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CysK Plays a Role in Biofilm Formation and Colonization by *Vibrio fischeri*

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A biofilm, or a matrix-embedded community of cells, promotes the ability of the bacterium *Vibrio fischeri* to colonize its symbiotic host, the Hawaiian squid *Euprymna scolopes*. Biofilm formation and colonization depend on *syp*, an 18-gene polysaccharide locus. To identify other genes necessary for biofilm formation, we screened for mutants that failed to form wrinkled colonies, a type of biofilm. We obtained several with defects in genes required for cysteine metabolism, including *cysH*, *cysJ*, *cysK*, and *cysN*. The *cysK* mutant exhibited the most severe wrinkling defect. It could be complemented with a wild-type copy of the *cysK* gene, which encodes *O*-acetylserine sulfhydrolase, or by supplementing the medium with additional cysteine. None of a number of other mutants defective for biosynthetic genes negatively impacted wrinkled colony formation, suggesting a specific role for CysK. CysK did not appear to control activation of Syp regulators or transcription of the *syp* locus, but it did influence production of the Syp polysaccharide. Under biofilm-inducing conditions, the *cysK* mutant retained the same ability as that of the parent strain to adhere to the agar surface. The *cysK* mutant also exhibited a defect in pellicle production that could be complemented by the *cysK* gene but not by cysteine, suggesting that, under these conditions, CysK is important for more than the production of cysteine. Finally, our data reveal a role for *cysK* in symbiotic colonization by *V. fischeri*. Although many questions remain, this work provides insights into additional factors required for biofilm formation and colonization by *V. fischeri*.

The ability of bacteria to grow in biofilms, or communities of cells embedded in a surface-associated, self-produced matrix, permits them to survive environmental assaults and colonize a variety of biotic and abiotic surfaces (1, 2). The biofilm matrix typically contains polysaccharides, proteins, and environmental DNA (eDNA), which together provide a protected environment and permit adherence to surfaces. Production of a biofilm depends on the ability of bacteria to recognize and respond to appropriate environmental cues and produce and export a variety of substances that are assembled to permit a three-dimensional (3D) community architecture from which bacteria can ultimately depart.

The complex nature of this process is apparent from the study of numerous bacteria, including the facultative symbiont *Vibrio fischeri*. This marine microbe forms a transient biofilm on the surface of a specialized organ of its host, the squid *Euprymna scolopes* (3). *V. fischeri* cells disperse from this transient biofilm to migrate into pores leading to the internal sites where they multiply to a high cell density and establish a long-term association with the squid (reviewed in reference 4). Mutants defective for the production of this transient biofilm fail to efficiently colonize their squid host (5–8).

Some components of the *V. fischeri* matrix have been identified (Fig. 1). Notably, the 18-gene *sy*mbiosis *p*olysaccharide locus, *syp*, encodes proteins that are responsible for synthesizing and exporting a polysaccharide believed to be the major component of the biofilm matrix (7–9). *syp* mutants fail to form biofilms or efficiently colonize squid. In addition, putative matrix proteins have been identified: BmpA, BmpB, and BmpC (10, 11). These proteins are required for full development of the 3D architecture associated with biofilm formation, and the BmpA protein itself is secreted into the matrix, where it may directly influence architecture. Finally, the presence of outer membrane vesicles in the biofilm matrix has been noted and associated with induction of biofilm formation (11, 12).

A complex two-component regulatory system controls transcription of *syp* and *bmpA*, *bmpB*, and *bmpC* as well as other downstream events required for biofilm formation (Fig. 1). Specifically, RscS, a sensor kinase, indirectly controls the *syp* locus and biofilm formation via a second sensor kinase, SypF, and two downstream response regulators, one of which is the direct DNA binding protein SypG. Mutants defective for these regulators exhibit defects in biofilm formation and colonization (5, 7, 10, 13–16).

Although biofilm formation is readily observed in the context of squid colonization, it is necessary to overexpress *rscS* or another *syp* regulator to observe biofilm phenotypes in laboratory culture (7, 8, 13, 17). Whereas wild-type *V. fischeri* cells form smooth colonies, those that overexpress *rscS* form colonies with a wrinkled morphology, indicative of matrix production dependent on both *syp* and *bmp* (11). Similarly, *rscS*-overexpressing cells form a pellicle, or biofilm, at the air/liquid interface of static liquid cultures, while wild-type cells remain planktonic. Defects in these two bio-

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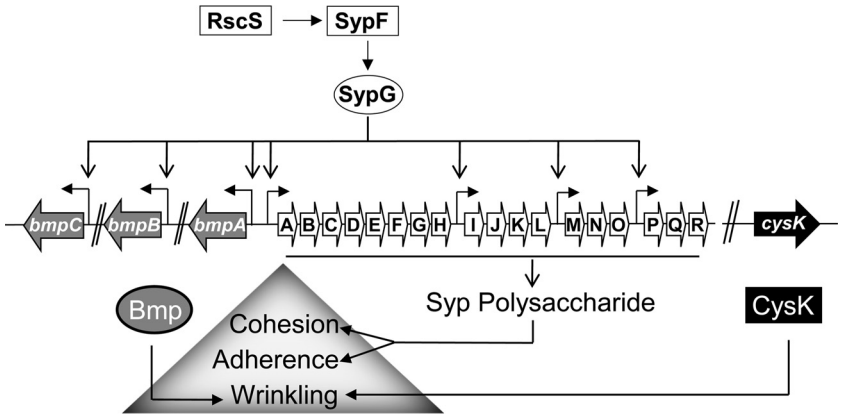

FIG 1 Model for *syp*-dependent wrinkled colony formation and maturation. Biofilm formation, including wrinkled colony formation and pellicle formation (not shown), is controlled via a complex two-component regulatory system comprised of four regulators, the sensor kinases RscS and SypF and the response regulators SypG and SypE (not shown). Activated SypG induces transcription of the *syp* genes, which encode the proteins that synthesize, modify, and export the Syp polysaccharide, and the *bmp* genes, which encode proteins that promote wrinkling of colonies and pellicles. These phenotypes also require cysteine, and CysK in particular. *cysK*, or *VF_1893*, is located on chromosome I, whereas the other indicated genes are located on chromosome II.

film phenotypes, such as those that occur when a *syp* gene is mutated, correlate with a defect in symbiotic colonization (6, 7, 9), making these phenotypes useful for predicting colonization fitness for a particular mutant.

To identify additional genes involved in biofilm formation and, potentially, symbiotic colonization, we mutagenized a biofilm-competent strain of *V. fischeri* and screened for transposon insertion mutants that failed to form wrinkled colonies. This screen identified biofilm mutants defective for genes in the cysteine biosynthesis pathway (see Fig. S1 in the supplemental material). Specifically, we obtained mutants defective for *cysK*, *cysH*, *cysJ*, and *cysN*. In *Escherichia coli* and other bacteria, CysK (*O*-acetylserine sulfhydrolase A subunit) generates L-cysteine from *O*-acetyl L-serine and hydrogen sulfide. The other Cys proteins (H, J, and N) function in the pathway that converts sulfate to sulfide, with CysN converting sulfate into adenosine 5′-phosphosulfate (APS), which is converted sequentially to 3′-phosphoadenylyl phosphosulfate (PAPS) (CysC), sulfite (CysH), and sulfide (CysJ and CysI) (see Fig. S1 in the supplemental material) (18). Our subsequent characterization determined that CysK is the key enzyme in the cysteine biosynthetic pathway involved in promoting biofilm formation. This work thus adds another element to the complex control over biofilm formation exerted by *V. fischeri*.

MATERIALS AND METHODS

Media. For routine culturing and biofilm experiments, *V. fischeri* strains were grown in Luria-Bertani salt (LBS) medium (19). Tris minimal medium (TMM) (20) (50 mM Tris [pH 7.5], 0.2% *N*-acetylglucosamine, 0.1% NH_4Cl, 0.0058% K_2HPO_4, 10 μM ferrous ammonium sulfate, 300 mM NaCl, 10 mM KCl, 50 mM $MgSO_4$, 10 mM $CaCl_2$), supplemented with a small amount of Casamino Acids (0.03%) to facilitate growth of *V. fischeri* and, where indicated, 1 mM cysteine, was used to examine the growth of *V. fischeri cys* mutants. All *E. coli* strains were grown in Luria-Bertani (LB) medium (21). Solid media were made using agar to a final concentration of 1.5%. Antibiotics were added to cultures when appropriate to the following final concentrations: ampicillin at 100 μg ml^{-1} (*E. coli*), tetracycline (Tc) at 15 μg ml^{-1} (*E. coli*) or 5 μg ml^{-1} (*V. fischeri*), chloramphenicol (Cm) at 12.5 μg ml^{-1} (*E. coli*) or 5 μg ml^{-1} (*V. fischeri*), kanamycin (Kn) at 50 μg ml^{-1} (*E. coli*) or 100 μg ml^{-1} (*V. fischeri*), and erythromycin (Em) at 5 μg ml^{-1} (*V. fischeri*). Along with any necessary antibiotics, thymidine was added to a final concentration of 0.3 mM for *E. coli* strain π3813. Cysteine was added to media where noted to the indicated final concentration, generally 1 mM.

Strains and plasmids. *E. coli* strains GT115 (InvivoGen, San Diego, CA), Tam1λpir (Active Motif, Carlsbad, CA), DH5α, and π3813 (22) were used for cloning and conjugation. *V. fischeri* strains used in this study are shown in Table 1, while plasmids and primers are shown in Tables S1 and S2 in the supplemental material, respectively. The *V. fischeri* strains were derived from strain ES114, a wild-type isolate from *Euprymna scolopes* (23), or from strain MJM1198 (described below). Except where noted, derivatives of *V. fischeri* were generated via conjugation (24). To generate Tn*7* insertions in *V. fischeri*, tetraparental matings were carried out as previously described (25). For the overexpression of *sypG*, plasmid pEAH73 was used. Specific *cys*::Tn mutations were introduced into MJM1198 or ES114 using natural transformation (26) as described previously (27), with the following modifications: cells were grown overnight at 28°C in LBS and subcultured in LBS at 25°C prior to subculturing in TMM, chromosomal DNA was extracted using sodium perchlorate and phenol-chloroform, and Em was used as antibiotic selection.

All plasmids were constructed using standard molecular biological techniques, with restriction and modification enzymes obtained from New England BioLabs (Beverly, MA) or Fermentas (Glen Burnie, MD), or via PCR with KOD HiFi (EMD Millipore) or *Taq* (Promega). Deletions of *cysH*, *cysK*, *sypE*, and *sypJ* were generated using suicide plasmids pVAR67, pVAR68, pVAR17, and pKPQ4, respectively, and the approach of Le Roux et al. (22). The *cysH* deletion construct pVAR67 was generated by amplifying DNA flanking *cysH* with primer pairs 1515/1516 and 1517/1518, fusing the flanking sequences using PCR SOEing (28), cloning the joined sequences into PCR cloning vector pJET1.2 (Fermentas), and then finally subcloning into pSW7848 (22). The *cysK* deletion construct pVAR68 was generated similarly, using primer pairs 1507/1508 and 1509/1510 and the final destination plasmid pKV363 (12), a derivative of pSW7848. pPAS2 was generated using PCR to amplify wild-type *cysK* sequences with primer pair 1893/1894, followed by Gibson Assembly (New England BioLabs, Beverly, MA, USA) to introduce $cysK^+$ into pARM47 (5) from which the *sypE* gene was deleted.

A number of strains were generated via Tn mutagenesis. Two different transposons were used, a Cm^r version of Tn*5*, which was delivered by pMJM30, and Mariner, which was delivered by pMarVF1 (27) (see Table S1 in the supplemental material). To generate pMJM30, PCR was performed on pKD3 DNA with primers MJM-108F and MJM-109R. These primers amplified the FLP recombination target (FRT)-flanked Cm^r cassette, adding XmaI restriction sites. PCR was performed on the Em^r Tn*5* delivery vector pMJM10 (29) with primers MJM-132 and MJM-133.

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

Strain	Relevant genotype	Reference
ES114	Wild type	23
KV3299	Δ*sypE*	13
KV4926	Δ*sypE* attTn*7*::P*sypA*-lacZ	This study
KV6305	Δ*sypE* Δ*cysH*	This study
KV6307	Δ*sypE* Δ*cysH* attTn*7*::P*sypA-lacZ*	This study
KV6410	Δ*sypE* Δ*cysK*	This study
KV6608	Δ*sypE* Δ*sypL*	This study
KV7379	*cysH*::Tn-Mariner (Em^r)	This study
KV7381	*cysJ*::Tn-Mariner (Em^r)	This study
KV7383	*cysK*::Tn-Mariner (Em^r)	This study
KV7409	MJM1198 *cysN*::Tn-Mariner (Em^r) (NT)[a]	This study
KV7502	Δ*sypE* Δ*cysK* attTn*7*::P*sypA-lacZ*	This study
KV7522	Δ*sypE* Δ*cysK* attTn*7*::*cysK*$^+$	This study
KV7712	Δ*sypE* Δ*sypJ*	This study
KV7735	Δ*sypE cysK*::Tn-Mariner (Em^r)	This study
KV7744	Δ*sypE* Δ*cysK* Δ*sypJ*	This study
MJM1198	IG (*glpR-rscS*)::Tn*5* (Cm^r)[b]	This study
MJM1555	*cysN*::Tn-Mariner (Em^r)	This study
MJM1602	MJM1198 *cysH*::Tn-Mariner (Em^r)	This study
MJM1603	MJM1198 *cysK*::Tn-Mariner (Em^r)	This study
MJM1604	MJM1198 *cysK*::Tn-Mariner (Em^r)	This study
MJM1661	*trpE2*::Tn-Mariner (Em^r)	This study
MJM1663	*trpB*::Tn-Mariner (Em^r)	This study
MJM1664	*purC*::Tn-Mariner (Em^r)	This study
MJM1665	*metE*::Tn-Mariner (Em^r)	This study
MJM1666	*lysA*::Tn-Mariner (Em^r)	This study
MJM1667	*ilvD*::Tn-Mariner (Em^r)	This study
MJM1669	*ilvA*::Tn-Mariner (Em^r)	This study
MJM1670	*thiC*::Tn-Mariner (Em^r)	This study
MJM1671	*purL*::Tn-Mariner (Em^r)	This study
MJM1672	*ilvB*::Tn-Mariner (Em^r)	This study
MJM1673	*purD*::Tn-Mariner (Em^r)	This study
MJM1675	*metC*::Tn-Mariner (Em^r)	This study
MJM1946	MJM1198 *sypB*::Tn-Mariner (Em^r)	27
MJM1952	MJM1198 *sypQ*::Tn-Mariner (Em^r)	27
MJM1962	MJM1198 *rscS*::Tn-Mariner (Em^r)	This study
MJM1963	MJM1198 *cysH*::Tn-Mariner (Em^r)	This study
MJM1964	MJM1198 *cysH*::Tn-Mariner (Em^r)	This study
MJM1965	MJM1198 *cysJ*::Tn-Mariner (Em^r)	This study
MJM1966	MJM1198 *cysK*::Tn-Mariner (Em^r)	This study
MJM1967	MJM1198 *cysN*::Tn-Mariner (Em^r)	This study

[a] NT, natural transformation was used to generate this strain.
[b] IG, intergenic region; the insert was located between *rscS* and divergently transcribed gene *glpR*.

These primers amplified the plasmid backbone and transposon ends, adding BspEI restriction sites. After digestion of the FRT-flanked Cm^r cassette with XmaI, the PCR product was introduced into the BspEI sites of the transposon delivery vector and selected on LB medium containing Cm, yielding the candidate delivery vector pMJM30. The resulting plasmid was designed to encode a Kn^r backbone and a Cm^r Tn (replacing the Em^r Tn cassette on pMJM10). Subsequent characterization revealed that the vector was Kn^s. This vector was ultimately discarded; however, a pilot transposition experiment had been conducted, and 400 putative hops into *V. fischeri* ES114 were isolated. One of these colonies displayed a highly unusual morphology and appeared to be dried out. Upon restreaking, a highly wrinkled colony morphology was apparent and resembled the colony morphology observed in strains that overexpress the biofilm regulator RscS (7). We isolated the strain, now termed MJM1198, and determined the site of insertion by cloning the Tn and flanking sequences and sequencing from the Tn using primer 170Ext2.

Sequencing and bioinformatics analyses. Semiarbitrarily primed PCR was performed on transposon insertion mutants to identify the exact transposon junction site for the pMarVF1 mariner-derived mutants (44, 45). Genomic DNA from each mutant was prepared using the Qiagen DNeasy Blood & Tissue kit, including the Gram-negative pretreatment. GoTaq (Promega) was used to amplify the transposon-chromosome junction in two sequential reactions of 50-μl volumes. The first reaction mixture contained 10 μl buffer (colorless), 4 μl deoxynucleoside triphosphate (dNTP) mix (2.5 mM each), 0.5 μl each of primers Arb1 and MJM-440, 0.25 μl GoTaq, 27.75 μl H_2O, and 7 μl candidate genomic DNA (gDNA; 28.6 ng/μl). PCR products were purified using the QIAquick PCR purification kit, eluted in 50 μl H_2O, and used for the second reaction. The second reaction mixture contained 10 μl buffer (colorless), 4 μl dNTP mix (2.5 mM each), 0.5 μl each of primers Arb2 and MJM-477, 0.25 μl GoTaq, 33.75 μl H_2O, and 1 μl purified round 1 product. The PCR products were purified using the QIAquick PCR purification kit and submitted for Sanger sequencing at the Northwestern University Genomics Core Facility with primer MJM-477. The sequences were trimmed after the transposon junction, and the resulting sequence was assessed using the Basic Local Alignment Search Tool (BLAST) (30, 31) to identify the site of insertion.

Growth evaluation. Growth of wild-type and mutant *V. fischeri* was assayed in LBS or TMM, lacking or containing 1 mM cysteine. For both conditions, *V. fischeri* strains were first inoculated into LBS medium with a single colony and grown overnight at 28°C with shaking. Then, the cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.005 in 20 ml LBS (or 0.02 in TMM) and incubated at 28°C with shaking. Every 60 min, the OD_{600} was measured using a spectrophotometer until the growth of the cells reached a plateau. The OD_{600} values were plotted over time. These experiments were performed at least 3 times for each set of strains.

Wrinkled colony assay. *V. fischeri* strains were cultured overnight (14 to 16 h) at 28°C with shaking in LBS medium, and then the culture was diluted 1:100 into fresh LBS and grown under the same conditions for 2 to 3 h the next day. The subcultures were then standardized to an OD_{600} of 0.2, and 10-μl aliquots were spotted onto LBS agar plates. For experiments with derivatives of MJM1198 that overexpressed *rscS* from the chromosome, the LBS plates were incubated at 23°C. For experiments that used the pSypG overexpression vector pEAH73, LBS plates containing Tc were incubated at 28°C. As appropriate, cysteine was added to the medium. The spots were then monitored over time to assess wrinkled colony formation. When appropriate, the spots were disturbed with a toothpick to assess their adherence to the agar. Each assay was performed at least 3 or 4 times. Images of spotted colonies were acquired at the indicated times using a Zeiss Stemi 2000-C dissecting microscope.

Syp polysaccharide extraction and immunoblotting analysis. Cells were grown overnight at 25°C shaking in LBS. A total of 1.5 ml of each culture was centrifuged at 10,000 × *g* for 10 min, and the cell pellets were washed with 70% filter-sterilized Instant Ocean. Pellets were resuspended in 400 μl of a 5 mM EDTA–50 mM sodium phosphate, dibasic solution. Saturated phenol (300 μl) was added to each sample. The samples were subsequently incubated at 65°C for 15 min, placed on ice for 5 min, and then centrifuged at 10,000 × *g* for 10 min. The aqueous phase was extracted and applied to a MicroSpin G-25 column (GE Healthcare Life Sciences) and then lyophilized overnight. Each sample was resuspended in 50 μl of sample buffer (65 mM Tris [pH 6.8], 10% glycerol). Ten microliters of each sample was resolved on a 10% SDS-PAGE gel. Protein transfer and Western blotting were performed using standard protocols with antibiofilm antibody generated and treated as described previously (12).

Cell mixing assay. Cell mixing experiments were carried out as follows. *V. fischeri cysK* mutant strain KV6509 was grown overnight in LBS containing Tc. Similarly, the indicated *V. fischeri* and *E. coli* strains were grown overnight in LBS and LB, respectively; for the *cysE* mutant complemented with the *cysE* plasmid, Cm was added for plasmid maintenance. A 300-μl aliquot of KV6509 was mixed with 100 μl of the indicated strain. The mixtures were concentrated by centrifugation, and the supernatant

was removed. The mixtures were resuspended in 10 μl of fresh LBS broth, and 10-μl aliquots were spotted onto LBS agar plates. The plates were incubated at 28°C for the indicated periods of time, after which images were acquired using a Zeiss Stemi 2000C dissecting microscope.

Pellicle assay. Strains were grown (in triplicate) with shaking in LBS-Tc containing or lacking 1 mM cysteine at 28°C overnight and then diluted to an OD_{600} of 0.2 in 2 ml of fresh medium in 24-well microtiter dishes. Cultures were then incubated statically at 28°C for 48 h. The strength of each pellicle was evaluated by disrupting the air-liquid interface with a sterile toothpick after the indicated incubation period. Images of pellicles were acquired at the indicated times using a Zeiss Stemi 2000-C dissecting microscope.

Colonization assay. Cultures of the *cysK* mutant and its parent grown in seawater tryptone (SWT) broth to early log phase were used to inoculate newly hatched juveniles of the squid *E. scolopes*. The squid were incubated with *V. fischeri* for 3 h or overnight. Following ~24 h of inoculation, the squid were sacrificed by homogenization and the homogenates were diluted and plated onto SWT plates. The colonies that arose were counted to determine the number of bacteria that had colonized the animals (CFU per squid). Experiments involving *E. scolopes* were carried out using approaches described in an Animal Component of Research Protocol (ACORP) approved by Loyola University's Institutional Animal Care and Use Committee (IACUC).

RESULTS

Generation of a biofilm-overproducing *V. fischeri* strain by Tn mutagenesis. In the course of Tn mutagenesis experiments designed to evaluate the efficacy of a newly generated Tn*5* delivery plasmid (see Materials and Methods), we identified a single mutant, MJM1198, that formed a colony with a wrinkled morphology. Subsequent sequence analysis revealed that the Tn (and its delivery vector) were inserted upstream of the *rscS* gene, which encodes the sensor kinase that is the upstream activator of *syp*-dependent biofilm formation (Fig. 1). Although the mechanism by which the Tn induces biofilm formation is unknown, we hypothesize that a promoter located within the transposon increases the transcription of *rscS*. This insertion was indeed the cause of the wrinkled colony morphology, as the introduction of mutations in either *rscS* or the RscS-regulated *syp* locus resulted in smooth colonies (27) (Table 1). Because our past work investigating biofilm formation has been dependent on the use of plasmids, and thus antibiotics, to induce biofilm formation of *V. fischeri*, this strain provides a new tool that facilitates the examination of biofilm formation.

Biofilm-defective mutants contain insertions in cysteine biosynthetic genes. We used MJM1198 as the parent strain in a screen for transposon mutants with defects in the formation of wrinkled colonies. In a screen of ~20,000 mutants, we isolated several that contained insertions in genes predicted to be required for cysteine biosynthesis, including *cysH*, *cysJ*, *cysK*, and *cysN*. For both *cysH* and *cysK*, three independent insertions were obtained (Tables 1 and 2; see also Fig. S2 in the supplemental material); as each set of these mutants behaved the same in subsequent experiments, we show here only one representative for the *cysH* and *cysK* mutants. The names of these genes indicate that they are bidirectional best hits with the genes of the same names from *E. coli* strain K-12 MG1655 (32), and our own alignments indicate the same (see Fig. S3 in the supplemental material). We thus hypothesize that the *V. fischeri*-encoded proteins carry out the same functions as their *E. coli* counterparts (see Fig. S1 in the supplemental material). In support of this idea, the *cys* mutants grew poorly relative to their parent (closed circles) in minimal medium containing only small amounts of amino acids, a growth defect that was reversed by added cysteine (Fig. 2A and B). In contrast, in the complex medium used for the biofilm experiments (LBS), the strains exhibited similar, but relatively mild, growth defects, indicating that cysteine is being supplied under these conditions (Fig. 2C and D).

TABLE 2 List of *cys* genes identified in the screen for wrinkled colony mutants

Gene name	VF no.	Position of Tn insertion[a]	ORF position (%)[b]	Strain name
cysH	VF_0312	322464	35	MJM1602
cysH	VF_0312	322825	83	MJM1963
cysH	VF_0312	322821	82	MJM1964
cysJ	VF_0310	320251	88	MJM1965
cysK	VF_1893	2129201	37	MJM1603
cysK	VF_1893	2129252	42	MJM1604
cysK	VF_1893	2128949	11	MJM1966
cysN	VF_0321	331610	80	MJM1967

[a] Position of Tn insertion in GenBank accession no. CP000020.2 (nucleotide position).
[b] Relative position of the insertion in the open reading frame (ORF), from 5′ (0%) to 3′ (100%).

We next used a semiquantitative method (33) for evaluating the relative defects of these strains in biofilm formation by assaying the development of wrinkled colonies over time in the absence and presence of added cysteine (1 mM) (Fig. 3 and 4 and data not shown). We observed the greatest defect for the *cysK* mutant, which never developed fully wrinkled colonies (Fig. 3E). The *cysJ* and *cysH* mutants both exhibited delays in development (Fig. 3C and D), with that of *cysH* being more substantial, but ultimately formed wrinkled colonies similar to those of the control. The defects of these three mutants were suppressed by the addition of cysteine to the medium (Fig. 4). Finally, in contrast to the other *cys* mutants, the *cysN* mutant did not exhibit a discernible defect in wrinkled colony formation (Fig. 3F). While this mutant was isolated as a smooth colony in the initial screen, our subsequent evaluation of this mutant revealed that its defect could not be suppressed by the addition of cysteine (data not shown). We suspected that it carried a secondary mutation, and indeed we determined this to be the case following the reintroduction of the *cysN*::Tn insertion into the parent background. We proceeded to use this newly constructed strain for subsequent experiments. From these observations, we conclude that defects in *cys* biosynthetic genes exert different effects on wrinkled colony formation—ranging from none to severe—and that *cysK* is the most important *cys* gene for this phenotype.

Wrinkled colony formation is not impacted by mutations in other biosynthetic pathways. We next evaluated the impact on wrinkled colony formation of a variety of mutations in other biosynthetic genes, including those required for biosynthesis of tryptophan, methionine, and lysine, among others. None of these mutations impacted the ability of RscS to induce wrinkled colony formation (Table 3). We conclude that cysteine may have a specific role in controlling biofilm formation.

CysK functions downstream of RscS signaling. To begin to assess the level at which cysteine plays a role in wrinkled colony formation, we asked whether cysteine provides a signal important for the activity of RscS, the sensor kinase that indirectly activates transcription of the *syp* locus and promotes biofilm formation

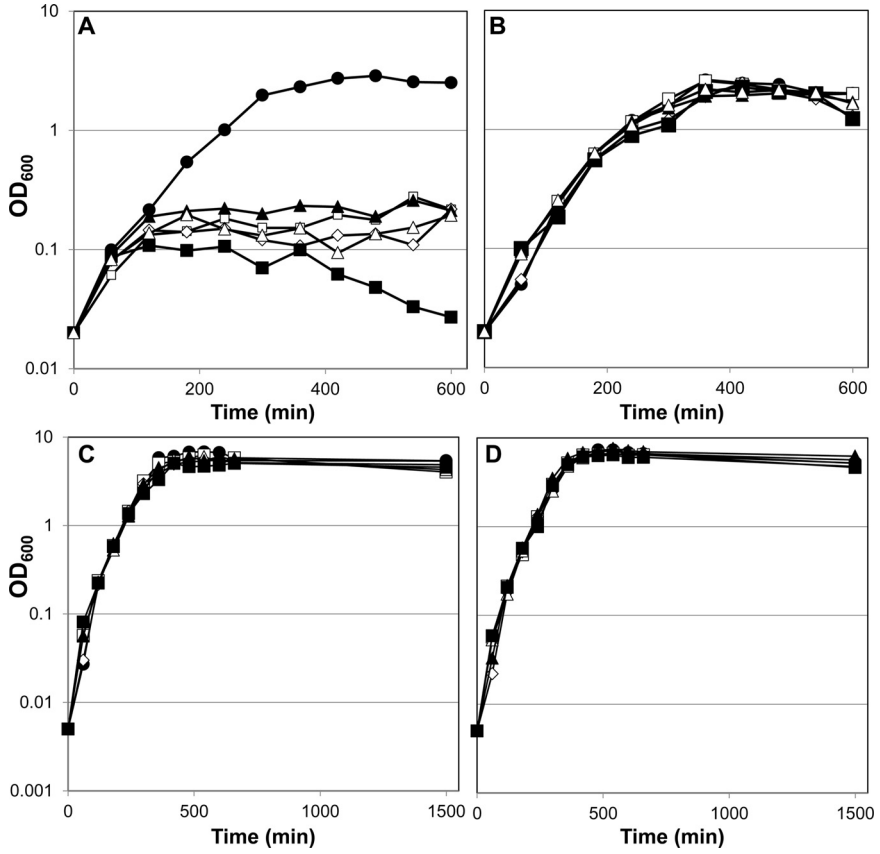

FIG 2 Growth of the *cys* Tn mutants. (A and B) Cells were grown in LBS medium and then subcultured into Tris minimal medium either lacking (A) or containing (B) added 1 mM cysteine. (C and D) Cells were grown and then subcultured in LBS lacking (C) or containing (D) 1 mM cysteine. The strains assayed are as follows: parent strain MJM1198 (filled circles), *cysH*::Tn (MJM1964) (open squares), *cysJ*::Tn (MJM1965) (open diamonds), *cysK*::Tn (MJM1966) (closed squares), *cysN*::Tn (MJM1967) (closed triangles), and *cysN*::Tn [NT] (KV7409) (open triangles). These data are representative of at least three independent experiments.

(Fig. 1). The requirement for RscS can be overcome by overproduction of the downstream response regulator, SypG, which directly activates *syp* transcription and promotes biofilm formation in strains lacking the biofilm inhibitor SypE (5, 13). We therefore generated deletions of *cysH* and *cysK* in a strain that lacks SypE and then overexpressed *sypG* and evaluated biofilm formation. These strains behaved like their respective Tn mutants: the Δ*cysH* mutant exhibited a mild defect in wrinkled colony development (data not shown), while the Δ*cysK* mutant exhibited a more severe defect (Fig. 5; see also Fig. S4 in the supplemental material). Finally, the defect of the Δ*cysK* mutant was complemented by the addition of cysteine (Fig. 5C) or by the insertion of a wild-type copy of *cysK* in the chromosome (Fig. 5D). These data indicate that the deletion of *cysK* fully accounts for the wrinkled colony defect and that this phenotype is connected to cysteine metabolism. We conclude that cysteine functions to control wrinkled colony formation at a level distinct from activation by RscS.

Transcription of the *syp* locus is unaffected by *cysK* mutation. A key player in *V. fischeri* biofilm formation is the *syp* locus, which generates the polysaccharide component of the matrix. Therefore, one possibility is that mutation of cysteine biosynthetic genes negatively impacts transcription of the *syp* locus. To test this idea, we assayed transcription of the *sypA* gene in a strain defective for either *cysK* or *cysH*. As previously observed (13), overexpression of *sypG* induced *sypA* transcription (Fig. 6). This induction was not substantially changed by the deletion of *cysH* or *cysK* (Fig. 6). These data suggest that the loss of cysteine biosynthesis impacts biofilm formation at a level distinct from *syp* transcription.

The *cysK* mutant retains the ability to produce Syp-PS. Although the formation of smooth colonies generally indicates a lack of biofilm formation, recent work from our lab has demonstrated that biofilm-induced but smooth-appearing colonies may, in fact, retain some biofilm properties. Notably, mutants defective for the production of a set of putative matrix proteins, Bmp (Fig. 1), formed smooth but cohesive colonies that could be moved as a unit across the plate surface (11). The cohesive properties were attributed to the production of Syp polysaccharide (Syp-PS), as disruption of representative *syp* genes abolished this phenotype. Therefore, we wondered if the *cysK* mutant produces smooth colonies with cohesive properties, as this phenotype would suggest that Syp-PS is produced. To evaluate colony cohesiveness, cultures of the *sypG*-overexpressing Δ*cysK* mutant and *cysK*$^+$ control strains were spotted onto LBS plates and monitored over time. At specific times, cohesion was evaluated by disrupting the spots with a flat toothpick. Unexpectedly, the positive-control strain adhered to the agar surface (Fig. 7A and B), a property distinct from that previously observed for a biofilm-induced (RscS-expressing) strain (11). This phenotype was not apparent at early stages (e.g., less than about 12 h of development); at 9 h, for example, the colony of the positive control was smooth and not adherent: the

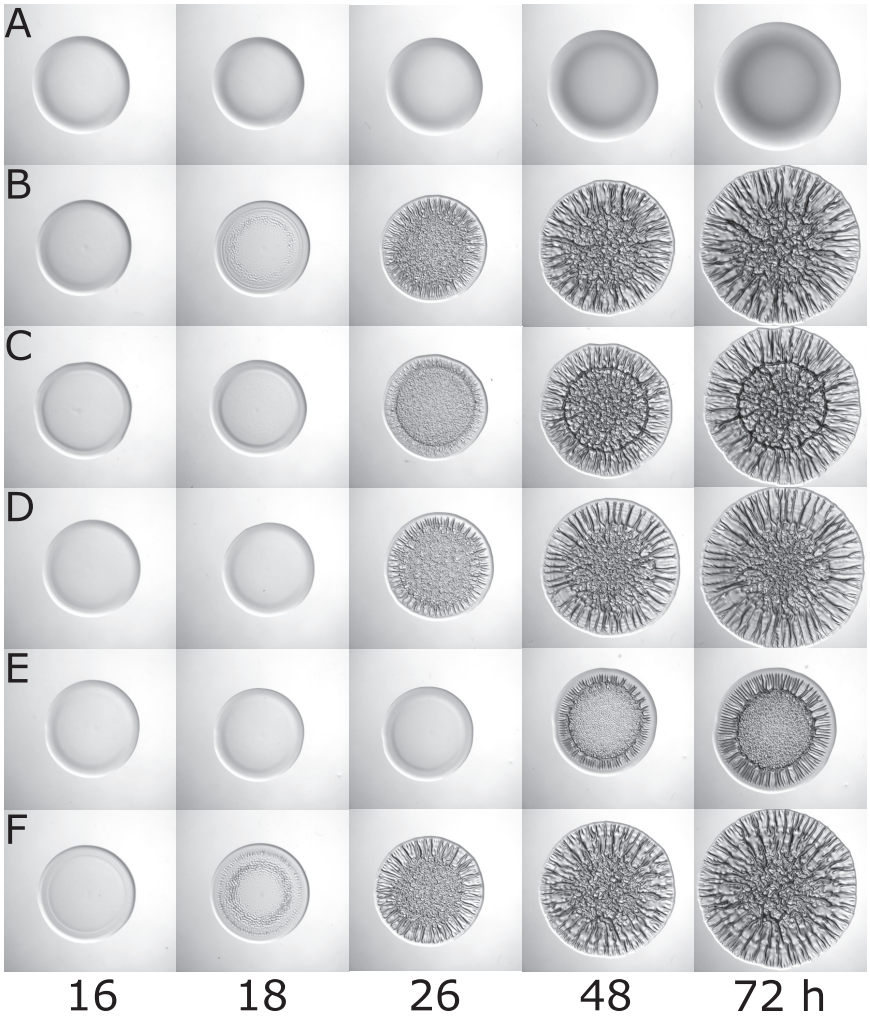

FIG 3 Wrinkled colony formation by *cys* Tn mutants. A time course assay of wrinkled colony formation was performed using strains that overexpress *rscS* from the chromosome. Ten microliters of freshly growing cultures was spotted onto LBS plates, which were then incubated at room temperature. Wrinkled colony formation was observed over time, and images were acquired at the indicated times. (A) Negative control (ES114); (B) *cys*$^+$ parent strain (MJM1198); (C) *cysH*::Tn (MJM1964); (D) *cysJ*::Tn (MJM1965); (E) *cysK*::Tn (MJM1966); (F) *cysN*::Tn [NT] (KV7409). These data are representative of at least three independent experiments.

toothpick readily passed through the colony, disrupting only the cellular material directly in its path. After about 12 h, most of the colony adhered to the surface of the agar plate, an adherent phenotype that did not diminish over time. Like the previously observed colony-cohesive phenotype, the ability of the control strain to adhere to the agar surface depended on the *syp* locus and thus the production of Syp-PS: disruption of a representative *syp* gene (*sypJ*) resulted in strains that failed to adhere to the agar surface (see Fig. S5 in the supplemental material). Despite its smooth phenotype, the *cysK* mutant exhibited a similar pattern: the colony was initially nonadherent and then attached to the plate and remained attached at later times (Fig. 7C and D). Like its parent, the ability of the *cysK* mutant to adhere to the agar surface depended on an intact *syp* locus (see Fig. S5 in the supplemental material). From these data, we conclude that the *cysK* mutant retains its parent's ability to adhere to the surface of the agar plate. Thus, despite the inability of this mutant to produce wrinkled colonies, it retains the ability to produce Syp-PS.

We next assessed Syp-PS production more directly using an antibody-based assay that we had previously developed with antibiofilm antibodies (12). We found that the *cysK* mutant consistently exhibited a reduction in the intensity of bands detected by the antibiofilm antibodies (Fig. 7E). Complementation with the *cysK* gene largely restored the phenotype to that of the parent strain. We conclude that the *cysK* mutant retains the ability to produce Syp-PS, but either at reduced levels or, potentially, with antigenically distinct properties. Either way, this result provides an explanation for the defect of the *cysK* mutant in producing colonies with 3D architecture.

Mixtures of strains complement the *cysK* defect. *E. coli* can secrete cysteine (34–36). Therefore, we asked if *E. coli* could supply cysteine to *V. fischeri* and complement the wrinkled colony defect of the SypG-expressing *cysK* mutant. Indeed, spotting a mixture of *V. fischeri* and *E. coli* resulted in the formation of a wrinkled colony (Fig. 8A). Surprisingly, however, we observed the same result when we used mutants of *E. coli* that were *cys* auxotrophs, including *cysK*, *cysH*, and *cysJ* mutants (Fig. 8A and data not shown). These data suggest that it is not cysteine, *per se*, that suppresses the

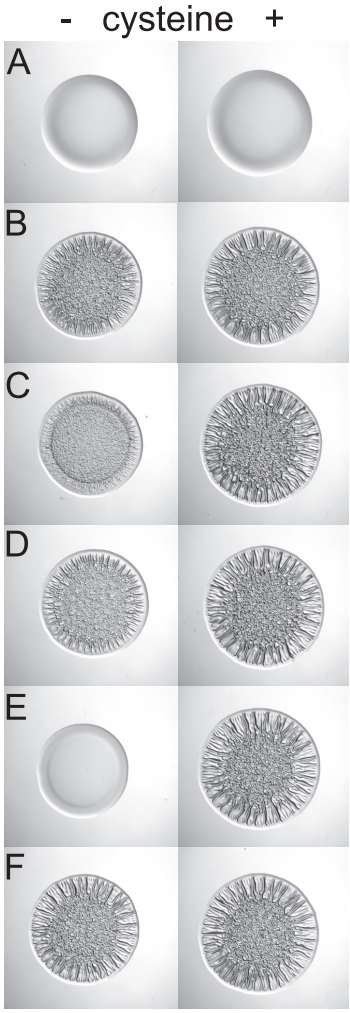

FIG 4 Complementation of wrinkled colony formation by exogenous addition of cysteine. The ability of exogenous cysteine to complement the wrinkled colony formation defect of *cys*::Tn mutants was assessed by adding 1 mM cysteine to the medium. Wrinkled colony formation was observed over time; the images shown here were taken at 26 h, and the no-cysteine control images are the same as those shown in Fig. 3 (26 h). (A) Negative control (ES114); (B) *cys*$^+$ parent strain (MJM1198); (C) *cysH*::Tn (MJM1964); (D) *cysJ*::Tn (MJM1965); (E) *cysK*::Tn (MJM1966); (F) *cysN*::Tn [NT] (KV7409). These data are representative of at least three independent experiments.

wrinkling defect. There was one *cys* mutant that behaved differently: an *E. coli cysE* mutant failed to promote wrinkled colony formation by *V. fischeri*. This defect was complemented by a plasmid-borne copy of the *cysE* gene (Fig. 8A). These data suggest that *E. coli* supplies a CysE-dependent product to promote wrinkling of *V. fischeri*.

We therefore wondered if *V. fischeri* could also secrete something to promote wrinkled colony formation of *V. fischeri*. We found that mixed cultures of the biofilm-defective *cysK* mutant with wild-type strain ES114 or various *cys* mutants (not induced for biofilm formation) could also produce wrinkled colonies (Fig. 8B). Thus, as was the case for *E. coli*, the production (and presumably secretion) by *V. fischeri* of something other than cysteine suppresses the wrinkled colony defect. An exception was *cysK*, which failed to promote wrinkled colony formation. Thus, in *V. fischeri*, some activity of CysK is necessary to promote biofilm formation.

CysK is required for pellicle formation. Another *syp*-dependent biofilm phenotype is the formation of pellicles at the air/liquid interface of static cultures. We therefore asked if the *cys* mutants exhibited a defect in this phenotype as well. We thus grew the mutants in the complex medium LBS overnight with shaking and then subcultured them into LBS and incubated them statically. Consistent with their moderate defects in wrinkled colony formation, the *cysH*, *cysJ*, and *cysN* mutants were competent to produce pellicles (see Fig. S6 in the supplemental material; also, data not shown). Consistent with their more severe defect in wrinkled colony formation, both the *cysK* Tn (see Fig. S6 in the supplemental material) and the Δ*cysK* (Fig. 9) mutants produced relatively poor pellicles with little biomass and that lacked 3D architecture. Moreover, although pellicle development was increased when the medium was supplemented with cysteine, normal pellicle formation did not occur, even when cysteine was supplied during both the overnight growth and the subsequent static growth (Fig. 9B and C). In contrast, the complemented Δ*cysK* mutant was competent to form a pellicle (Fig. 9D). We conclude that *cysK*, but not cysteine *per se*, is required for full pellicle formation.

The *cysK* mutant exhibits a defect in colonizing squid. Previous studies have revealed a correlation between the failure of *V. fischeri* mutants to form wrinkled colonies and pellicles with their inability to colonize the squid *E. scolopes* (e.g., see references 7, 8, and 9). In addition, previous work by Graf and Ruby (37) examining colonization by amino acid auxotrophs identified a cysteine biosynthesis mutant as defective for colonization. Thus, we hypothesized that the Δ*cysK* mutant would exhibit a colonization defect.

To test this hypothesis, we inoculated newly hatched squid with either the *cysK* mutant or a *cysK*$^+$ strain for 3 h and then incubated them for an additional ~21 h and assessed the resulting colonization. Whereas the parent strain colonized to an average of about 10^5 CFU per squid, the *cysK* mutant exhibited a severe defect in its ability to colonize (Fig. 10A). To assess this phenotype further, we inoculated another set of animals for an extended period of time (24 h) with the same two strains. Under these conditions, the *cysK* mutant was nearly as proficient as the *cys*$^+$ strain at colonizing (Fig. 10B). We conclude that the *cysK* mutant has an

TABLE 3 List of biosynthetic mutants tested[a]

Gene	VF no.	Position of Tn insertion[b]	Strain name
ilvA	VF_2560	2883584	MJM1669
ilvB	VF_2556	2878984	MJM1672
ilvD	VF_2559	2880810	MJM1667
lysA	VF_2485	2786078	MJM1666
metC	VF_1186	1318558	MJM1675
metE	VF_1721	1940121	MJM1665
purC	VF_1498	1657684	MJM1664
purD	VF_2395	2683076	MJM1673
purL	VF_0652	713926	MJM1671
thiC	VF_0032	37875	MJM1670
trpB	VF_1028	1132573	MJM1663
trpE2	VF_2602	1138072	MJM1661

[a] All mutants listed displayed a wrinkled colony phenotype upon RscS overexpression with pKG11 compared to the positive-control wild-type strain containing pKG11.
[b] Position of Tn insertion in GenBank accession no. CP000020.2 (nucleotide position).

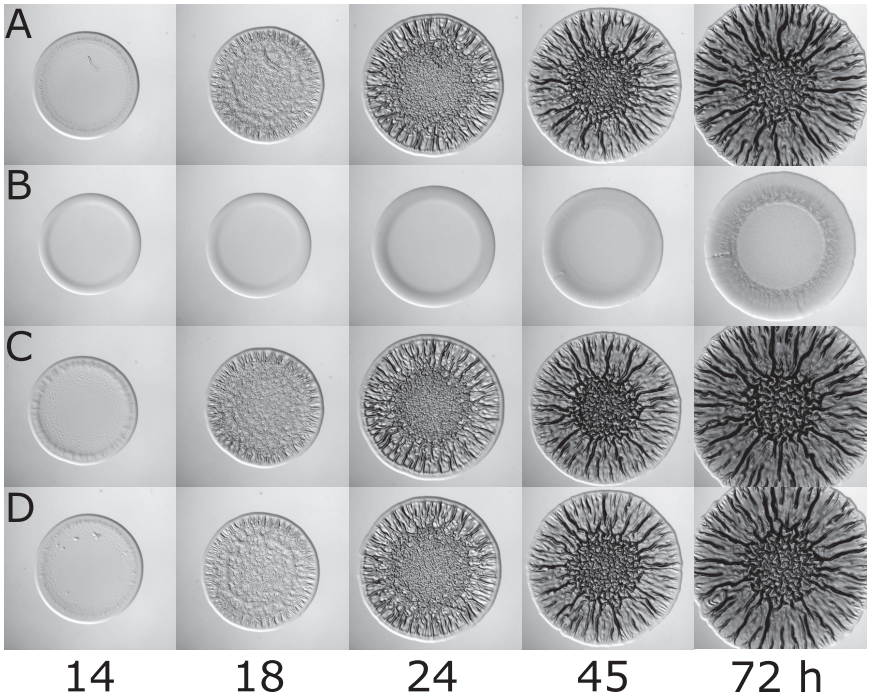

FIG 5 Complementation of the *cysK* mutant with cysteine or *cysK*$^+$. A time course assay of wrinkled colony formation was performed to assess the biofilm phenotype of the *sypG*-expressing Δ*cysK* mutant and its complemented derivative. Ten microliters of freshly growing cultures was spotted onto LBS plates containing Tc, which were then incubated at 28°C. Wrinkled colony formation was observed over time, and images were acquired at the indicated times. The strains used are pEAH73-containing derivatives of the following: (A) positive control, KV3299; (B and C) Δ*cysK* mutant, KV6410; (D) Δ*cysK cysK*$^+$ strain, KV7522. In panel C plates, 1 mM cysteine was added to the plate.

early colonization defect that can be overcome by an increase in the time of inoculation.

DISCUSSION

In this study, we identified and used a new tool for investigating biofilm formation by *V. fischeri*, namely, strain MJM1198, which constitutively forms biofilms. We used this strain to identify unknown biofilm factors and found a role for cysteine biosynthesis in general, and CysK in particular, in promoting biofilm formation by *V. fischeri*. Tn insertions in several *cys* biosynthetic genes, but not in any of a variety of other biosynthetic genes, negatively impacted wrinkled colony formation. This work thus adds cysteine biosynthetic genes to a growing list of genes involved in biofilm development by *V. fischeri*.

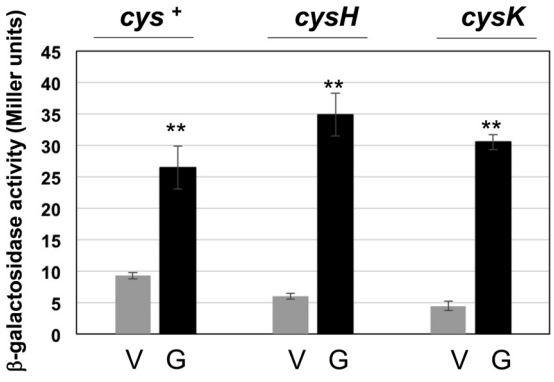

FIG 6 Mutations in *cysH* or *cysK* do not diminish *syp* transcription. Reporter strains (P*sypA-lacZ*) containing the vector control pKV69 (V) or the *sypG*-expressing plasmid pEAH73 (G), as indicated by the gray and black bars, respectively, were grown in LBS containing Tc. The strains contained wild-type copies of the *cys* genes or carried a Δ*cysH* mutation or Δ*cysK* mutation, respectively, as indicated. The strains used are plasmid-containing derivatives of the following strains: KV4926, KV6307, and KV7502. These experiments were performed at least twice with similar results. The asterisks indicate that the SypG-induced levels are significantly different from the uninduced levels ($P < 0.05$).

Cysteine biosynthetic genes have been shown to play roles in the biofilms formed by other bacteria, including *E. coli* and *Providencia stuartii* (38). In these organisms, cysteine appears to negatively regulate biofilm formation, as mutations in *cysE* enhance biofilm formation and cause higher biomass production. The enhanced biofilms of the *E. coli cysE* mutant could be reduced by exogenous addition of *O*-acetyl serine (OAS), the product of CysE (38). However, the mechanism by which this occurs and whether OAS is the natural signal controlling biofilm formation remain unknown. Intriguingly, *cysE* might be an essential gene in *V. fischeri*: in a saturating mutagenesis, no insertions in the *cysE* gene were recovered (27), and in our preliminary experiments we failed to generate a deletion mutant of *cysE*. This gene is not essential in *E. coli*, and additional work is necessary to determine if it is essential in *V. fischeri*. If it were, such a result would suggest a divergence of the pathways in the two organisms.

E. coli encodes multiple proteins that facilitate export of cysteine and other metabolites of the cysteine biosynthetic pathway, including YfiK, YdeD, and CydC/CydD (34–36). It is possible that *V. fischeri* can also secrete cysteine, as it encodes the latter two proteins (VF_0900 and VF_0901) (but not the former two). Our

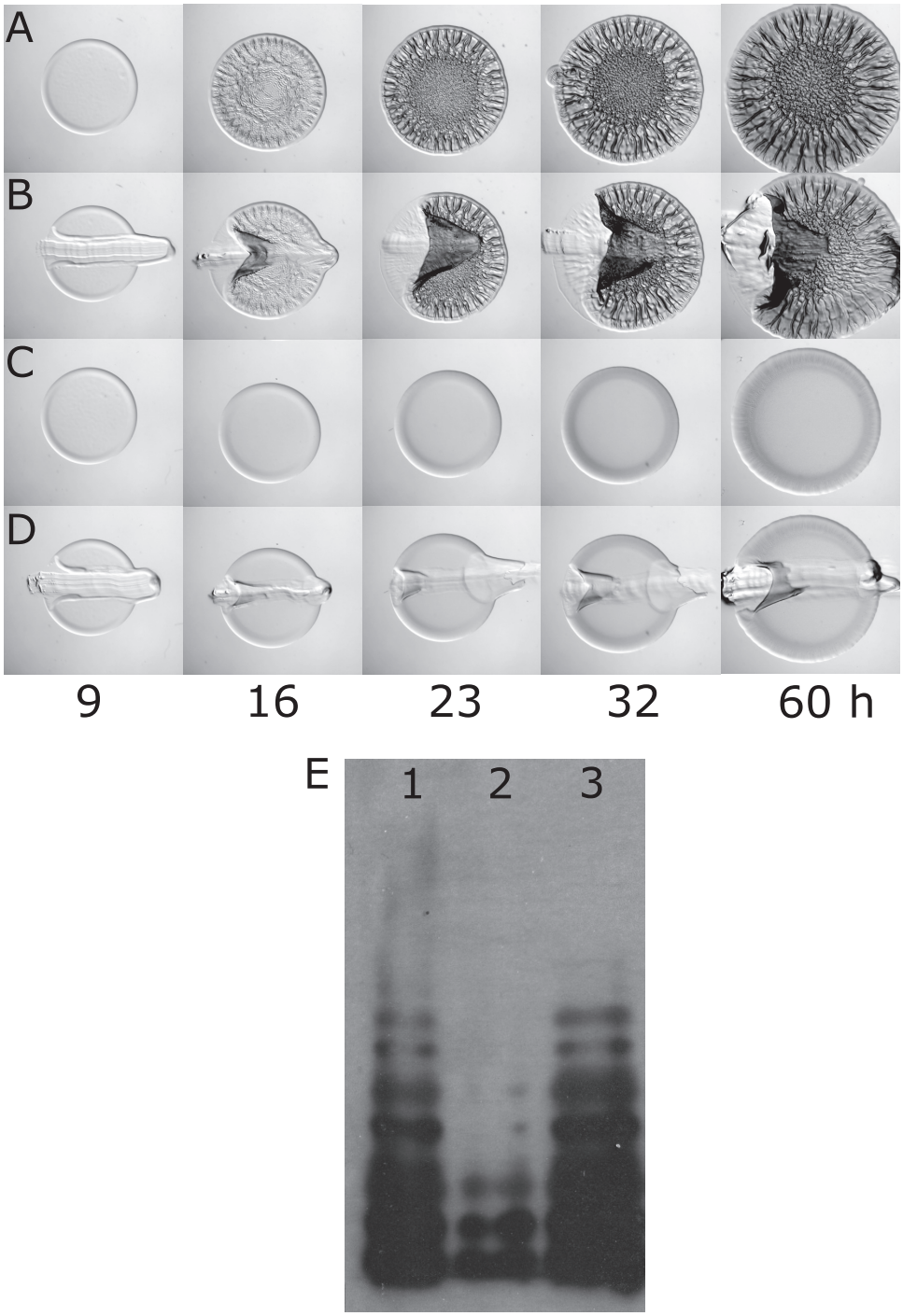

FIG 7 The *cysK* mutant retains at least a partial ability to produce Syp-PS. The agar adherence properties of the Δ*cysK* mutant and its parent were determined by growing cultures of the pEAH73-containing (*sypG*-overexpressing) derivatives of Δ*sypE* strain (KV3299) and Δ*sypE* Δ*cysK* strain (KV6410) and spotting them onto LBS agar containing Tc. The spots were disturbed with a toothpick at the indicated times. Shown are representative images of undisturbed (A) and disturbed (B) spots of p*sypG*/Δ*sypE* and undisturbed (C) and disturbed (D) spots of p*sypG*/Δ*sypE* Δ*cysK*. (E) The ability of the *cysK* mutant to produce Syp-PS was assessed with a Western blot approach using antibiofilm antibodies. The lanes contain extracts from pEAH73-containing (*sypG*-overexpressing) strains as follows: 1, Δ*sypE* strain (KV3299); 2, Δ*sypE* Δ*cysK cysK*$^+$ strain (KV6410); and 3, Δ*sypE* Δ*cysK cysK*$^+$ strain (KV7522).

experimental evidence using mixed cultures supports the hypothesis that *V. fischeri* can secrete cysteine and/or other molecules to impact biofilm formation: mixtures of wild-type *V. fischeri* with the induced but biofilm-defective *cysK* mutant of *V. fischeri* permitted the wrinkled colony formation by the latter strain. However, the same was true when *cys* mutants were used in place of the wild-type strain, indicating that some metabolite other than cysteine or else another secreted molecule was responsible for

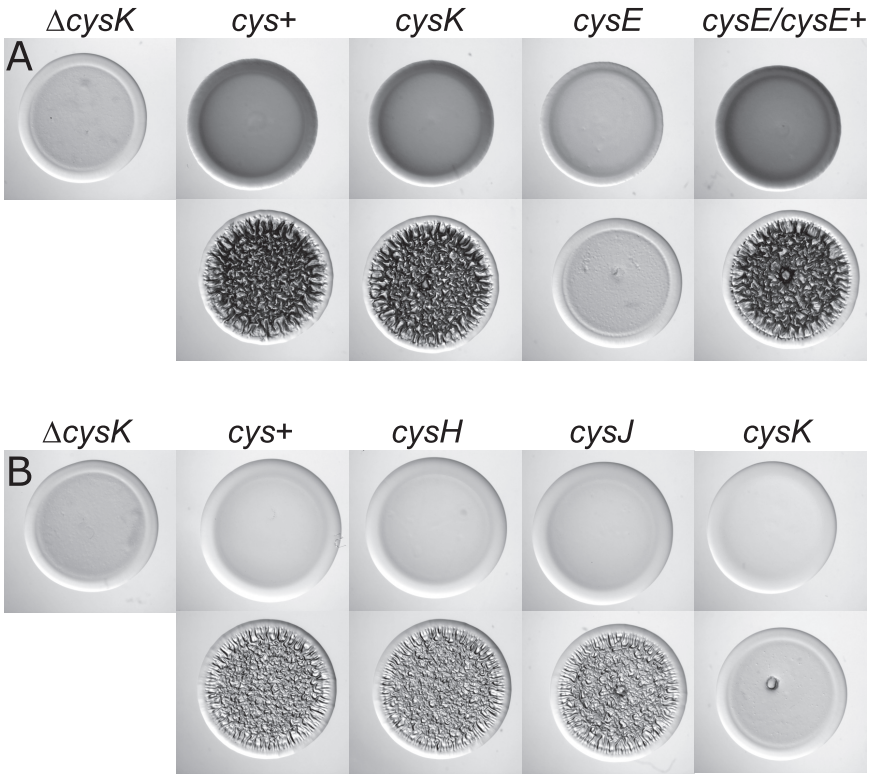

FIG 8 Mixtures of the *cysK* mutant with *V. fischeri* and *E. coli* produce wrinkled colonies. The ability of wild-type and *cys* mutant strains of *E. coli* and *V. fischeri* to suppress the wrinkled colony defect of the Δ*cysK* mutant was assessed with mixed culture spotting experiments. Cultures of the pEAH73-containing (*sypG*-overexpressing) derivative of Δ*sypE* Δ*cysK* mutant (KV6410) were mixed with cultures of *E. coli* (A) or *V. fischeri* (B) and spotted onto LBS plates. The plates were incubated 26 h (A) or 24 h (B). The strains shown were spotted alone or in combination with pEAH73-containing KV6410 and are as follows: *E. coli*, *cys*+ strain (BW25113), *cysK*, *cysE*, and p*cysE*$^+$/*cysE*; *V. fischeri*, *cys*$^+$ (wild-type *V. fischeri* strain ES114), *cysH* (KV7379), *cysJ* (KV7381), and *cysK* (KV7383).

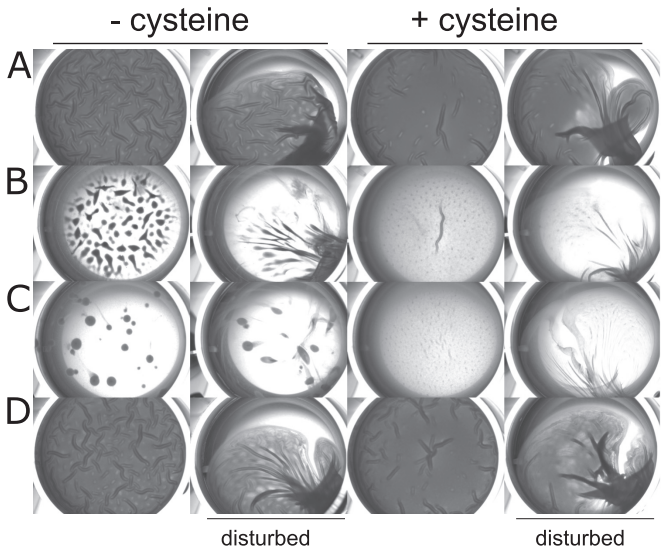

FIG 9 CysK, but not cysteine, complements the pellicle defect of the *cysK* mutant. Cultures of the *cys* mutants and parent strains were grown statically in LBS Tc lacking or containing added cysteine. The strains include pEAH73-containing derivatives of Δ*sypE* strain (KV3299) (A), Δ*sypE* Δ*cysK* strain (KV6410) (B), Δ*sypE cysK*::Tn strain (KV7735) (C), and Δ*sypE* Δ*cysK cysK*$^+$ strain (KV7522) (D).

promoting biofilm formation. We obtained similar results using *E. coli*: mixtures of the biofilm-induced *cysK* mutant with *E. coli*, both wild-type and *cys* mutant strains, also permitted robust wrinkled colony formation, with one exception, a *cysE* mutant. The *cysE* mutant should be defective for the production of *O*-acetyl serine and *N*-acetyl serine, but as these metabolites should be present in the *V. fischeri cysK* mutant, it remains unclear what *E. coli* is doing to promote wrinkled colony development.

Another puzzle lies in the specific requirement for *cysK* in biofilm formation. All the mutants that we isolated exhibited defects in the ability to grow in a medium with limiting amounts of cysteine, a defect that was suppressed by added cysteine, and thus we concluded that all of these putative *cys* genes are indeed important for *cys* biosynthesis. Although the *cysK* mutant consistently exhibited a slightly more severe growth defect than the other *cys* mutants, its growth defect in LBS, the medium that we used for the biofilm experiments, was not severe and cannot alone account for the more severe biofilm defect of this mutant. Surprisingly, while cysteine could complement the wrinkled colony defect, it could not complement the pellicle defect, while the *cysK* gene was able to complement both defects. These data suggest that CysK has a function independent of cysteine biosynthesis. In other organisms, CysK physically interacts with at least two proteins, CysE and CdiA. The interaction between CysK and CysE results in the formation of the cysteine synthase complex that produces cysteine (39, 40), while CysK binding to the CdiA cytotoxin is required for contact-dependent growth inhibition in *E. coli* (41). Determining

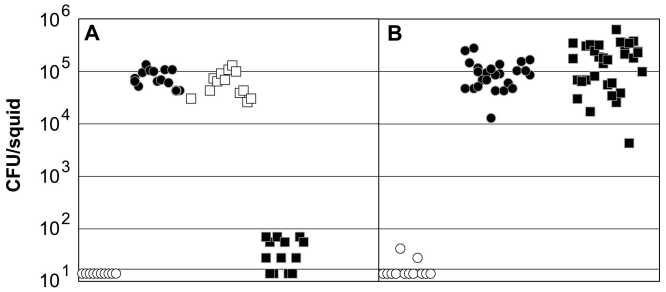

FIG 10 Colonization by the *cysK* mutant. To determine if *cysK* is required for colonization, squid were unexposed (aposymbiotic, open circles) or were inoculated with the *cys*$^+$ wild-type strain (ES114) (closed circles), the *cys*$^+$ parent (KV3299) (open squares), or the *cysK* mutant (KV6410) (closed squares) for 3 h (A). The same strains, with the exception of KV3299, were used to inoculate squid for 16 h (B). Every symbol represents an individual animal. The line at the bottom of the graph represents the limit of detection, 14 CFU/squid.

if *V. fischeri* CysK interacts with other proteins is thus an important future direction.

During the course of investigating the relationship between CysK and Syp-PS production, we identified a novel phenotype, adherence of the cells to the agar surface. This phenotype did not depend on *cysK*, as both the smooth-colony-forming *cysK* mutant and the wrinkling-positive control behaved similarly. Agar adherence is distinct from another plate phenotype that we recently observed for biofilm-induced *bmp* mutants, which form smooth colonies that are cohesive (self-adherent rather than agar adherent) (11). In the earlier study, we never observed adherence of our positive control to the agar surface. The two studies are difficult to compare, as our methods for inducing biofilm formation were different. However, the two distinct phenotypes both depend on the ability of *V. fischeri* to produce Syp-PS, indicating that a range of biofilm phenotypes can be attributed to the production of this polysaccharide. In current work, we have identified medium conditions that promote or fail to promote agar adherence (C. R. DeLoney-Marino, J. M. Ondrey, and K. L. Visick, unpublished data). Together, these findings, along with our recent documentation of colony cohesion (11) and our previous demonstration that *syp* genes are conserved and functional even in *V. fischeri* strains that do not encode the regulator RscS (42, 43), provide insights into the factors that influence biofilm development in the context of a solid abiotic surface like agar. These results may therefore suggest specific pathways that influence adhesion to abiotic surfaces in the marine environment.

Our work demonstrates that the *cysK* gene is required for initiation of squid colonization: when the squid were inoculated with the *cysK* mutant for a short duration (3 h), little to no colonization was observed. This result was perhaps not surprising, given a previous study that reported that an uncharacterized *cys* mutant colonized to only 5% of the wild-type strain colonization level (37). Both studies could be interpreted to mean that the squid does not supply cysteine to the bacteria, and therefore *cys* mutants cannot grow to a high cell density in the symbiotic organ. However, this conclusion cannot be made, as our subsequent experiment assessing colonization after a prolonged (overnight) inoculation demonstrated that the *cysK* mutant could, in fact, colonize and therefore must be supplied with cysteine. We thus interpret our studies and the older study to mean that the *cysK* mutant fails to properly initiate colonization, likely due to a defect in host-relevant biofilm formation. We imagine that the extended inoculation time might permit one or a few bacteria to enter the symbiotic organ (albeit less efficiently without first forming a biofilm), after which they can use host-supplied cysteine to colonize to high levels. This work thus provides an explanation for an old finding that a *cys* mutant exhibits a colonization defect.

Overall, this study provides a new tool and demonstrates a specific role for CysK in biofilm formation by *V. fischeri*. It also suggests that CysK functions in biofilm formation on at least two levels, one of which is to provide cysteine. Finally, this work revealed another form of biofilm, a finding that will permit a deeper understanding of how these complex microbial communities develop.

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