. The cultures were grown statically for 24 h at room temperature, and then 1-ml aliquots were concentrated by centrifugation, resuspended in Z buffer and lysed with chloroform. β-Galactosidase activity was measured using *o*-nitrophenyl-β-D-galactopyranoside (ONPG), and reactions were stopped using sodium carbonate as described previously (48). Lowry assays were performed to standardize the β-galactosidase activity to protein concentration (49).

Colonization assay. Wild-type *V. fischeri* cells that lacked or contained an Em^r resistance marker were inoculated into SWT broth that lacked or contained 0.2% arabinose and grown for 2 to 3 h. The Em^r marker has been previously shown to exert little impact on colonization proficiency (50). The cell number was then estimated using a spectrophotometer, and approximately equal numbers of cells were mixed and added to seawater containing newly hatched juvenile squid. Following ~24 h of inoculation, the squid were homogenized, and the homogenates were diluted and plated onto SWT plates. Colonies that arose were transferred to plates containing or lacking Em to determine the percentage of arabinose-exposed bacteria present in the colonized animal.

RESULTS

L-Arabinose induces biofilm formation by *V. fischeri*. During experiments that used an arabinose-inducible promoter controlling the *ccdB* toxin, we observed that *V. fischeri* cells grown in the presence of L-arabinose formed pellicles on the surfaces of static liquid cultures within 24 h of inoculation. This phenotype did not depend on toxin production or the arabinose-inducible promoter, as wild-type *V. fischeri* cells similarly responded to the addition of arabinose (Fig. 1). Although the pellicles were easy to visualize, it was difficult to document their presence using the imaging system available to us (Fig. 1A). However, we found that if we disturbed the pellicles by repeatedly dragging a pipette tip across the pellicle surface, the disrupted pellicle could be visualized and thus documented (Fig. 1B). In the absence of arabinose, no pellicle formed, and thus, no disturbance could be visualized following the use of the pipette tip (Fig. 1C), although frequently small spots of cells could be observed at the air/liquid interface. This technique also permitted us to qualitatively assess the arabinose-induced pellicle. The pellicles were readily disturbed and did not hold together tightly (i.e., they were not very self-cohesive or sticky) or stick tightly to the plastic surface of the well. However, full resuspension of the pellicles required several rounds of pipetting, suggesting that the cells were, in fact, adherent to each other, possibly through a biofilm matrix. The pellicles were primarily on the surface of the culture, rather than attached to the surface of the plastic well.

To understand this phenomenon further, we tested a wide range of arabinose concentrations for their ability to induce pellicle formation (0.025% to 1.6%). Pellicles formed in the presence of 0.2%, 0.4%, and 0.6% arabinose, but not at levels below those concentrations (see Fig. S1A in the supplemental material). At levels around 0.8% arabinose and above, growth of *V. fischeri* was inhibited (see Fig. S1A; also data not shown). Because the smallest amount of arabinose that consistently induced the formation of pellicles was 0.2%, we chose that amount for our subsequent studies.

We next asked whether other sugars or carbon sources similarly induced pellicle production by *V. fischeri*. We assayed a variety of sugars and carbon sources for their ability to induce pellicle production under the same conditions (at final concentrations of both 0.2% and 0.4%) (Table 2). Neither one of two closely related sugars, xylose and ribose, induced pellicle production. Similarly, D-arabinose was unable to induce pellicle production. Indeed, of

TABLE 2 Carbon sources tested for the ability to induce pellicle formation

Carbon source[a]	Pellicle production[b]
D-Arabinose	−
L-Arabinose	+
Cellobiose	−
Fructose	−
Galactose	−
N-Acetylglucosamine	−
Glucose	−
Glycerol	−
Maltose	−
Mannitol	−
Mannose	−
Raffinose	−
Ribose	−
Succinate	−
Sucrose	−
Xylose	−[c]

[a] Added to LBS medium to a final concentration of 0.2% or 0.4%.
[b] The absence of pellicle production (−) and the formation of pellicles (+) are indicated.
[c] Growth was partially inhibited by 0.4% xylose.

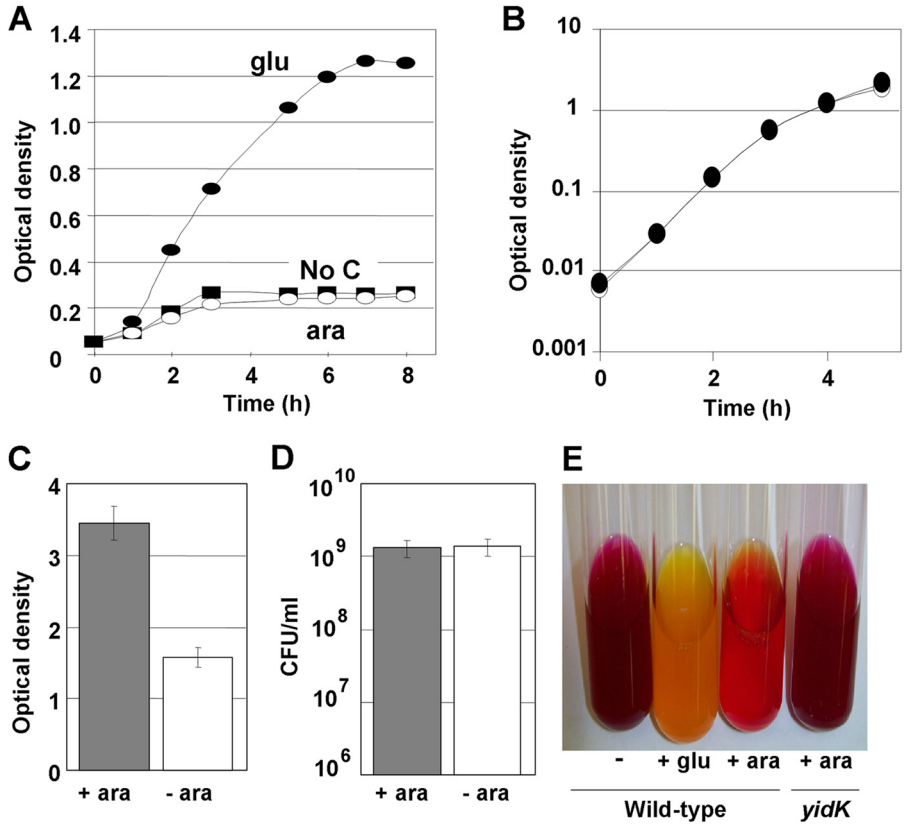

FIG 2 Wild-type *V. fischeri* responds but fails to grow with arabinose as a carbon source. (A and B) Optical density measurements of wild-type *V. fischeri* strain ES114 grown with shaking in HMM containing 0.2% glucose (glu) (black ovals), 0.2% arabinose (ara) (white ovals), or no additional carbon source (No C) (black rectangles) (A) or with shaking in LBS (black circles) or LBS with 0.2% arabinose (white circles) (B). (C) Optical density measurements of strain ES114 grown statically in LBS in the presence (+) and absence (−) of 0.2% arabinose. (D) Quantity (CFU/ml) of ES114 following static growth in LBS in the presence and absence of 0.2% arabinose. The error bars in panels C and D show the standard deviations. (E) Cultures of ES114 and *yidK*::Tn mutant strain KV5837 grown overnight with shaking in SWT broth containing phenol red and with no addition (−) or with the addition of 0.2% glucose (+ glu) or 0.2% arabinose (+ ara).

the 16 carbon sources tested, only L-arabinose induced pellicle production. Thus, production of this pellicle was highly specific.

***V. fischeri* cells do not grow on L-arabinose.** To determine whether arabinose impacts growth of *V. fischeri*, we monitored the optical density of *V. fischeri* cells in shaking cultures over time. When added as the carbon source to HEPES minimal medium, L-arabinose did not support the growth of the bacteria (Fig. 2A). Furthermore, the addition of arabinose did not increase the aerobic growth of *V. fischeri* in LBS, the complex tryptone-based medium used in the pellicle experiments (Fig. 2B). When grown statically, the optical density measurements obtained from wild-type cells in the presence of arabinose were about twofold higher than the measurements in cells grown without arabinose (Fig. 2C). However, these measurements appeared not to accurately reflect cell number, as plating assays suggested that near equivalent numbers of cells were present under the two conditions (Fig. 2D). Finally, we asked whether we could detect any metabolism of this sugar using the pH indicator phenol red in the unbuffered SWT medium. When *V. fischeri* was grown overnight in SWT containing phenol red, the cultures exhibited a bright red color (Fig. 2E). The addition of glucose as the positive control resulted in a color change from red to bright yellow, indicating acidification of the medium. Growth of *V. fischeri* in the presence of 0.2% arabinose resulted in a slight, but reproducible color change from red to orange. Together, these data indicate that pellicle production is not a result of increased growth of *V. fischeri* in the presence of arabinose and that *V. fischeri* responds to the presence of a sugar that it cannot productively metabolize for growth.

Arabinose-induced pellicle formation is independent of *syp* but partially dependent on *bcs*. One polysaccharide locus extensively characterized for its role in biofilm formation in *V. fischeri* is *syp* (20). Therefore, we asked whether pellicle formation induced by arabinose depended on the *syp* locus and found that it did not: pellicle production was unaffected by mutations in either one of two regulatory *syp* genes, *sypG*, which encodes a positive putative transcription factor (39), or *sypE*, which encodes a regulatory protein that functions both positively and negatively to control *syp*-dependent biofilm formation (23) (see Fig. S2 in the supplemental material). Similarly, mutants with deletions in either one of two structural *syp* genes, *sypC* and *sypN*, which are critical for *syp*-dependent biofilm formation (34, 39), produced pellicles in response to the addition of arabinose (data not shown). These data suggest that *syp* is not required for pellicle production induced by arabinose.

Another polysaccharide locus known to contribute to biofilm formation is the cellulose locus *bcs* (*VF_A0887-881*) (25, 26). To test the role of the cellulose locus, we generated a Δ*bcsA* mutant and evaluated its ability to form a pellicle. In most of our experi-

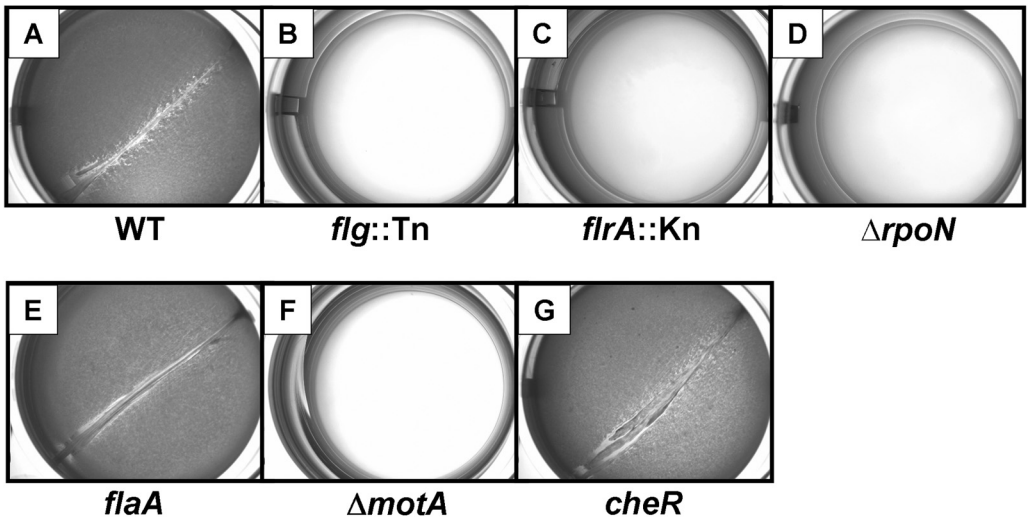

FIG 3 Nonmotile mutants exhibit defects in pellicle production. (A to G) Pellicle production by the indicated strains following static growth in LBS containing 0.2% arabinose is depicted. A pipette tip was used to disturb the surface of each culture. Wild-type (WT) strain ES114 (A), *flg*::Tn mutant KV5365 (B), *flrA* mutant DM127 (C), *rpoN* mutant KV5005 (D), *flaA* mutant DM143 (E), *motA* mutant KV5836 (F), and *cheR* mutant KV1172 (G).

ments, the pellicle formed by the *bcsA* mutant was not intact and/or had fallen to the bottom of the well within 24 h (see Fig. S3 in the supplemental material). Occasionally, however, the pellicle appeared similar to that of the wild-type strain. It is possible that the exact temperature of the room, or another poorly controlled factor, may contribute to the variability of the mutant phenotype. A role for the cellulose locus was further confirmed when we performed a screen for pellicle-defective mutants, which we describe in greater detail below. We focused primarily on those mutants with apparent null phenotypes, but we also isolated and characterized three mutants with a "fallen" phenotype similar to the Δ*bcsA* mutant (see Fig. S3). These three mutants contained insertions in the *bcs* locus. Two insertions were located in *bcsE*, which encodes a putative protease; this gene is necessary for cellulose biosynthesis in *Salmonella enteritidis* (16). A third insertion was located within the intergenic region between *bcsE* and the divergently transcribed operon that includes the genes for cellulose synthase (*bcsA* and *bcsB*) (see Fig. S3). We conclude from these data that cellulose is not required for production of a pellicle in response to arabinose but that it contributes substantially to the stability/structure of the pellicle.

Motility is required for pellicle formation. To determine the factors contributing to the production of pellicles in response to arabinose, we undertook a random transposon (Tn) mutagenesis screen. Our screens of approximately 8,000 mutants yielded about 50 that were reproducibly defective in producing a pellicle in response to arabinose. We chose to characterize one of the biofilm-defective *V. fischeri* mutants with a null phenotype, KV5365 (compare Fig. 3A and B), by complementing it with a library of cloned *V. fischeri* chromosomal DNA. From cells that became proficient to form a pellicle, we isolated and sequenced a portion of the complementing plasmid, pKPQ21. Sequence analysis revealed that the cloned DNA contained flagellar genes *flgJ-L*, suggesting that the Tn insertion in KV5365 caused the strain to be nonmotile. Indeed, we confirmed that the mutant was nonmotile and verified that pKPQ21 restored motility and biofilm formation (data not shown). We subsequently determined that 21 other biofilm-defective mutants, from 8 independent sets, were nonmotile or had severely reduced motility, based on the soft agar motility assay (data not shown). These data suggested that motility is critical to the formation of a pellicle in response to arabinose.

To further investigate the role of motility, we evaluated the ability of various flagellar mutants in our collection to form biofilms. Nonmotile mutants with defective motility regulator genes *flrA* (27) and *rpoN* (51) were also defective in forming biofilms (Fig. 3C and D). In contrast, a wild-type-appearing pellicle was formed by a *flaA* flagellin mutant; this mutant retains its ability to swim due to the presence of multiple flagellin genes carried by *V. fischeri* (28) (Fig. 3E). We next asked whether rotation of the flagella was important by assaying the biofilm-forming ability of a *motA* mutant. This nonmotile mutant exhibited a defect as severe as the nonflagellated *flrA* mutant, indicating that rotation of the flagella is important for pellicle formation (Fig. 3F). Finally, we asked whether chemotaxis was important. We found that a *cheR* mutant (30) exhibited no defect in biofilm formation (Fig. 3G). Together, these data indicate that motility is important for biofilm formation, but reduced motility does not substantially impair biofilm formation, and the ability to perform chemotaxis appears not to be critical for this phenotype.

The response to arabinose depends on a diverse set of genes. We next determined the locations of the Tn insertions in the biofilm-defective mutants that did not exhibit defects in motility and identified insertions in a variety of genes (Table 3). In several cases, we obtained multiple insertions in the same gene or locus; however, despite a relatively large screen of 8,000 mutants, a number of genes were identified only once. We note that, because we did not obtain multiple independent insertions, we cannot conclude that all of the genes listed in Table 3 are involved in pellicle production. In addition, some of the insertions mapped within operons, making it unclear whether the gene of interest, a neighboring gene, or both are involved in the observed phenotype. We thus chose to verify the roles of three of these loci by additional studies.

First, we evaluated the role of *VF_1812*, a gene in which we

TABLE 3 Locations of Tn insertions in pellicle-defective, but motile, mutants

Strain	Location of insertion[a]	Phenol red phenotype
KV5944	*VF_0311* (*cysI*)	Orange
KV6001	*VF_0360* (*mshM*)	Orange
KV6000	*VF_0361* (*mshN*)	Orange
KV5948	IG (*VF_0365-0366*) (*mshB-mshA*)	Orange
KV5999	*VF_0435* (*gshB*)	Orange
KV5629	*VF_0696* (*acfD*)	Red
KV5635	*VF_0804* (*asnB*)	Orange
KV5943	*VF_0819* (*sdhC*)	Orange
KV6002	*VF_1037* (*ainS*)	Orange
KV5998	IG (*VF_1631-1632*) (*hns-mipA*)	Orange
KV5942	*VF_1812*[b]	Orange
KV5807	*VF_1812*	Orange
KV5805	*VF_1896* (*ptsI*)	Red
KV5633	*VF_1896* (*ptsI*)	Red
KV5634	*VF_2291* (*aroB*)	Orange
KV5945	*VF_A0351* (*yidK*)	Red
KV5804	*VF_A0685* (*talB*)	Orange
KV5632	*VF_A0703* (*gcvP*)	Orange
KV5630	*VF_A0859*[c]	Red
KV6028	*VF_A0860*[d]	Red
KV5631	*VF_A1015* (*rpoQ*)	Red

[a] Putative or known gene names indicated in parentheses.
[b] Putative long-chain fatty acid transport gene.
[c] Putative methyl-accepting chemotaxis protein gene.
[d] Hypothetical protein gene.

obtained two independent insertions (Fig. 4A and Table 3). This gene encodes a putative long-chain fatty acid transport protein precursor. The two mutants failed to produce a pellicle in the presence of arabinose (Fig. 4B to D). To verify the involvement of *VF_1812* in pellicle production, we cloned a wild-type copy of *VF_1812*, to generate pSMM2, and asked whether it could complement the observed defect. Arabinose-induced pellicle production was restored to both *VF_1812* mutant strains by the presence of pSMM2, but not by the vector control (Fig. 4E to H). These data suggest that *VF_1812* is necessary for the ability of *V. fischeri* to form pellicles in response to arabinose.

A second set of mutants that failed to respond to arabinose with the production of a pellicle contained insertions in a large locus predicted to be involved in the production of a pilus (*VF_0355-371*) (52). One of these insertions was located just upstream of a gene (*VF_0366*; *mshA*) that is predicted to encode a putative mannose-sensitive hemagglutinin (the Tn was inserted between *mshB* and *mshA*). Two other insertions were located further upstream, in putative pilus genes *VF_0360* (*mshM*) and *VF_0361* (*mshN*) (Fig. 5A). All three mutants failed to produce a pellicle in response to arabinose (Fig. 5B to D and data not shown). To test the involvement of this locus, we first asked whether the addition of mannose or the mannose analog methyl-α-D-mannopyranoside also could inhibit the formation of pellicles in response to arabinose. We found that mannose addition diminished biofilm formation, while addition of the analog eliminated pellicle formation; however, the analog also seemed to impair growth of *V. fischeri* cells, making it difficult to interpret these data (data not shown). We therefore generated an in-frame deletion of *mshA*. This mutant similarly exhibited a defect in biofilm formation under these conditions (Fig. 5E). Thus, this locus confers the ability to produce pellicles in response to arabinose during static growth.

As we characterized the mutants described above (*VF_1812* and *msh* mutants), we noticed that they grew poorly under static

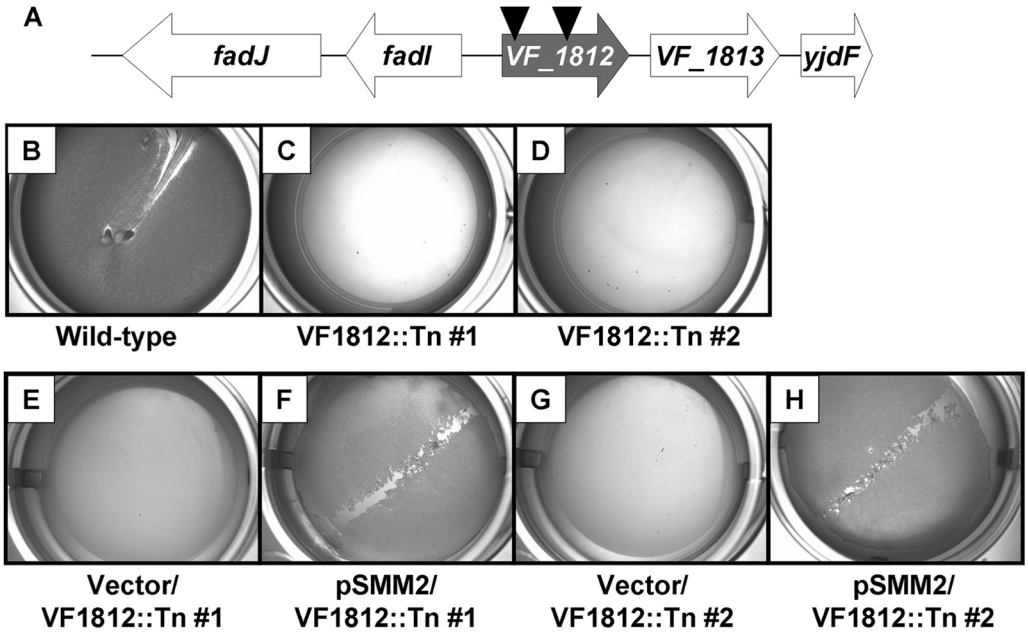

FIG 4 *VF_1812* mutants exhibit defects in pellicle production. (A) Map of the region of the chromosome surrounding *VF_1812*. The two black triangles above *VF_1812* represent the approximate sites of insertions in strains KV5807 and KV5942. (B to H) Pellicle production by the indicated strains following static growth in LBS containing 0.2% arabinose is depicted. A pipette tip was used to disturb the surface of each culture. Wild-type strain ES114 (B), *VF_1812* mutants KV5807 (C) and KV5942 (D) and *VF_1812* mutants KV5807 and KV5942 carrying vector control plasmid pVO8 or complemented with *VF_1812* expressed from plasmid pSMM2, as indicated (E to H).

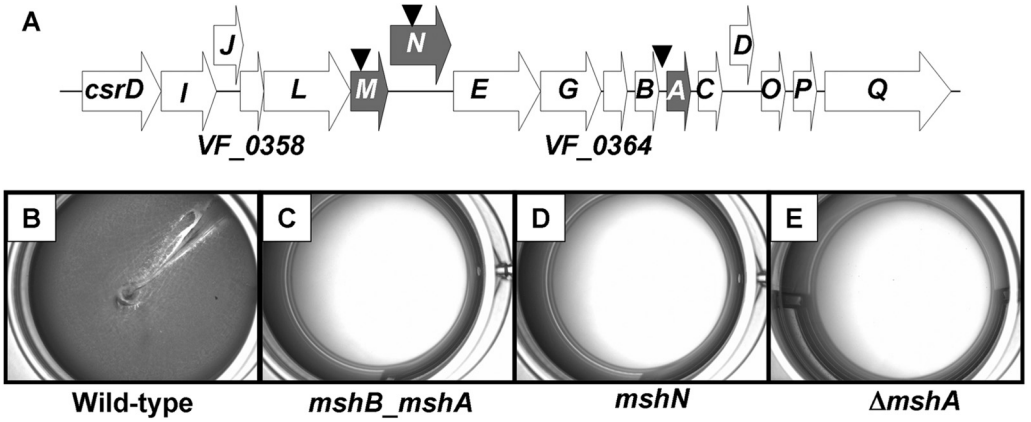

FIG 5 A putative pilus locus plays a role in pellicle production. (A) Map of the region of the chromosome containing the putative *msh* pilus operon (*VF_0355-VF_0371*). The three black triangles above the genes represent the approximate sites of insertions in the three genes. (B to E) Pellicle production by the indicated strains following static growth in LBS containing 0.2% arabinose. A pipette tip was used to disturb the surface of each culture. Wild-type strain ES114 (B), *msh* mutant strain KV5948 (C), *mshN* mutant KV6000 (D), and Δ*mshA* mutant KV6004 (E).

conditions upon the addition of arabinose (see Fig. S4A and S4B in the supplemental material). These strains grew to wild-type or near-wild-type levels in the absence of arabinose, indicating that these strains do not have a general growth defect (see Fig. S4A and S4B). Thus, the failure of these strains to generate a pellicle could be attributed, at least in part, to a failure to grow robustly in the presence of arabinose. While the exact cause of this growth defect has yet to be determined, these data suggest that arabinose may be toxic to *V. fischeri*, at least under the static growth conditions of our assay. These genes could thus be considered important for resistance to arabinose toxicity.

The third mutant we characterized contained an insertion in the *yidK* gene. In contrast to the above examples, the *yidK* mutant exhibited normal static growth in the presence of arabinose, except that it failed to form a pellicle (Fig. 6; see Fig. S4C in the supplemental material). The *yidK* gene, the first in an apparent operon of four genes, encodes a putative galactose transporter YidK (*VF_A0351*) (Fig. 6A). This mutant failed to grow with galactose as a carbon source, indicating that this locus is indeed important for galactose utilization (data not shown). To evaluate the role of *yidK*, we generated an in-frame deletion mutant. We found that this mutant similarly exhibited a defect in pellicle production in response to arabinose (Fig. 6B to D) as well as in its ability to grow with galactose as a carbon source (data not shown). In addition, we found that a mutant with a defective galactose utilization gene *galK* (*VF_A0355*) (31) was also defective for pel-

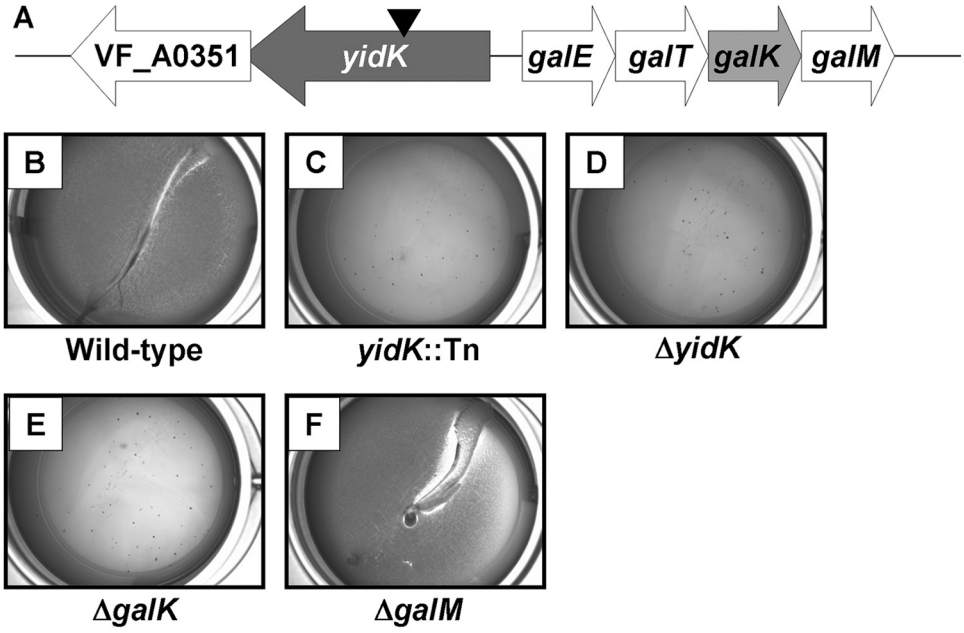

FIG 6 Pellicle production depends on galactose metabolism genes. (A) Map of the region of the chromosome surrounding *yidK*. The black triangle above *yidK* represents the approximate site of insertion in *yidK*. (B to F) Pellicle production by the indicated strains following static growth in LBS containing 0.2% arabinose. A pipette tip was used to disturb the surface of each culture. Wild-type strain ES114 (B), *yidK*::Tn mutant KV5945 (C), Δ*yidK* mutant KV5837 (D), *galK* mutant KV1356 (E), and Δ*galM* mutant KV5849 (F).

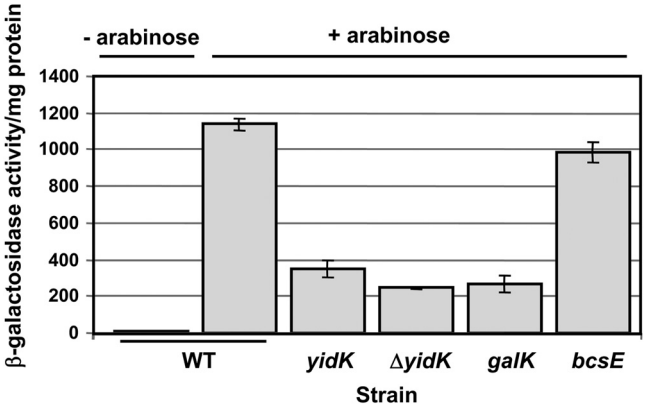

FIG 7 Arabinose induces an arabinose-responsive reporter in the wild-type strain but induces reduced levels in *yidK* and *galK* mutants. β-Galactosidase activity of wild-type and mutant strains carrying the arabinose-inducible *lacZ* reporter plasmid following growth in the absence or presence of 0.2% arabinose. pKV143-containing wild-type strain ES114 in the absence (−) or presence (+) of arabinose, pKV143-containing *yidK*::Tn mutant KV5837, Δ*yidK* mutant KV5945, *galK* mutant KV1356, and *bcsE*::Tn mutant KV5939, all grown statically overnight in the presence of arabinose.

licle production in response to arabinose (Fig. 6E); GalK is predicted to function as a galactose kinase to phosphorylate galactose (53). In contrast, a mutant with a defective *galM* gene, located downstream of *galK* and predicted to encode an aldose-1-epimerase (53), was not defective for pellicle production (Fig. 6F); however, it also retained the ability to grow on galactose as a carbon source (data not shown). Finally, the biofilm defect of the *galK* mutant could be complemented by a plasmid that contained *galK* and flanking sequences (data not shown). We conclude from these studies that genes involved in the uptake and/or use of galactose are required for *V. fischeri* to form biofilms in response to arabinose.

Arabinose likely exerts its impact from an intracellular location. To more globally characterize these mutants, we asked whether any were defective in the metabolic response to arabinose using the phenol red assay. The cultures of several Tn mutants, including the *yidK* mutant (but not the *VF_1812* or *msh* mutant), retained the starting red color of the indicator dye (Fig. 2E and Table 3). In addition, cultures of the unmarked *galK* and *yidK* mutants also retained the red starting color of the indicator dye in this assay (data not shown). These data suggest that a subset of the mutants failed to respond to and/or catabolize arabinose. These experiments thus permitted the grouping of these mutants into two classes: those that appear to be involved in arabinose uptake/metabolism and those that do not (Table 3).

To further evaluate the role of arabinose, we asked whether we could detect arabinose uptake by *V. fischeri*. We thus generated and introduced into *V. fischeri* a reporter construct that contained the arabinose-inducible promoter, P_{BAD}, fused upstream of a promoterless *lacZ* gene. Then, we asked whether wild-type *V. fischeri* containing this plasmid, pKV143, could respond to the addition of arabinose by the production of β-galactosidase during static growth in LBS medium. Whereas little to no detectable β-galactosidase activity was observed in the absence of added arabinose, the addition of 0.2% arabinose induced measurable amounts of β-galactosidase activity from the reporter plasmid (Fig. 7). These data support the conclusions of the phenol red experiments that arabinose can enter *V. fischeri*, and thus likely exerts its effect from the inside.

We also assayed reporter activity of the *yidK* and *galK* mutants and, as a control, the *bcsE* mutant. The *bcsE* mutant exhibited β-galactosidase activity similar to that of the wild-type strain in the presence of arabinose (Fig. 7), indicating that loss of cellulose production does not impact uptake of arabinose. In contrast, both *yidK* mutants and the *galK* mutant exhibited a reduced level of β-galactosidase activity (Fig. 7). These data suggest that YidK and/or the galactose utilization pathway may contribute to arabinose uptake. However, the β-galactosidase activity of all three mutants remained significantly above that produced by the wild-type strain in the absence of arabinose. These data suggest that YidK may be involved in arabinose transport but that it may not be the only factor promoting uptake.

Finally, in further support of the possibility that YidK may contribute to arabinose uptake, we observed that the Δ*yidK* mutant was able to grow in the presence of high concentrations of arabinose: whereas concentrations of arabinose at or above 0.8% impaired growth of wild-type cells, the *yidK* mutant exhibited no growth defect even at levels as high as 1.6% (see Fig. S1B in the supplemental material; also data not shown). At high concentrations of arabinose (1.4% and 1.6%), however, the *yidK* mutant formed a pellicle (see Fig. S1B). These data support the idea that YidK may serve as an arabinose transporter. If it does, then there must be other avenues through which arabinose can enter the cell, particularly when high concentrations of arabinose are present.

Arabinose addition impairs symbiotic colonization. To begin to address the biological relevance of arabinose with respect to *V. fischeri*, we asked whether arabinose impacted the ability of *V. fischeri* to form a symbiosis with its host, the squid *E. scolopes*. Specifically, we inoculated newly hatched juvenile squid with a mixture of unmarked (Em^s) and marked (Em^r) wild-type *V. fischeri* cells that had been subcultured in media containing and lacking 0.2% arabinose, respectively, for approximately 2 to 3 h prior to their inoculation (see Materials and Methods). The next day, we determined the relative amounts of the two strains in each squid. Surprisingly, cells that had been grown in the presence of arabinose prior to inoculation were substantially outcompeted by those that had not been exposed to arabinose (Fig. 8). We conclude that the physiological changes that occur due to exposure to arabinose are not advantageous to *V. fischeri* during initiation of colonization of its squid host.

DISCUSSION

We report here that *V. fischeri* exhibits a novel response to arabinose, the production of a pellicle in static cultures. This response is unique to L-arabinose, as D-arabinose and even structurally similar sugars failed to induce a biofilm. Furthermore, this response is surprising, as *V. fischeri* does not use arabinose as a carbon source. These results thus highlight the ability of bacterial cells to recognize and respond to their surroundings.

Typically, a major component of biofilms is an exopolysaccharide (EPS). However, both our targeted mutagenesis and our random Tn mutation approaches failed to identify an EPS locus that was absolutely required for pellicle production. Notably, the well-studied *syp* locus did not play any observable role. In contrast, although not absolutely required, the cellulose locus *bcs* made an important contribution to the stability or structure of the pellicle. Cellulose similarly plays a relatively minor role in pellicle produc-

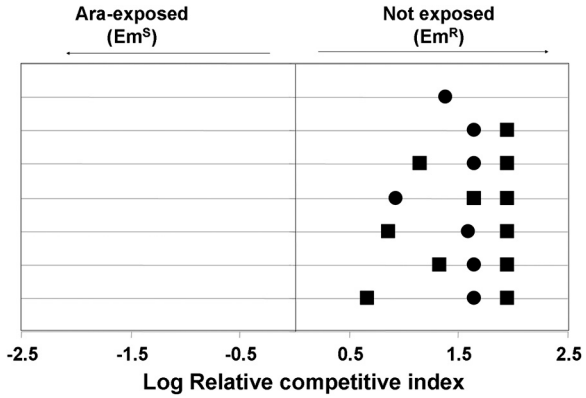

FIG 8 Symbiotic colonization is impaired by exposure to arabinose. Newly hatched juvenile *E. scolopes* squid were inoculated with a 1:1 mixture of Em^s wild-type cells (ES114) and Em^r wild-type cells (KV4389). Prior to inoculation, cells were grown in SWT (KV4389) and SWT containing 0.2% arabinose (ES114). The log relative competitive index (RCI) is plotted on the *x* axis. The positions of the symbols on the *y* axis have no significance; the symbols are merely spaced out. Each symbol represents the value for a single animal. Squares represent data from one experiment, while circles represent data from a separate experiment.

tion by *Erwinia chrysanthemi* (54). Some bacteria incorporate arabinose or a derivative into their EPS, including *Vibrio parahaemolyticus* (55), *Acinetobacter junii* (56), and *Streptococcus phocae* (57). Whether *V. fischeri* does this as well remains to be determined.

Our mutagenesis studies revealed an important role for motility in pellicle production by *V. fischeri*. The ability to move was critical, as nonmotile mutants, including a *motA* mutant, were all defective for pellicle production. In contrast, a *flaA* mutant, which exhibits reduced motility (28), formed wild-type-appearing pellicles. In addition, a *cheR* mutant that is motile but unable to perform chemotaxis was competent to form pellicles, suggesting that pellicle formation requires motility but not chemotaxis. Flagellar motility is similarly required for biofilm formation by other bacteria (7–10). However, we found the requirement for flagellar motility somewhat surprising, as we previously reported that *V. fischeri* is poorly motile under conditions in which little to no magnesium is present, as is the case with LBS broth, the condition that we used here (58). The addition of magnesium to LBS did not, however, increase pellicle formation but rather appeared to decrease it (unpublished data). Together, our data suggest that pellicle production depends on bacterial movement but is not dependent on optimal motility.

We also identified a number of other factors that may contribute to the response to arabinose (Table 3) and confirmed the requirement for a subset of these, *VF_1812*, *msh*, and *yidK*. In further support for a role in biofilm formation for the *msh* locus, *mshA* was recently identified as being involved in biofilm formation by another wild-type *V. fischeri* strain, ETJB1H (11). The roles of these genes in promoting pellicle production in response to arabinose, however, remain unclear. Mutants defective for either *VF_1812*, which encodes a putative long-chain fatty acid transport protein precursor, or members of the putative *msh* pilus locus retained their ability to alter the color of the phenol red indicator dye, suggesting that they were not defective in arabinose transport or catabolism. Both mutants also exhibited a defect in static growth when arabinose was added. These data suggest that these mutants have increased sensitivity to the presence of arabinose. At this time, we have not yet distinguished between cause and effect: does poor growth result in the failure to form a pellicle, or does the failure to form a pellicle result in poor growth? For the *msh* mutants, it is tempting to speculate both that pili are involved in the production of a pellicle and that pellicle production protects the cells from arabinose toxicity. Whether the *msh* locus does, in fact, function in pilus production and whether these hypothetical pili are directly involved in pellicle production remain to be determined.

The toxicity of a sugar that cannot be used as a carbon source has been observed previously. *Bacillus subtilis* contains the genes for galactose utilization but does not transport galactose and therefore does not grow on this sugar (59). Disruption of the *B. subtilis galE* gene, which encodes a protein that serves as an epimerase to convert UDP-galactose to UDP-glucose, caused the resulting mutant to be susceptible to galactose; in the presence of this sugar, the strain rapidly died. Although transport of galactose itself could not be detected, the presence of compounds derived from the addition of radiolabeled galactose was observed, indicating that sufficient levels of galactose entered the cell to promote toxicity in the absence of *galE* (59). In particular, accumulation of small amounts of UDP-glucose seemed to account for the growth inhibition of the *galE* mutant. Perhaps a similar mechanism of toxicity occurs in *V. fischeri* upon the addition of arabinose to specific mutants.

In contrast to *VF_1812* and *msh* mutants, the *yidK* mutant did not exhibit a growth defect in the presence of arabinose. Furthermore, the *yidK* mutant appeared to be "blind" to the presence of arabinose due to the following observations. (i) Unlike the wild-type strain, the *yidK* mutant exhibited no growth defects in the presence of large amounts (≥0.8%) of arabinose. (ii) The *yidK* mutant failed to alter the color of the phenol red indicator dye. (iii) The *yidK* mutant containing the arabinose-inducible reporter exhibited a decrease in the response to the presence of arabinose. One possibility to account for these results is that YidK contributes to the transport of arabinose into the cell. The YidK protein is highly similar to the *Vibrio parahaemolyticus* sodium/glucose cotransporter vSGLT (85% identical and 94% similar; NCBI Protein Database accession number BAA11215 [60]). The *V. parahaemolyticus* protein is known to transport both glucose and galactose along with sodium (61, 62). It can also transport sodium in the presence of fucose. Whether it can transport arabinose is unknown. In *Sinorhizobium meliloti*, a connection between galactose and arabinose transport was shown. In this organism, the arabinose transporter AraABC facilitates the transport of galactose, although it is not wholly responsible for galactose uptake (63). In *V. fischeri*, YidK is required for growth on galactose; whether it also plays a role in arabinose transport remains to be determined.

The requirement for galactose kinase, GalK, is even less clear. However, a *galK* mutant exhibits a deficiency in arabinose uptake that is similar to that of the *yidK* mutant, based on results from both the phenol red assay and the arabinose-inducible reporter assay. This deficiency presumably accounts for the defect of this mutant in pellicle production in response to arabinose. Understanding the mechanism of action of both GalK and YidK requires additional study.

We obtained a number of mutants with insertions in genes predicted to be involved in metabolism, for example, *cysI* (sulfite

reductase), *gshB* (glutathione synthetase), *asnB* (asparagine synthetase), and *gcvP* (glycine dehydrogenase). Of note, we obtained two insertions in *ptsI*, encoding phosphotransferase system (PTS) enzyme I (EI), phosphoenolpyruvate-protein phosphotransferase. The PTS plays an important role in sugar transport. In support of such a role, both mutants exhibited a defect in the phenol red assay (Table 3). In *V. cholerae*, PTS enzyme EI also plays a role in biofilm formation, but it serves a negative function: deletion of the gene promotes surface adhesion (64). Thus, *ptsI* may be playing different roles in the two organisms or may be playing different roles under different conditions. Confirmation that this gene and the others identified in our screens play a role in pellicle production awaits further analysis. However, if most or all of the genes we identified are, in fact, involved, then it appears that the response to arabinose is complex.

It is not clear why *V. fischeri* exhibits a response to arabinose, a carbon source it cannot productively utilize. The response—the production of a biofilm—may provide resistance to external stresses, potentially even arabinose itself. Surprisingly, however, our results suggest that exposure to arabinose is detrimental to the early stages of colonization by *V. fischeri*, at least in the context of its symbiosis with the squid *E. scolopes*. With this result, the question becomes where do *V. fischeri* cells become exposed to arabinose? It remains possible that *V. fischeri* cells are exposed to arabinose at a later stage in squid colonization; however, animal tissues are not generally considered a good source of arabinose. In contrast, a significant biological source of arabinose is the plant cell wall, which contains hemicellulose, a heteropolymer composed of a variety of sugars, including L-arabinose (65). Thus, one formal possibility is that the response of *V. fischeri* to arabinose promotes an interaction with plants. Alternatively, *V. fischeri* could respond to arabinose present in seaweed: one study showed that the polysaccharide extracted from the green seaweed *Chaetomorpha anteninna* contained 57% arabinose (66). As seaweeds are known to produce antimicrobial agents (67, 68), it is possible that recognition of arabinose could upregulate a protective response by *V. fischeri*. Although we are unaware of any documented interactions between *V. fischeri* and seaweed, other vibrios, such as *V. vulnificus* and *V. parahaemolyticus*, have been found associated with seaweed (69, 70). Intriguingly, the ability of the microbe *Pseudoaltermonas tunicata* to adhere to the green alga *Ulva australis* depends upon an *msh*-like locus (71).

We did not observe any changes in colony morphology when we grew wild-type *V. fischeri* cells on plates containing arabinose (unpublished data). However, at this time, we cannot conclude that the cells are not responding to arabinose under these conditions. This is an important consideration, as the use of arabinose-inducible expression plasmids is common in microbiology research. Thus, in addition to providing important insights into the biology of *V. fischeri*, this study also serves as a cautionary note for researchers considering the use of arabinose-inducible promoters. However, we have used arabinose to induce *ccdB* toxin production during the selection of recombinants in experiments designed to generate deletion mutants, and the strains remain susceptible to subsequent rounds of arabinose treatments during the course of generating multiple deletions (72). Therefore, the use of arabinose as a tool in *V. fischeri* remains feasible, with the caveat that it appears toxic under certain conditions.

In summary, this work identifies a novel signal and new pathways involved in the production of a biofilm by *V. fischeri*. The finding that this seemingly innocuous sugar, arabinose, impairs growth of *V. fischeri* mutants provides new insights into the biology of *V. fischeri*. Significantly, the ability of arabinose to impair symbiotic colonization by wild-type cells opens up an important new area of investigation for symbiosis research.

ACKNOWLEDGMENTS

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Supplemental materials
Arabinose induces biofilm formation by *Vibrio fischeri*
Karen L. Visick*, Kevin P. Quirke, and Sheila McEwen

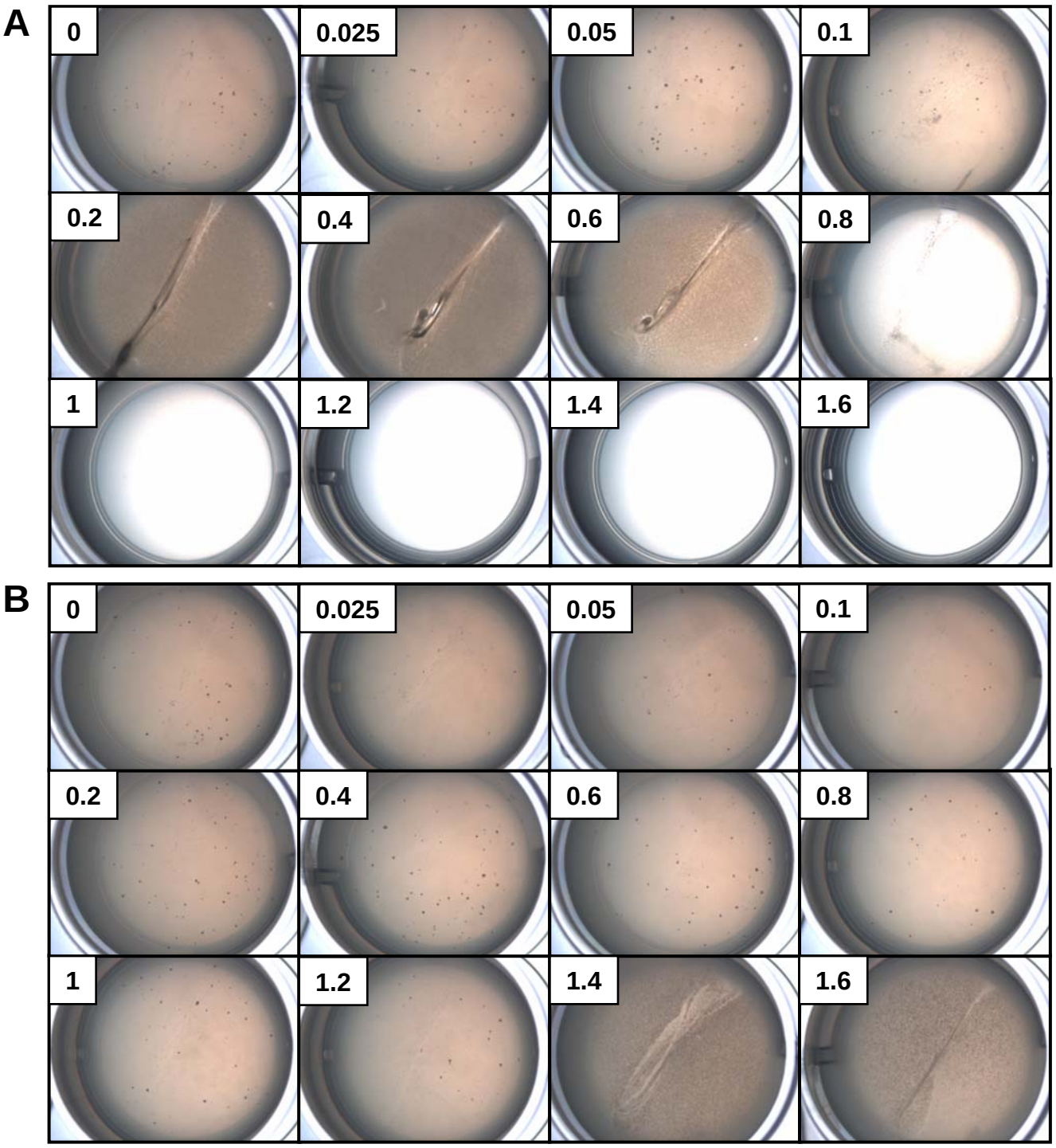

Fig. S1. Pellicle production in response to different amounts of L-arabinose. Wild-type strain ES114 (A) and Δ*yidK* strain KV5837 (B) were grown overnight in LBS, then diluted into LBS containing different final percentages of L-arabinose, as indicated; cultures were then incubated for 24 h. Pellicles were visualized by disturbing the air/liquid interface with a pipet tip. At concentrations of L-arabinose around and above 0.8%, the growth of wild-type *V. fischeri* was inhibited, as indicated by the white color of the images.

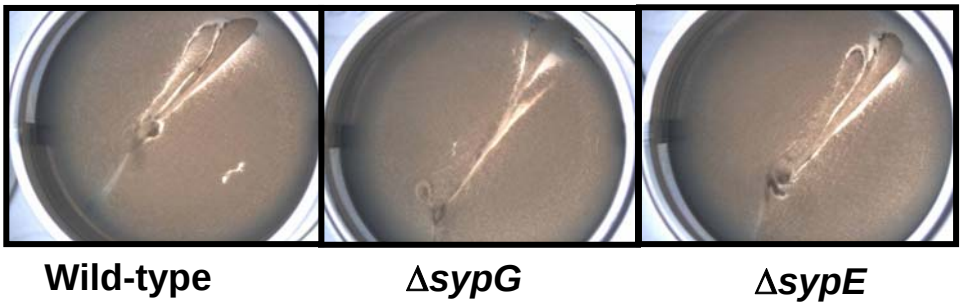

Fig. S2. Pellicle production by *syp* mutants. Wild-type strain ES114 and syp mutants Δ*sypG* and Δ*sypE* were grown overnight in LBS, then diluted into LBS containing 0.2% L-arabinose; cultures were then incubated for 24 h. Pellicles were visualized by disturbing the air/liquid interface with a pipet tip.

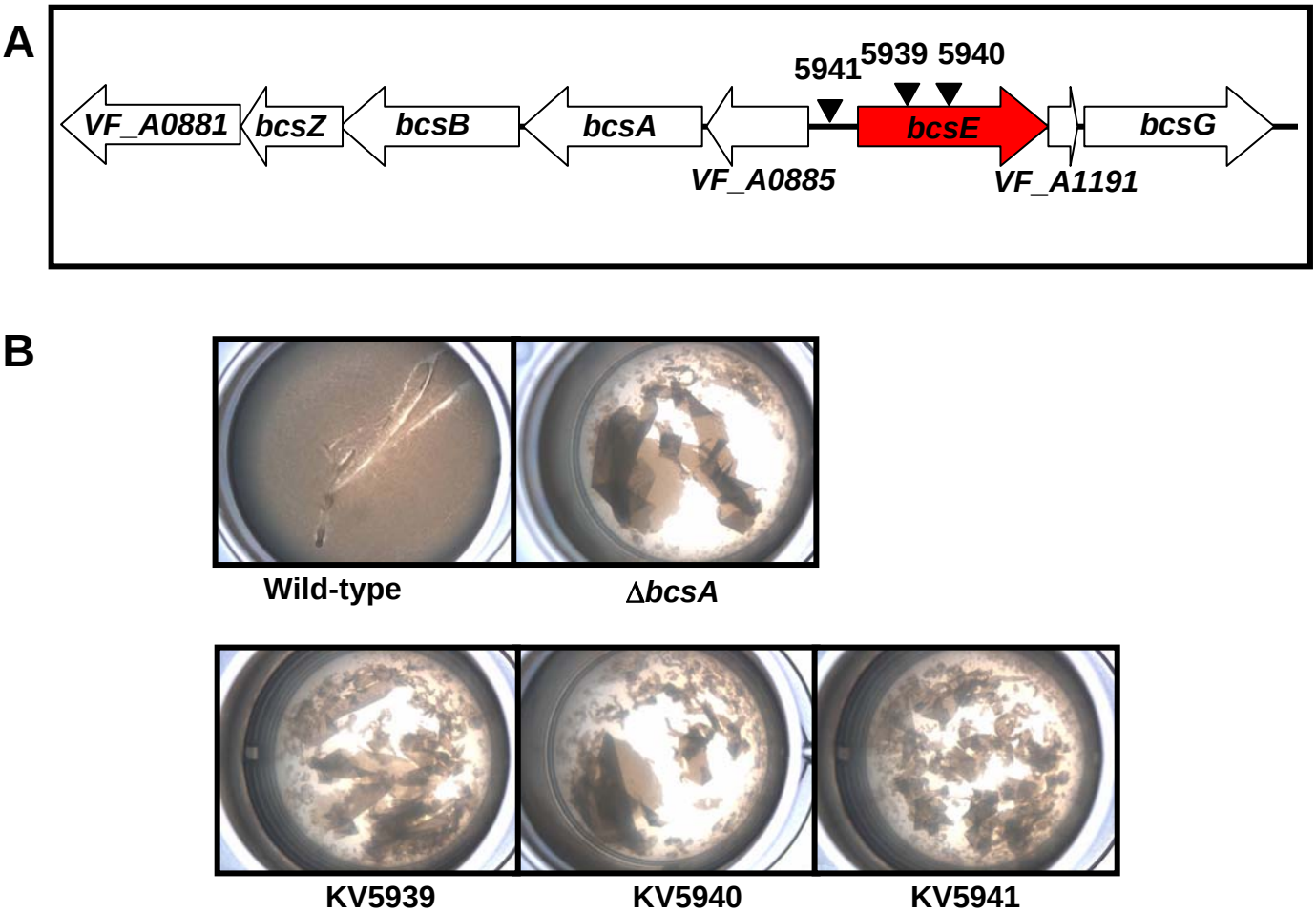

Fig. S3. Pellicle production by *bcs* mutants. (A). Schematic of the cellulose locus. The inverted triangles indicate the sites of insertions in strains KV5939, KV5940, and KV5941. (B) Pellicle production by wild-type and *bcs* mutants. Wild-type strain ES114, Δ*bcsA* mutant KV5366, and *bcs* Tn mutants KV5939, KV5940, KV5941 were grown overnight in LBS, then diluted into LBS containing 0.2% L-arabinose and incubated for 24 h. Where necessary, pellicles were visualized by disturbing the air/liquid interface with a pipet tip.

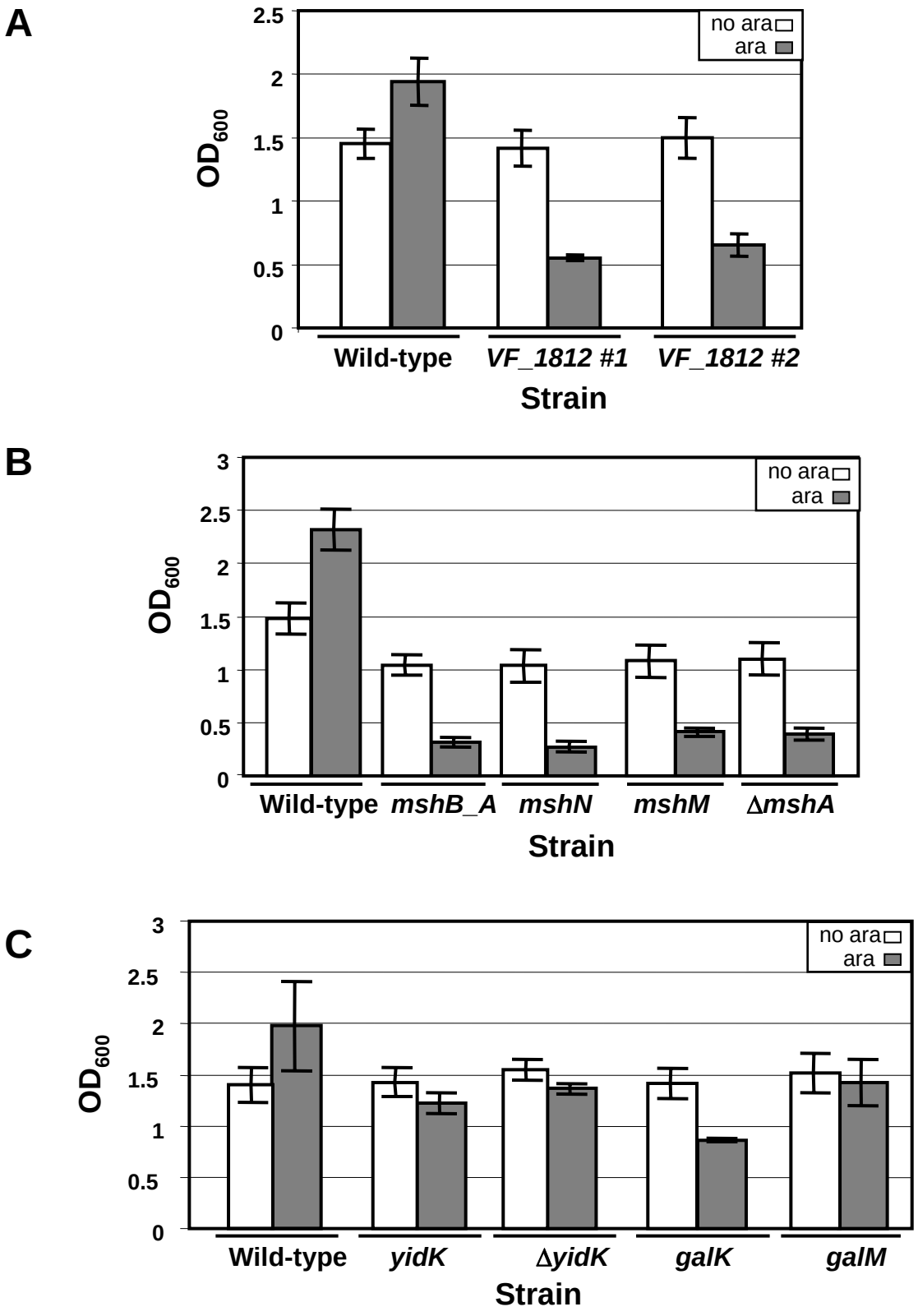

Fig. S4. Optical density measurements of *V. fischeri* static cultures. The indicated *V. fischeri* strains were grown statically overnight in LBS lacking (open bars) or containing (grey bars) 0.2% arabinose. (A). Wild-type strain ES114 and *VF_1812* mutants KV5807 (*VF_1812* #1) and KV5942 (*VF_1812* #2). (B) Wild-type strain ES114, and *msh* mutants KV5948 (*mshB_A*), KV6000 (*mshN*), KV6001 (*mshM*), and KV6004 (Δ*mshA*). (C) Wild-type ES114 and *yid*/*gal* mutants KV5837 (*yidK*), KV5945 (Δ*yidK*), KV1356 (*galK*), and KV5849 (*galM*).

Supplemental Table S1. Plasmids used in this study

Plasmid	Description	Source or reference
pBAD/His/LacZ	*araC*, pBAD-*lacZ*; Ap^R	Invitrogen
pCNW1	pVO8-based *galK*$^+$ plasmid	(1)
pEVS104	Conjugal helper plasmid; Kn^R	(2)
pEVS78	Mobilizable low copy plasmid, Cm^R	(2)
pEVS170	Tn*5* delivery plasmid, Em^R Kn^R	(3)
pKPQ21	pVO8 containing BglII fragment encompassing *flgJ-L* genes	This study
pKPQ22	pKV363 containing ~600 bp sequences flanking both sides of *bcsA* (*VF_A0884*), generated using primers 1297, 1298, 1317, and 1318	This study
pKPQ23	pKV363 containing ~600 bp sequences flanking both sides of *motA* (*VF_0714*), generated using primers 1449, 1450, 1451, and 1452	This study
pKPQ24	pKV363 containing ~600 bp sequences flanking both sides of *yidK* (*VF_A0351*), generated using primers 1441, 1442, 1443, and 1444	This study
pKPQ25	pKV363 containing ~600 bp sequences flanking both sides of *galM* (*VF_A0356*), generated using primers 1445, 1446, 1447, and 1448	This study
pKPQ29	pKV363 containing ~600 bp sequences flanking both sides of *mshA* (*VF_0366*), generated using primers 1502, 1503, 1504, and 1505	This study
pKV143	pEVS78 containing arabinose-inducible *lacZ* derived from pBAD/His/LacZ	This study
pKV363	Suicide plasmid (*ori*-R6K); Cm^R	(4)
pMSM28	pKV363 containing ~600 bp sequences flanking both sides of *rpoN* (*VF_0387*), generated using primers 1229, 1230, 1231, and 1232	This study
pSMM2	pVSV105 containing *VF_1812*, generated using primers 1478 and 1479	This study
pVSV105	Mobilizable low copy plasmid, *ori*-R6K *ori*-pES213 *oriT*; Cm^R	(5)
pVO8	Mobilizable low copy plasmid, Em^R, Cm^R	(6)

Supplemental Table S2. Primers used in this study

Primer	Sequence[a]
908	GCACTGAGAAGCCCTTAGAGCC
1229	TGTAGGCTTCGTCGCTCGTG
1230	taggcggccgcacttagtatgTCCCATTTTTAGCTGAAGAGATGC
1231	catactaagtgcggccgcctaCGTAAGCGTTTACTTTAATTAATG
1232	GAAGCTTACATTGTTCATCGGG
1297	ATGGTTGGGCTAAGAGATGGC
1298	taggcggccgcacttagtatgGAATTCTTTAACGAATCTCGGTTG
1317	catactaagtgcggccgcctaGTCAATTTTTAGAGTGCAATCAG
1318	ATGTAATCGTACTTTGAGAGCTG
1441	ACTGCTTTTAATCCTGCGAAG
1442	taggcggccgcacttagtatgGATGATATCGACAATACCTAATCC
1443	catactaagtgcggccgcctaGCAGTGCTGTACTCAGTATTCTGG
1444	CGCAATATCACTGTTAGGACG
1445	AATACAACACTCGTCGCGAGC
1446	taggcggccgcacttagtatgCATGGTTTCGAACAATGTTGATTG
1447	catactaagtgcggccgcctaCAATTTATTGTTGATAGTTAAGGCC
1448	CGTCTAAAGATGCGAATGAAGG
1449	ATCAAAAGGTGTTAGTTCTGCG
1450	taggcggccgcacttagtatgACCAACTAAGCCTATTAACGTTGC
1451	catactaagtgcggccgcctaGACACTGGCGAAGAATAAGACTGG
1452	CTAATCTCGCGCTCTAGTGC
1478	GGTCGGATTTGTCGTCTATG
1479	CCTAGTCCCTTGAAGCGAAG
1502	GCGGTTAGCTATGATGGTAC
1503	taggcggccgcacttagtatgCTCGATAAGGGTGAAACCACCTTG
1504	catactaagtgcggccgcctaATCGTTACTGATACTGGTTGTTAATC
1505	GCTACGGCACCACTTGCCG

[a]Sequences in lower case letters represent non-native sequences incorporated into the PCR product