The symbiosis regulator RscS controls the *syp* gene locus, biofilm formation and symbiotic aggregation by *Vibrio fischeri*

Emily S. Yip,[1] **Kati Geszvain,**[1]
Cindy R. DeLoney-Marino[2] **and Karen L. Visick**[1]*
[1]*Department of Microbiology and Immunology, Loyola University Chicago, Maywood, IL, USA.*
[2]*Department of Biology, University of Southern Indiana, Evansville, IN, USA.*

Summary

Successful colonization of a eukaryotic host by a microbe involves complex microbe–microbe and microbe–host interactions. Previously, we identified in *Vibrio fischeri* a putative sensor kinase, RscS, required for initiating symbiotic colonization of its squid host *Euprymna scolopes*. Here, we analysed the role of *rscS* by isolating an allele, *rscS1*, with increased activity. Multicopy *rscS1* activated transcription of genes within the recently identified symbiosis polysaccharide (*syp*) cluster. Wild-type cells carrying *rscS1* induced aggregation phenotypes in culture, including the formation of pellicles and wrinkled colonies, in a *syp*-dependent manner. Colonies formed by *rscS1*-expressing cells produced a matrix not found in control colonies and largely lost in an *rscS1*-expressing *sypN* mutant. Finally, multicopy *rscS1* provided a colonization advantage over control cells and substantially enhanced the ability of wild-type cells to aggregate on the surface of the symbiotic organ of *E. scolopes*; this latter phenotype similarly depended upon an intact *syp* locus. These results suggest that transcription induced by RscS-mediated signal transduction plays a key role in colonization at the aggregation stage by modifying the cell surface and increasing the ability of the cells to adhere to one another and/or to squid-secreted mucus.

Introduction

Communication between bacteria and their plant or animal hosts is an essential component of both beneficial symbioses and pathogenic associations. Perhaps equally important are interactions within the community of bacterial cells as they colonize their host. For example, during *Vibrio cholerae* infection of the mouse intestine, interactions mediated by the TCP pilus allow the bacteria to aggregate into microcolonies, possibly affording protection from antimicrobial agents within the intestine (Kirn *et al.*, 2000). Notably, biofilm formation by *V. cholerae* increases its resistance to the toxicity of the bile acids found in the intestine (Hung *et al.*, 2006). Similarly, biofilm formation by *Pseudomonas aeruginosa* in the cystic fibrosis lung is thought to contribute to chronic infection by increasing resistance of the bacterial community to antibiotics (Whiteley *et al.*, 2001). In addition to pathogenic functions, biofilm formation is implicated in the maintenance of the complex microbial community found within the mammalian gut (Sonnenburg *et al.*, 2004); therefore, bacterial cell–cell interactions also appear to be a component of beneficial symbioses.

An elegant model system for studying bacteria–host and inter-bacterial interactions during colonization of a host is the symbiosis between the Hawaiian bobtail squid *Euprymna scolopes* and the marine bioluminescent bacterium *Vibrio fischeri* (reviewed in Nyholm and McFall-Ngai, 2004). The specialized symbiotic organ (the light organ) of *E. scolopes* is exclusively colonized by its bacterial symbiont, *V. fischeri*. Newly hatched squid lack symbionts but rapidly become colonized upon exposure to *V. fischeri* in the seawater. In the squid, symbiotic initiation is facilitated by ciliated cells located on the surface of the light organ that direct the flow of bacteria-containing seawater towards pores that lead into the organ. The squid also promotes colonization by secreting mucus on the surface of the light organ (Nyholm *et al.*, 2000). Early in the association, *V. fischeri* cells form a tight, dense aggregate in this mucus (Nyholm *et al.*, 2000). The cells then migrate from the aggregate into the pores and through ducts that lead to crypt spaces where finally they multiply to high cell density and luminesce (Nyholm and McFall-Ngai, 2004).

The specificity of the *Vibrio*–squid symbiosis is first manifested at the aggregation stage. Several species of Gram-negative bacteria, including the closely related *Vibrio parahaemolyticus*, can aggregate on the light organ surface (Nyholm *et al.*, 2000). However, when both

V. fischeri and *V. parahaemolyticus* are present, the symbiotic *V. fischeri* cells dominate in the aggregate (Nyholm and McFall-Ngai, 2003). These data suggest that *V. fischeri* actively promotes aggregation; however, the bacterial factors that contribute to this activity are unknown (Nyholm and McFall-Ngai, 2003).

Studies investigating the genetic requirements for the symbiosis have revealed a number of mutants defective for initiating colonization, some of which have been characterized with respect to aggregation (Nyholm *et al.*, 2000). A mutant defective for the flagellar master regulator, FlrA, forms smaller aggregates than those produced by wild-type *V. fischeri* (Millikan and Ruby, 2003). Hypermotile mutants, which are delayed in initiating colonization, also form smaller aggregates, typically after a lag period (Millikan and Ruby, 2002). While appropriate levels of motility appear to be required for optimal aggregation, it is unclear what other bacterial factors promote aggregation in the squid-secreted mucus. Furthermore, characteristics associated with bacterial aggregation in other bacteria – such as biofilm formation, wrinkled colony morphology and clumping of cells (Romling *et al.*, 1998; Del Re *et al.*, 2000) – have not been reported to date for *V. fischeri* cells grown in culture. This lack of culture phenotypes has hampered genetic investigation of the basis for aggregation within the squid mucus.

In bacteria, two-component regulatory systems detect environmental signals and respond by autophosphorylation of a sensor kinase and subsequent transfer of the phosphate to a response regulator. Signalling through these systems, in many cases, regulates transcription of a target gene(s) (reviewed in Stock *et al.*, 2000). Previously, we reported that a mutant defective for the putative sensor kinase RscS exhibited a severe defect in colonization (Visick and Skoufos, 2001). However, known symbiosis traits, including motility and bioluminescence, were unaltered under laboratory conditions. Subsequently, we reported the identification of the symbiosis polysaccharide (*syp*) gene locus (Yip *et al.*, 2005) involved in symbiotic initiation. Mutation of *syp* genes causes over a 1000-fold decrease in symbiotic colonization. The 18 genes (*sypA-R*) in this cluster encode proteins with predicted roles as glycosyltransferases and other polysaccharide metabolism and export functions. In addition, four genes encode putative regulatory proteins such as response regulators. Consistent with a possible role of *syp* in glycosylation of a surface molecule(s), we found that multicopy expression of a *syp*-encoded putative response regulator, SypG, caused a substantial increase in biofilm formation by cells grown in both static and shaking liquid cultures.

Here, we demonstrate that one role of RscS is regulation of the *syp* gene cluster. Multicopy expression of an allele of *rscS* with increased activity induced *syp* tran-

scription and dramatic *syp*-dependent cell–cell aggregation phenotypes, including the formation of wrinkled colonies on solid media and pellicles in static liquid culture. We then show the relevance of these phenotypes to symbiosis by identifying a role for *rscS* in promoting aggregation of *V. fischeri* cells in squid-secreted mucus on the light organ surface. Our results reveal central roles for RscS and *syp* in cell–cell aggregation by this bacterium, both in culture and during symbiotic initiation, and provide a basis for understanding one of the earliest known stages of symbiotic colonization of squid by *V. fischeri*.

Results

RscS controls the syp *gene cluster*

Our preliminary data showed that the putative sensor kinase, RscS, could autophosphorylate, suggesting it did indeed function as a sensor kinase (E.S. Yip and K.L. Visick, unpublished). However, this gene was not linked to a response regulator, and our attempts to identify *rscS*-controlled genes through global screens with a wild-type copy of *rscS* on the multicopy plasmid pLMS33 (Visick and Skoufos, 2001) were unsuccessful. We therefore hypothesized that RscS might exhibit only a low basal level of activity under laboratory culture conditions and that isolation of an *rscS* allele with increased activity could make it possible to detect *rscS*-dependent phenotypes and gene regulation. We further speculated that one potential target of RscS could be the *syp* cluster, which we recently identified as essential for symbiotic colonization (Yip *et al.*, 2005). Like disruption of *rscS*, *syp* mutations caused severe initiation defects but no discernible alterations in traits required for symbiosis. Furthermore, the *syp* genes were not detectably transcribed under laboratory conditions unless a *syp*-encoded regulator, SypG, was expressed from a multicopy plasmid (Yip *et al.*, 2005). Therefore, we asked whether we could identify an allele of *rscS* capable of inducing *syp* transcription.

To isolate mutations in *rscS*, we passaged pLMS33 through an *E. coli* mutator strain and we introduced the resulting library of randomly mutagenized plasmids into reporter strains. To monitor the induction of *syp* transcription, we employed strains containing a promoterless *lacZ* gene carried on Tn*10* inserted in either *sypD* or *sypN* (KV1635 and KV1601 respectively; Table 1) which are located within two different putative operons of the *syp* cluster (Yip *et al.*, 2005). We then screened for blue colonies, and found them at a frequency of about 1 in 1000. We confirmed that the plasmid was responsible for the elevated *syp* transcription for six such plasmids, and following subcloning experiments (see *Experimental procedures*), we identified two alleles, *rscS1* and *rscS2*, for

Table 1. Strains and plasmids.

Strains	Genotype or characteristics	Reference
E. coli		
CC118λ*pir*	Δ(*are-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE* (Am) *recA1* λ*pir*	Herrero *et al.* (1990)
CC130	F-, *ara*D139, Δ(*ara-leu*)7697 Δ*lac*-74 *gal*U *gal*K *rps*L Δ (*pho*A-*pro*C) *pho*R *tsx*::Tn*5 mut*D5	Manoil and Beckwith (1985)
DH5α	*endA1 hsdR17* (r$_K^-$ m$_K^+$) *glnV44 thi-1 recA1 gyrA* (Nal^r) *relA* Δ(*lacIZYA-argF*) U169 *deoR* [φ80d*lac*Δ(*lacZ*)M15]	Woodcock *et al.* (1989)
TOP10F′	F′ [*lacI^R* Tn10 (Tet^R)] *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ* ΔM15 Δ*lacX74 rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *en*dA1 *nup*G	Invitrogen
V. fischeri		
ES114	Wild type	Boettcher and Ruby (1990)
ESR1	Rif^R	Graf *et al.* (1994)
KV905	pKV69/ESR1	Visick and Skoufos (2001)
KV1066	pKV111/ES114	This study
KV1223	pKV111/Rif^R*rscS*::*erm*	This study
KV1228	pKV111/Rif^R	This study
KV1339	pKV111/ES114 *rscS*::*erm*	This study
KV1421	ES114 att Tn*7*::*erm*	O'Shea *et al.*, 2006
KV1601	Rif^R*sypN*::Tn*10lacZ*	Yip *et al.* (2005)
KV1635	Rif^R*sypD*::Tn*10lacZ*	Yip *et al.* (2005)
KV1838	ES114 *sypN*::pTMB54	Yip *et al.* (2005)
KV1844	pKV69/ES114	Visick and Skoufos (2001)
KV1845	pLMS33/ES114	This study
KV1956	pKG11/ES114	This study
KV1958	pKV69/KV1601	Yip *et al.* (2005)
KV1959	pLMS33/KV1601	This study
KV1960	pKG11/KV1601	This study
KV1962	pKV69/KV1635	Yip *et al.* (2005)
KV1963	pLMS33/KV1635	This study
KV1964	pKG11/KV1635	This study
KV1980	pKG11/ESR1	This study
KV1992	pKG11/KV1838	This study
KV2273	pKG13/KV1601	This study
KV2274	pKG13/KV1635	This study
KV2435	pKV69/KV1421	This study
KV2437	pKG11/KV1421	This study
KV2608	pVSV208/ES114	This study
KV2613	pKG11/KV2608	This study
KV2617	pKV69/KV2608	This study
KV2682	pESY37/ES114	This study
KV2683	pESY37/KV1838	This study
KV2685	pKV69/KV2682	This study
KV2686	pKV69/KV2683	This study
KV2688	pKG11/KV2682	This study
KV2689	pKG11/KV2683	This study
KV2888	pESY48/KV1838	This study
KV2976	pKV69/KV2888	This study
KV2977	pKG11/KV2888	This study

| Plasmids	Characteristics or construction	Reference
pCR2.1-TOPO	Commercial cloning vector; Amp^R, Kan^R	Invitrogen
pESY36	pCR2.1-TOPO + 4.6 kb fragment of *sypMNO*; Amp^R, Kan^R	This study
pESY37	pVSV105 (KpnI) + 1.3 bp BamHl/XmnI fragment from pKV111 containing *gfp*; Cm^R	This study
pESY47	pEVS107 (KpnI) + 4.6 kb KpnI fragment from pESY36 encoding *sypMNO*; Kan^R, Em^R	This study
pESY48	pESY47 (EcoRV) + 1.2 kb fragment encoding Cm^R; Kan^R, Cm^R	This study
pEVS107	Mini-Tn*7* delivery plasmid; mob; Kan^R, Em^R	McCann *et al.* (2003)
pKG11	pKV69 (SalI) + 3 kb SalI fragment from mutagenized pLMS33 containing *rscS1* allele; Cm^R, Tet^R	This study
pKG13	pKV69 (SalI) + 3 kb SalI fragment from mutagenized pLMS33 containing *rscS2* allele; Cm^R, Tet^R	This study
pKV69	Mobilizable vector; Cm^R, Tet^R	Visick and Skoufos (2001)
pKV111	Mobilizable vector containing *gfp*; Cm^R	Stabb and Ruby (2002)
pLMS33	pKV69 containing wild-type *rscS*; Cm^R, Tet^R	Visick and Skoufos (2001)
pVSV105	Mobilizable vector; Cm^R	Dunn *et al.* (2006)
pVSV208	Mobilizable vector containing *rfp*; Cm^R	Dunn *et al.* (2006)
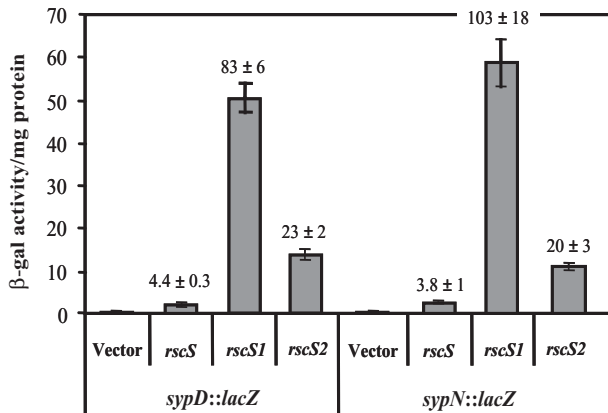
Fig. 1. Transcription of *syp* genes by *rscS*. After 24 h growth at 22°C in HMM, β-galactosidase activity levels were determined for *syp*::*lacZ* reporter strains carrying vector (pKV69), *rscS* (pLMS33), *rscS1* (pKG11) or *rscS2* (pKG13) (see *Experimental procedures*). Error bars represent the standard deviation of three replicate cultures. Numbers above the bars indicate the fold induction relative to the β-galactosidase activity produced by the vector control strain.

which the mutation mapped within the subcloned *rscS* portion of the plasmid (carried by pKG11 and pKG13 respectively). The effect of these two alleles on *syp* transcription was quantified by β-galactosidase assays. The presence of multicopy *rscS1* or *rscS2* increased the amount of β-galactosidase activity generated from the *sypN*::*lacZ* fusion strain by 103 ± 18-fold and 20 ± 3-fold, respectively, relative to the vector control (Fig. 1). Similar levels of induction were seen for the *sypD*::*lacZ* fusion strain (Fig. 1).

Sequence analysis of *rscS1* and *rscS2* revealed mutations within or near the putative *rscS* ribosome binding site, while *rscS1* contained a second, silent mutation at codon Leu25 (see *Experimental procedures*). Because the *rscS1* allele exerted the greatest effect on *syp* transcription, we used the plasmid (pKG11) carrying this allele in all of our subsequent investigations. Semi-quantitative RT-PCR revealed no difference in the amount of *rscS* transcripts produced by pKG11 relative to pLMS33 (data not shown); therefore, the mutations present in *rscS1* do not appear to affect transcription of the gene and thus we hypothesize that they affect translation. We verified that *syp* induction and the resulting *syp*-dependent phenotypes induced by multicopy *rscS1* in culture (described below) did indeed depend upon the *rscS* gene carried by pKG11 (data not shown). Furthermore, because the *rscS1* allele encodes wild-type protein, we anticipate that any observed effect on *syp* transcription is likely to represent a normal activity of RscS. Indeed, when we introduced multicopy wild-type *rscS* into the *syp* reporter strains, we observed a small but reproducible increase in β-galactosidase activity (approximately fourfold) relative to vector control (Fig. 1). Semi-quantitative RT-PCR

experiments verified *rscS1*-induced expression of *sypD* and *sypN* in a wild-type background and demonstrated *rscS1*-dependent induction of at least two other genes within the cluster (*sypB* and *sypE*; K. Geszvain and K.L. Visick, unpubl. data). Therefore, *rscS1*-mediated induction of the *syp* cluster is neither an artefact of the particular mutations present within *rscS1* nor a consequence of the Tn*10lacZ* insertion present in the reporter strains.

From these data, we conclude that the sensor kinase RscS regulates expression of the *syp* cluster. Furthermore, these experiments have generated mutagenized alleles of *rscS* that can be employed to increase RscS expression, allowing us to investigate the role of the regulator in culture and during symbiosis.

RscS induces novel aggregation phenotypes in culture

Given the large increase in *syp* transcription induced by *rscS1* relative to wild-type *rscS*, we anticipated that phenotypic effects of RscS activity would be more readily observed in strains carrying *rscS1*. Indeed, we found that *rscS1* induced a novel phenotype following prolonged incubation on complex solid medium. Normally, *V. fischeri* colonies are round, smooth and wet-appearing, as are colonies carrying the vector (Fig. 2Ai). Colonies containing *rscS1*, however, appeared dry, flat and wrinkled (Fig. 2Aiii). These wrinkled colonies formed a film that could be peeled off the plate in a continuous sheet (data not shown). This phenotype depended upon *rscS1*-mediated induction of the *syp* cluster. When a representative *syp* gene, *sypN*, which encodes one of six putative *syp* glycosyltransferases (Yip *et al.*, 2005), was disrupted, *rscS1* failed to induce wrinkling (Fig. 2Aiv). Wrinkling of the *sypN* mutant was restored upon complementation with the *sypMNO* operon (data not shown), further demonstrating the dependence on *syp* of this phenotype. Finally, wrinkling could be weakly induced by pLMS33 (Fig. 2Aii), indicating that this phenotype reflects the natural activity of RscS.

Wrinkled colony morphology is frequently associated with increased cell aggregation phenotypes (Branda *et al.*, 2005); therefore, we screened cells for such phenotypes. First, we observed that after prolonged static growth in liquid Hepes minimal media (HMM), wild-type cells containing *rscS1* formed a pellicle at the air/liquid interface that exhibited high tensile strength. In fact, the pellicle was sufficiently strong to retain the culture when the tube was completely inverted (Fig. 2Biii). Disruption of *sypN* resulted in the loss of this thick pellicle while complementation with *sypMNO* restored it (data not shown), demonstrating that this phenotype similarly depended upon *syp*. Second, during growth with shaking in HMM, we observed that *rscS1* cultures formed stringy aggregates of cells (Fig. 2Ciii), which were difficult to disrupt
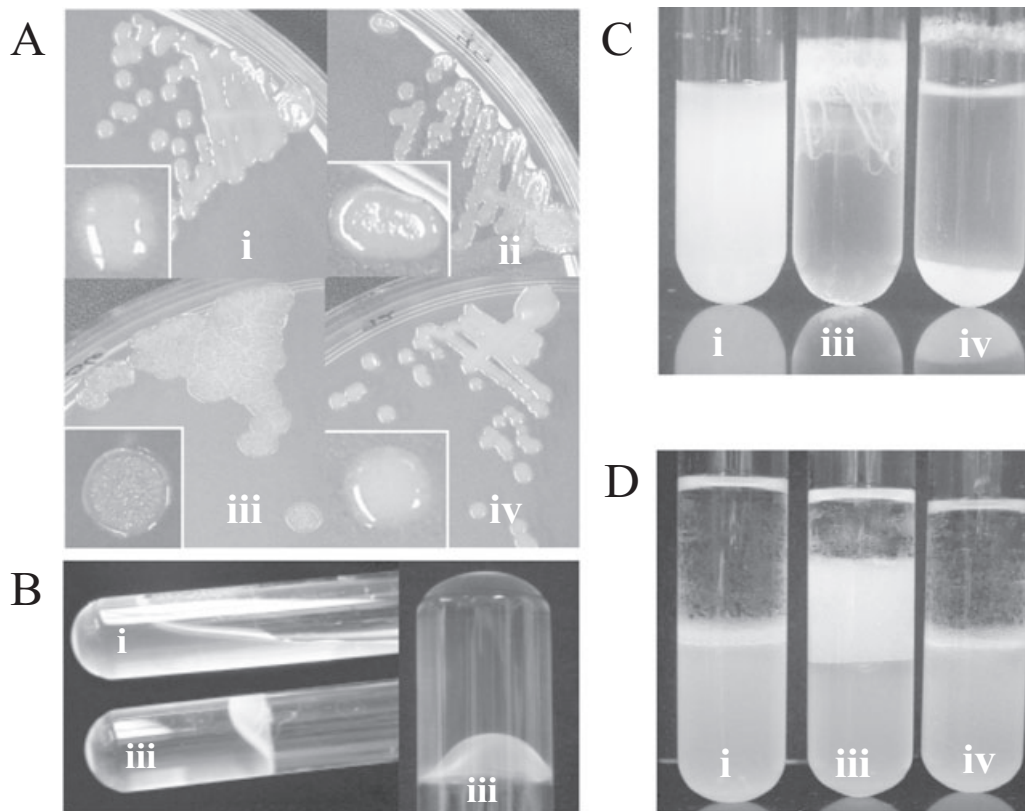
Fig. 2. Culture phenotypes of *V. fischeri* expressing *rscS*. (i) vector (pKV69) in ES114 (KV1844), (ii) *rscS* (pLMS33) in ES114 (KV1845), (iii) *rscS1* (pKG11) in ES114 (KV1956), (iv) *rscS1* (pKG11) in *sypN* (KV1992).
A. Growth phenotypes on solid media. Strains were streaked onto LBS with Tet and incubated at RT for 48 h. Inserts show a close-up view of a representative colony for each strain.
B. Pellicle formation in static cultures. Cultures were grown in HMM at RT for 7 days, then photographed after inverting the tubes 90 (left panel) and 180 degrees (right panel).
C. Growth phenotypes in shaking cultures. Cultures were grown in HMM with shaking at 22°C for 24 h. The experiment was performed in triplicate; representative results are shown.
D. Hydrophobicity of *V. fischeri* cultures. The organic solvent hexadecane was added to cultures that had been grown in HMM at 28°C for 24 h. After vortexing, cells were allowed to partition into the lower, aqueous phase or the upper, hydrophobic phase. The experiment was performed in triplicate; representative results are shown.

even with vigorous vortexing. This phenotype suggested that RscS greatly increases the 'stickiness' of the cell surface, allowing the cells to form aggregates even during agitation. In a *sypN* mutant, *rscS1* did not induce the strings of aggregated cells seen in the wild-type background; however, the cells settled on the bottom of the tube (Fig. 2Civ) rather than remaining suspended as in the vector control (Fig. 2Ci). Together, these data demonstrate that RscS induces *syp*-dependent cell–cell aggregation.

In some organisms, such as Lactobacilli and *Bifidobacterium longum*, autoaggregation and adhesion have been correlated with hydrophobic cell surfaces (Wadstrom *et al.*, 1987; Del Re *et al.*, 2000). Given the aggregation phenotypes induced by *rscS1*, we hypothesized that *rscS1* might increase hydrophobicity of *V. fischeri* cells. When cultures of wild-type *V. fischeri* carrying vector were vortexed with an equal volume of hexadecane (an organic

solvent), the bulk of the cells remained in the aqueous phase (Fig. 2Di; bottom layer). However, for cells carrying *rscS1*, a substantial proportion of the cells partitioned to the organic, hexadecane layer (Fig. 2Diii; top, 'creamy' layer). The shift to the organic phase induced by *rscS1* was largely lost in the *sypN* mutant (Fig. 2Div). These data suggest that RscS-induced expression of the *syp* locus results in an altered, hydrophobic cell surface that might contribute to the above aggregation phenotypes.

RscS promotes the formation of thick biofilm on a glass surface

To further understand the nature of the RscS-mediated aggregation phenotypes, we asked whether multicopy *rscS1* enhanced the ability of *V. fischeri* to form biofilms. We used confocal microscopy to quantify the biomass of biofilms produced by GFP-labelled strains carrying either
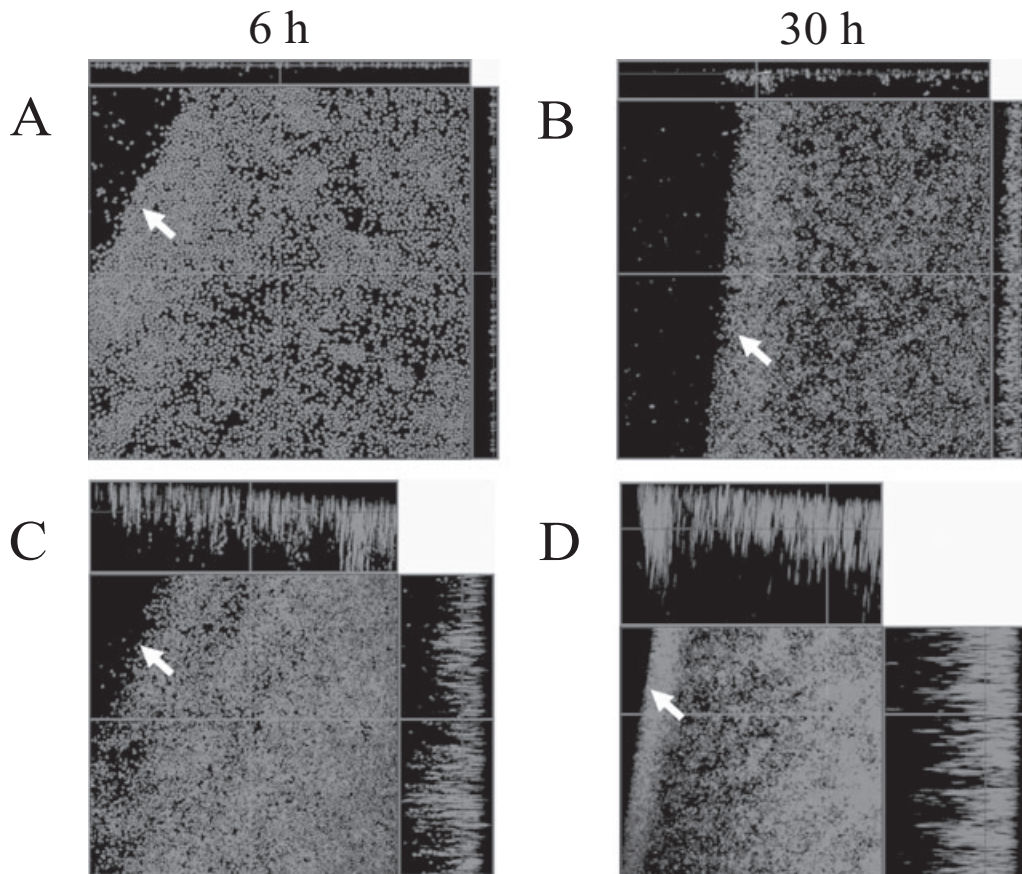
6 h   30 h

Fig. 3. Confocal microscopy of biofilm formation on a glass surface. Development of *V. fischeri* biofilms formed on glass coverslips was visualized by confocal microscopy. Representative views of the *xy* plane and *z* sections were shown for GFP-labelled (A and B) vector-containing (KV2685) and (C and D) *rscS1*-expressing wild-type *V. fischeri* cells (KV2688) at 6 h and 30 h post inoculation. Arrows indicate the air–liquid interface.

rscS1 or the vector control. These strains were grown statically in HMM medium in microtiter wells. To facilitate microscopy, glass coverslips were submerged vertically into medium immediately following inoculation, then removed either 6 or 30 h later. At 6 h, both vector control and *rscS1*-containing cells showed adherence to the glass surface. Even at this early time point, however, *z* sections showed that the thickness of the adherent cells was substantially greater for *rscS1*-containing cells (~18 μm) relative to the control (~2 μm) (Fig. 3A and C). At 30 h, the difference between the two cell types was even more dramatic (Fig. 3B and D). Whereas the biofilm of vector control cells was approximately 7 μm thick, that of the *rscS1*-containing cells was approximately 45 μm. Furthermore, at this time point, an even thicker region (about 56 μm) could be seen for the *rscS1* cells present at the air–liquid interface, where the pellicle eventually forms. These data suggest that multicopy *rscS1*, while not necessary for *V. fischeri* cells to adhere to a solid surface, substantially promotes the events that lead to the formation of a thick biofilm.

RscS induces a polysaccharide extracellular matrix

In nature, many bacteria reside in biofilms that are composed mainly of polysaccharides (Yildiz and Schoolnik, 1999; Enos-Berlage and McCarter, 2000; Zogaj *et al.*, 2001; Solano *et al.*, 2002; Friedman and Kolter, 2004a,b; Jackson *et al.*, 2004; Matsukawa and Greenberg, 2004). To investigate the nature of *rscS1*-dependent biofilms, we first performed both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) on colonies derived from *rscS1*-containing or vector control cells. SEM images revealed the existence of a smooth, sheet-like matrix produced by *rscS1*-expressing wild-type (Fig. 4B) but not vector control cells (Fig. 4A). Consistent with other culture phenotypes, the dense matrix induced by *rscS1* in the wild type was largely absent in the *sypN* mutant strain (Fig. 4C), indicating that matrix formation required an intact *syp* cluster.

We therefore further characterized the nature of the *rscS1*-dependent matrix by TEM using ruthenium red, which is commonly used to detect acidic polysaccharides
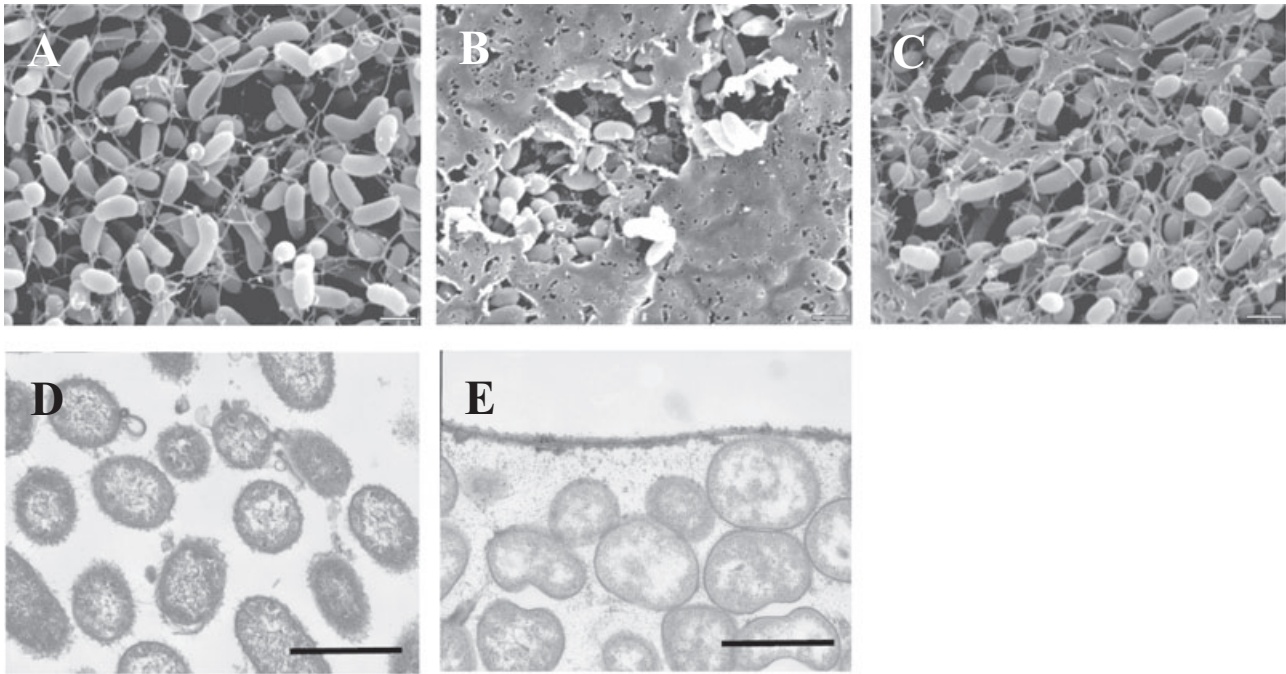
Fig. 4. SEM and TEM analysis of *V. fischeri* cells expressing *rscS1*. The production of an extracellular matrix was examined by SEM from (A) vector control wild-type cells (KV1844), (B) *rscS1*-expressing wild-type cells (KV1956) and (C) *rscS1*-expressing *sypN* mutant cells (KV1992) and by TEM analysis of (D) vector control and (E) *rscS1*-containing wild type cells stained with ruthenium red. Bars represent 1 μm.

(Luft, 1971). Colonies were fixed and stained with ruthenium red and thin sections examined by TEM. The *rscS1*-containing wild-type cells exhibited two types of ruthenium red-stained materials that were absent in vector control (Fig. 4D). First, an electron-dense layer was located at the colony surface which probably corresponds to the sheet-like matrix detected by SEM (Fig. 4E). Second, electron-dense material was also observed between cells, indicative of an extracellular matrix. Together, these data suggest that *rscS1* promotes the production of an extra-cellular matrix composed at least in part of acidic polysaccharides.

The observations by SEM and TEM of a matrix pro-duced by *syp*-expressing cells supported our hypothesis that the *syp* cluster functions to modify the cell surface. To begin characterization of this matrix, we extracted surface-associated polysaccharide from smooth colonies and wrinkled colonies formed by vector-containing cells and *rscS1*-containing cells, respectively, using a capsule extraction protocol established for *V. parahaemolyticus* (Enos-Berlage and McCarter, 2000). Extractions from *rscS1*-containing wild-type cells yielded a high-molecular-weight polysaccharide (Fig. 5A). This polysac-charide was absent from vector control and largely missing from *rscS1*-expressing *sypN* mutant extracts. These data support the hypothesis that the alteration induced by *rscS1* involves increased surface polysaccharide.

Purification of the large polysaccharide has proven dif-ficult to date due to its insolubility. Therefore, to further characterize its nature, we performed lectin binding assays with a panel of seven biotinylated lectins. A single lectin, Concanavalin A (ConA), reacted strongly with the *rscS1*-induced polysaccharide (Fig. 5B), while the other six lectins tested exhibited no binding (data not shown).
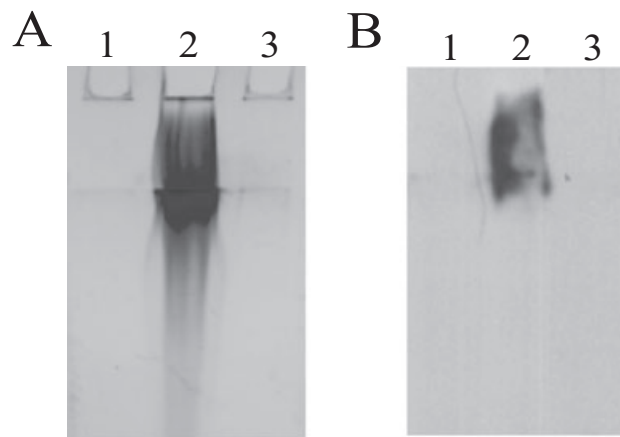

Fig. 5. Polysaccharide extraction of *V. fischeri* expressing *rscS1*. Polysaccharide extracts were resolved on SDS-PAGE gel and (A) stained with Stains-All or (B) transferred onto membrane and probed with the lectin ConA. Lane 1: vector control (KV905); 2: *rscS1*-expressing wild type (KV1980) and 3: *rscS1*-containing *sypN* mutant (KV1960).

ConA is specific for glucose and α-linked mannose residues, indicating that one (or more) of these sugars is present in the surface-associated polysaccharide produced by *rscS1*-containing wild-type *V. fischeri*.

RscS enhances colonization efficiency by promoting aggregation outside the light organ

V. fischeri cells lacking RscS exhibit a significant colonization defect (Visick and Skoufos, 2001). We therefore wanted to determine the relevance to symbiosis, if any, of phenotypes associated with multicopy expression of *rscS1*. Given the dramatic aggregation phenotypes induced in culture, we hypothesized that *rscS1* could either enhance or impede colonization through its role in cell–cell aggregation. Preliminary experiments, in which juvenile squid were incubated with cells containing either vector or *rscS1*, indicated that the *rscS1*-containing cells initiated the symbiosis earlier than vector-containing cells (data not shown). These data suggested that *rscS1* provided an advantage; however, the effect was difficult to quantify. As a more sensitive assay for colonization phenotypes, we performed competition assays. In these experiments, juvenile squid were inoculated with a mixture of two strains, one of which was marked with an erythromycin resistance (Em^R) gene on the chromosome. As a control, we competed vector-containing cells that were either erythromycin sensitive (Em^S) or resistant (Em^R) against each other at a ratio of about 1:1 and screened bacteria recovered from the light organ after colonization for antibiotic resistance. The majority of these animals (8 of 11) were colonized with a roughly equal number of each strain (Fig. 6, white circles), demonstrating that the Em^R cassette did not severely impact colonization fitness. Next, we inoculated squid with a mixture of vector-containing (Em^R) cells and *rscS1*-containing (Em^S) cells present at a ratio of about 2:1. All of the bacteria recovered from the light organ were Em^S and therefore were derived from the *rscS1*-containing cells (Fig. 6, black circles). Reciprocal experiments in which the Em^R marker was present in the *rscS1*-containing strain produced similar results: the *rscS1*-containing strain dominated in the light organ (data not shown). We conclude that multicopy expression of *rscS1* conferred a significant advantage to the cells during colonization.

To understand the mechanism by which RscS controls colonization proficiency, we asked at which stage of colonization RscS exerts its influence. Specifically, we asked whether the *rscS* gene was required for the ability of *V. fischeri* cells to aggregate in squid-secreted mucus, one of the earliest known stages of symbiotic colonization. We hypothesized that disruption of *rscS* would result in a loss of aggregates, while multicopy *rscS1* would enhance aggregate formation.
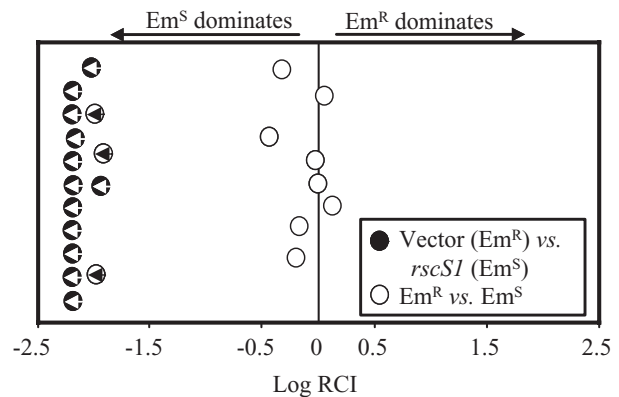

Fig. 6. Colonization by strains carrying multicopy *rscS1*. The log of the relative competitive index (log RCI) for each juvenile squid in the experiment is plotted on the *x*-axis. Log RCI is calculated as the log of the fraction of Em^R bacteria in the homogenate divided by the fraction of Em^R bacteria in the inoculum. Each circle represents a single animal. Circles with arrows represent animals in which no Em^R bacteria were present in the 100–200 colonies screened from the homogenate; therefore, the log RCI is below the limit of detection and the values plotted for these animals are approximate. In the competition between vector- and *rscS1*-containing cells, all of the colonies screened from the homogenate were the Em^S *rscS1*-containing strain (black circles). The position of the circles on the *y*-axis is merely for spacing.

To determine if *rscS* mutants exhibit a defect in aggregation, we inoculated newly hatched, aposymbiotic squid with either wild-type or *rscS* mutant strains constitutively expressing GFP. At times ranging from 2 to 6 h post inoculation, we dissected the animals and examined light organs for the presence of bacterial aggregates. Within 3–5 h of inoculation, animals exposed to GFP-labelled wild-type cells (KV1066 or KV1228) contained tightly packed aggregates of bacteria (~20 μm in diameter; Fig. 7A), either at or above a light organ pore, as observed previously (Nyholm *et al.*, 2000). In contrast, animals inoculated with similar numbers of *rscS* mutant cells (KV1339 or KV1223) did not contain such aggregates (Fig. 7B); in fact, of 49 animals, 46 contained no visible GFP-labelled cells, while the remaining three animals showed only small clusters of about three to five cells. These data suggest that the defect of the *rscS* mutant in initiating symbiotic colonization stems from an inability of the cells to interact with one another and/or with mucus on the surface of the light organ.

Next, we evaluated the effect of *rscS1* on the ability of *V. fischeri* to aggregate in squid mucus. Whereas vector control cells formed aggregates of approximately 10–20 μm in diameter, similar to what we and others have seen for wild-type cells (Fig. 7C; Nyholm *et al.*, 2000), *rscS1*-containing wild-type cells formed substantially larger aggregates (50–200 μm in diameter; Fig. 7D and E). These data strongly suggest that the aggregation phenotypes induced by *rscS1* in culture indeed reflect the role
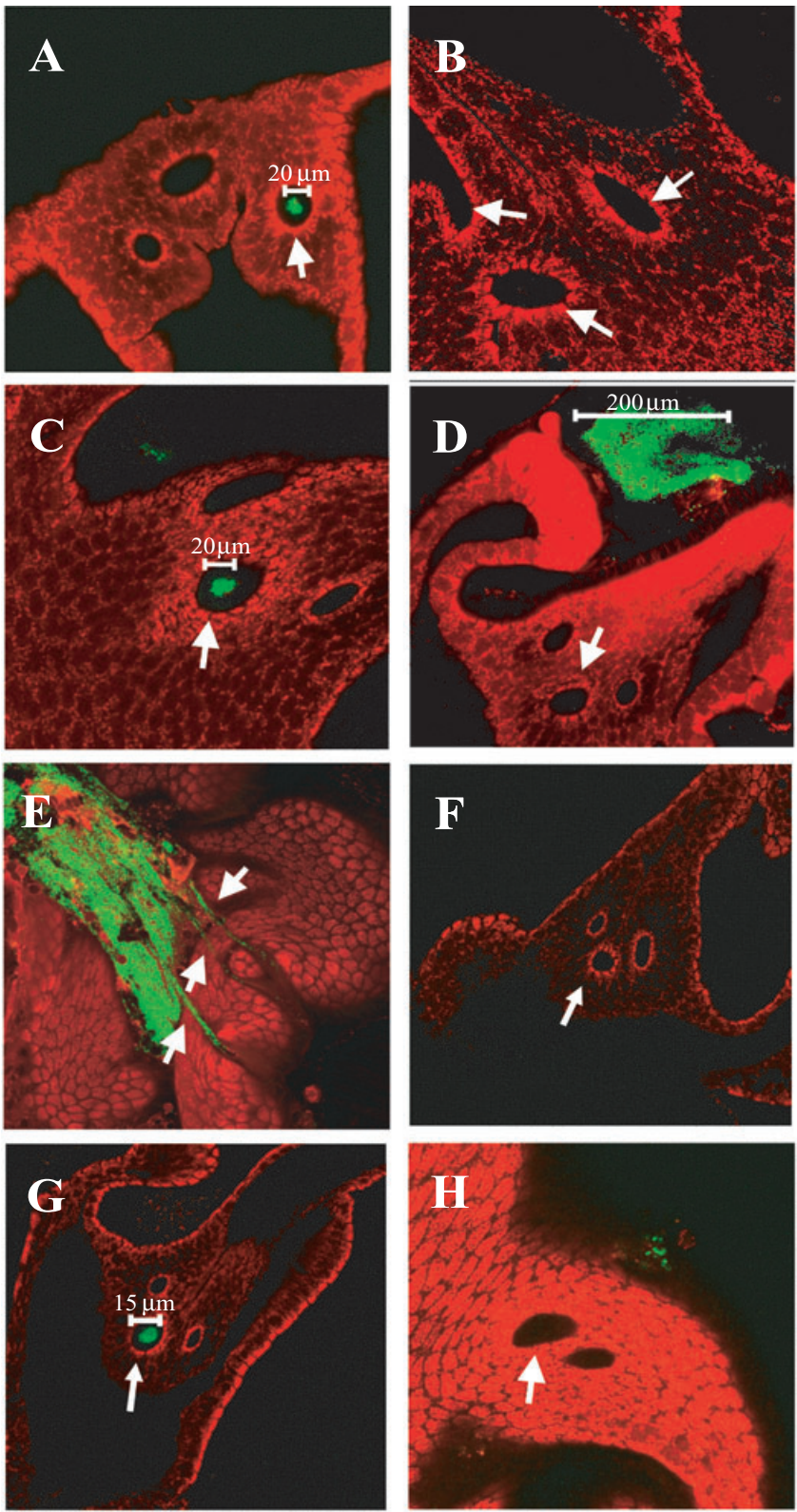
Fig. 7. Aggregate formation on the light organ by *V. fischeri*. Newly hatched juvenile squid were inoculated with GFP-labelled bacteria. After 2–6 h, animals were stained with Cell Tracker Orange (red colour) and the light organs examined by confocal microscopy. Representative images of aggregated *V. fischeri* cells at or near a light organ pore are shown. Animals were inoculated with (A) ES114 (KV1066), (B) *rscS* mutant cells (KV1339), (C) wild-type cells carrying the vector control (KV2685), (D and E) wild-type cells carrying *rscS1* (KV2688), (F and G) *rscS1*-expressing *sypN* mutant cells (KV2689), and (H) *sypN* mutant cells carrying vector (KV2686). Arrows indicate pores of the light organ.

of *rscS* in symbiosis. In further support of this hypothesis, we found that multicopy expression of *rscS1* in a *sypN* mutant largely eliminated the production of large aggregates: four of 28 animals contained no detectable *V. fischeri* (Fig. 7F), 12 contained only a few cells and the remaining 12 animals had aggregates that were substantially smaller than those produced by wild-type cells expressing *rscS1* (Fig. 7G; about wild type in size, 5–10 μm in diameter). These data demonstrate a role for *syp* in symbiotic aggregation. Furthermore, they are consistent with what we observed in culture: disruption of *sypN* reduces but does not eliminate aggregation induced by *rscS1*. Intriguingly, the *sypN* mutant carrying the vector control exhibited a more severe defect: of 18 animals examined, 15 contained no bacterial aggregates, while three contained only a few bacterial cells (Fig. 7H). These data further support a role for *sypN* in squid aggregation and suggest that *rscS1* might partially compensate for the loss of *sypN* function.

Discussion

The process by which *V. fischeri* establishes itself as the sole species within the symbiotic organ of the squid *E. scolopes* is poorly understood. Here, we demonstrate that a key step, aggregation on the surface of the squid light organ, is facilitated by the action of the bacterial regulator, RscS. Our experiments show that RscS promotes aggregation in culture and initiation of symbiotic colonization in the squid light organ by regulating expression of genes that alter the cell surface. Our data thus provide an important correlation between laboratory-based biofilm studies and natural interactions that occur during the course of colonization and as well as a basis for further genetic analysis of the regulatory pathway.

Through the isolation of an allele (*rscS1*) with increased activity, we demonstrated that RscS regulates expression of the recently discovered *syp* gene cluster (Yip *et al.*, 2005). Consistent with the predicted role of the *syp* genes in the production of an extracellular polysaccharide, induction of *syp* transcription by multicopy *rscS1* resulted in phenotypes associated with cell–cell aggregation in culture: wrinkled colony morphology, biofilm and pellicle formation, clumping in liquid culture, and increased hydrophobicity. In further support of this role, cells expressing *rscS1* produce an extractable extracellular matrix visible by SEM and TEM and recognized by the lectin ConA. With this RscS-induced cell surface alteration, the ability of *V. fischeri* to colonize its host was greatly enhanced, as demonstrated by (i) competition experiments in which *rscS1*-containing cells dramatically out-competed vector control cells and (ii) microscopy studies that uncovered the role of *rscS* in promoting bacterial aggregation on the surface of the light organ.

To determine the role of the *syp* cluster in the production of *rscS1*-dependent phenotypes, we disrupted a representative *syp* gene, *sypN*, and found that most of the phenotypes depended on *sypN*. We chose *sypN* because it is a structural gene (encoding a putative glycosyltransferase), the loss of which should impact primarily the product of the *syp* cluster, rather than producing pleiotropic effects as would deletion of a regulatory gene such as *sypG*. Indeed, mutations in other *syp* genes similarly disrupt *rscS1*-dependent phenotypes. For example, disruption of most of the *syp* genes either eliminated or reduced the formation of wrinkled colonies and pellicles (E.S. Yip and K.L. Visick, unpubl. data). However, disruption of *sypN* (or other genes in the cluster) did not always render *rscS1*-containing cells indistinguishable from vector control cells. For example, shaking liquid cultures of *rscS1*-containing *sypN* mutants exhibited a cell settling phenotype unlike vector control cells (Fig. 2C). This phenotype may result either from the activities of the remaining intact *syp* genes or from RscS-dependent regulation of genes outside of the *syp* cluster.

It is not yet clear how RscS exerts its impact on *syp*. As a sensor kinase, it is predicted to function by signalling to a response regulator, and the *syp*-encoded response regulator SypG seems a prime candidate for this target regulator. Both regulators control *syp* transcription (Yip *et al.*, 2005; Fig. 1) and *rscS1*-dependent *syp* induction requires SypG (K. Geszvain and K.L. Visick, unpubl. results). However, induction of the *syp* cluster through multicopy expression of *sypG* produces a different array of culture phenotypes from that which *rscS1* produces. First, multicopy *sypG* induces substantial adherence to the test tube when cells are grown with shaking (Yip *et al.*, 2005), while *rscS1* does not (data not shown). Second, unlike *rscS1*, multicopy *sypG* fails to induce wrinkled colonies (E.A. Hussa and K.L. Visick, unpubl. data). Although cognate response regulators and sensor kinases can induce distinct phenotypes (for example, see Yap *et al.*, 2005), it is also possible that these two regulators control the expression of distinct subsets of genes. The *syp* cluster includes two additional two-component regulators, a putative sensor kinase (SypF) and a putative response regulator (SypE) that is not predicted to bind DNA, suggesting that *syp* regulation may be complex.

Why is a sensor kinase necessary for one of the earliest steps of colonization? Presumably RscS recognizes an environmental signal and subsequently induces *syp* transcription such that surface modification can occur prior to aggregation. Potentially, a component of the seawater itself could serve as the signal for RscS. However, our preliminary experiments have provided no evidence for seawater-mediated induction of *syp* transcription (K. Geszvain and K.L. Visick, unpubl. data). Therefore, it is

likely that the environmental signal is found in proximity to the squid but external to the light organ.

The work presented here suggests that the *syp* cluster functions to modify the cell surface. Biotinylated lectin assays revealed that the surface polysaccharides produced by *syp* likely contain glucose or α-linked mannose residues. Intriguingly, the *rscS1*-induced matrix promotes the formation of a strong pellicle at the air–liquid interface similar to the cellulose-based pellicle produced by *Gluconacetobacter xylinum* (Ross *et al.*, 1991). Cellulose biosynthetic genes are present in the *V. fischeri* genome (Ruby *et al.*, 2005; K.L. Visick, unpubl. data); however, our preliminary data suggest that the *V. fischeri* matrix is not composed of cellulose, as the addition of cellulase did not dissolve it and *rscS1*-containing cells failed to bind either Congo Red or calcofluor (E.S. Yip and K.L. Visick, unpubl. data). It is worth noting that addition of a mannose analogue to the seawater interfered with the ability of *V. fischeri* to initiate the symbiosis (McFall-Ngai *et al.*, 1998), supporting a role for a mannose-based interaction between the bacteria and their host, or perhaps among the bacteria, during colonization.

The increased surface hydrophobicity we detected in cells expressing *rscS1* may be relevant to the function of RscS and the *syp* cluster in symbiosis. In a number of systems, bacterial hydrophobicity has been correlated with adherence to plant and animal tissues (reviewed in Doyle, 2000). In the oral bacterium *Streptococcus gordonii*, fibrils composed of CshA render the cell surface hydrophobic and are required for colonization of the oral cavity in mice (McNab *et al.*, 1999). In addition, the rugose phase variant of *V. cholerae* is more hydrophobic than the smooth variant due to the production of the VPS[ElTor] exopolysaccharide (Yildiz *et al.*, 2004). For *V. fischeri*, it is possible that hydrophobic interactions among the bacterial cells, between the bacterium and its host, or both, could provide a substantial advantage, because the initial interactions occur in the context of an aqueous seawater environment. Indeed, it has been estimated that seawater flows in and out of the mantle cavity of the juvenile squid every 0.3 s (Visick and McFall-Ngai, 2000), creating a difficult environment for specific, receptor–ligand type interactions to occur between the two organisms.

After aggregation, *V. fischeri* cells must migrate through ducts into the light organ crypts in order to establish the symbiotic association. This migration was apparently not impaired in *rscS1* cells, despite the increased cell–cell aggregation on the light organ surface that could potentially impair subsequent cell movement. However, in our aggregation experiments we detected *rscS1*-containing bacteria beginning to move into the ducts in continuous streams of cells (Fig. 7E). This streaming of the bacteria into the ducts has also been observed with wild-type cells (Nyholm *et al.*, 2000), indicating that colonization by *rscS1*-containing cells proceeds normally after aggregate formation. Indeed, the presence of *rscS1* does not impair the migration of *V. fischeri* through complex soft agar media (data not shown), supporting the conclusion that these cells can freely migrate away from the aggregate.

The ability of bacteria to form biofilm communities within their host organisms is thought to play a central role in both pathogenesis and symbiosis, by rendering the bacteria resistant to antimicrobial agents and enhancing adherence to host tissues, such as infection of lungs of cystic fibrosis patients by *P. aeruginosa* (Singh *et al.*, 2000). Mutants which decrease the ability of pathogenic bacteria to form biofilms in culture show a concomitant decrease in virulence in animal models of infection (Paranjpye and Strom, 2005; Kulasakara *et al.*, 2006). However, in many cases, these studies did not examine loss of biofilm within the host. Here, we report a mutualistic symbiosis where biofilm production in culture is directly linked to biofilm production in the host. Therefore, our work provides critical support for the importance of bacterial biofilm formation during the establishment of a natural bacteria–animal association.

Experimental procedures

Strains, plasmids and media

Strains used in this study are listed in Table 1. *V. fischeri* strains constructed in this work were generated by conjugation as previously described (Visick and Skoufos, 2001; DeLoney *et al.*, 2002). A bacterial isolate from *E. scolopes*, ES114 (Boettcher and Ruby, 1990), and its rifampicin-resistant derivative, ESR1 (Graf *et al.*, 1994), were the symbiosis-competent parent strains used in this study. The *E. coli* strains DH5α, TOP10 (Invitrogen, Carlsbad, CA) and CC118λ*pir* (Herrero *et al.*, 1990) were used as hosts for cloning and conjugation. *V. fischeri* strains were grown in complex media [seawater tryptone, SWT (Yip *et al.*, 2005) or LBS (Graf *et al.*, 1994; Stabb *et al.*, 2001)] or in HMM (Ruby and Nealson, 1977) containing 0.3% Casamino acids and 0.2% glucose (Yip *et al.*, 2005). The following antibiotics were added, as needed, to the final concentrations indicated: chloramphenicol (Cm), 5 μg ml⁻¹; erythromycin (Em) 5 μg ml⁻¹; and tetracycline (Tet), 5 μg ml⁻¹ in LBS, 30 μg ml⁻¹ in SWT and HMM. Agar was added to a final concentration of 1.5% for solid media. Juvenile *E. scolopes* were maintained in artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH). In some experiments, natural seawater collected in *E. scolopes*-free waters off the coast of Oahu, Hawaii, was used. This seawater does not contain sufficient *V. fischeri* cells to colonize the squid. For motility assays, bacteria were grown at 28°C to mid-exponential phase and inoculated onto SWT, TBS or TB-SW soft agar plates (DeLoney-Marino *et al.*, 2003).

Molecular techniques

Plasmids were constructed using standard molecular biology techniques, with restriction and modifying enzymes obtained

from New England Biolabs (Beverly, MA) or Promega (Madison, WI). Plasmids used or constructed in this study are shown in Table 1.

Random mutagenesis of rscS

A plasmid encoding wild-type *rscS*, pLMS33, was introduced into mutator strain CC130 (*mutD5*; Manoil and Beckwith, 1985; Schaaper, 1988) by transformation. Five independent transformations were performed to obtain a total of approximately 24 000 single colonies on selective media. Colonies were scraped off the plates into six separate pools; each pool of mutagenized plasmids was then conjugated into *syp* reporter strains KV1601 and KV1635 (Table 1). Transconjugants were selected for the presence of the plasmid and screened for induction of β-galactosidase expression on SWT Tet containing 80 μg ml⁻¹ Xgal (Molecular Probes). After incubation at room temperature (RT) for 2 days, approximately 200 blue (i.e. *syp*-expressing) colonies were found among the ~200 000 colonies plated. Plasmids were isolated from the six colonies that exhibited the greatest amount of *syp* expression (based on the intensity of blue colour), the *rscS* gene from these plasmids was subcloned into unmutagenized vector (pKV69, Table 1) and the resulting constructs were examined again for their ability to induce *syp* transcription. Two subcloned *rscS* alleles (*rscS1* and *rscS2*, encoded on plasmids pKG11 and pKG13 respectively) retained the ability to induce *syp* expression.

Sequencing

The mutations in *rscS1* and *rscS2* were identified by automated sequencing (MWG Biotech, Highpoint, NC) of the *rscS* gene present on pKG11 and pKG13 respectively, using primers specific to *rscS*. The *rscS1* allele contains a C to T transition in the sixth position of the putative ribosome binding site (AGGAGC to AGGAGT) and a C to T transition at the wobble position within a Leu codon at amino acid 25 (CTC to CTT). The *rscS2* allele contains a T to C transition immediately 3′ of the ribosome binding site (AGGAGCT to AGGAGCC).

β-Galactosidase assay

Strains were grown in HMM with Tet at 22°C overnight, then subcultured into fresh medium and grown for 24 h. Aliquots (1 ml) were removed and β-galactosidase activity in triplicate was assayed as described (Miller, 1972). Protein concentration was measured as described (Lowry *et al.*, 1951).

Hydrophobicity assay

Strains were grown in HMM with Tet at 28°C overnight, subcultured into fresh medium and grown for 24 h. Next, an equal volume of hexadecane (Acros Organics, NJ) was added to the culture tubes and vortexed until the suspension was homogeneous (at least 1 min). Then, the hydrophobic

(hexadecane) and hydrophilic fractions were allowed to separate. Partitioning of the cells to the hydrophobic layer was visualized as an increase in turbidity of the top layer (Rosenberg *et al.*, 1980; Araujo *et al.*, 1994).

Confocal microscopy

Cells expressing fluorescent proteins (RFP or GFP) were grown statically in HMM in 12 well microtiter plates with glass coverslips submerged partially into the culture medium. Coverslips were incubated with bacteria at RT for up to 30 h and removed at specific time points for biofilm examination. A Zeiss LSM 510 confocal microscope (65× objective) was used to collect *xy* plane and *z* sections (*xz* and *yz* plane) images of the biofilms. Images were prepared using the Zeiss LSM Image Browser software.

Scanning electron microscopy

Colonies grown on cellulose membranes overlaid on LBS Tet plates were prepared as described (Yildiz *et al.*, 2001) with the following modifications: cells were fixed in 2% glutaraldehyde in 0.45 M cacodylate buffer (pH 7.3), washed with 0.45 M cacodylate buffer, post-fixed with 1% osmium tetroxide for 1 h and washed again with cacodylate buffer. The samples were subjected to dehydration with ethanol, critical point drying and sputter-coating with gold-palladium. Finally, samples were examined with a scanning electron microscope (JEOL JSM-840A).

Transmission electron microscopy

Colonies were grown on LBS Tet plates at RT for 3 days and fixed as described (Luft, 1971; Patterson *et al.*, 1975) with the following modifications: colonies were fixed overnight in a solution containing 1.2% glutaraldehyde, 0.45 M cacodylate buffer (pH 7.3) and 0.1% (wt/vol) ruthenium red. After fixation, cells were washed, post-fixed with 1% osmium tetroxide for 1 h, washed again and subjected to serial dehydration with ethanol. Samples were embedded in resin, thin-sectioned and stained with uranyl acetate and lead citrate. Finally, the samples were examined with a transmission electron microscope (Hitachi H-600) operating at an accelerating voltage of 75 kV.

Polysaccharide extraction

Polysaccharides were extracted as described by Enos-Berlage and McCarter (2000) with the following modifications: bacterial strains were grown in a lawn on LBS Tet until *rscS1*-containing WT cells became wrinkled. Cells scrapped off of the plates were resuspended in 5 ml of phosphate-buffered saline (20 mM sodium phosphate, 100 mM sodium chloride, pH 7.3). Cell suspensions were shaken at 28°C for 90 min, vortexed and shaken for another 90 min. Cultures were centrifuged at 10 000 *g* for 15 min to remove cells and cellular debris. Supernatants were transferred to fresh tubes and incubated for 3 h at 37°C with MgCl₂, RNase (Sigma, St Louis, MO) and RQ1 DNase (Promega, Madison, WI) at

final concentrations of 10 mM, 50 µg ml^{-1} and 50 µg ml^{-1} respectively. Proteinase K (Fisher, Fair Lawn, NJ) was subsequently added to a final concentration of 200 µg ml^{-1} and the samples were incubated at 37°C overnight. Polysaccharides were extracted using phenol/chloroform and precipitated with ethanol. Samples were boiled in loading buffer containing 10% β-mercaptoethanol prior to gel electrophoresis. However, only a portion of the samples were solubilized and thus loaded onto the gels for Stains-All (Kelley and Parker, 1981) staining and lectin binding assays.

Lectin binding assay

Polysaccharide extracts were evaluated using a panel of biotinylated lectins. Extracts were subjected to electrophoresis on a Tris glycine-polyacrylamide (14%) gel and then transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The membrane was blocked with Tris-buffered saline containing 0.2% Tween (TBST) and 2% (v/v) bovine serum albumin for 1 h. After blocking, the membrane was incubated in TBST for 45 min with 1 µg ml^{-1} (final concentration) of one of seven biotinylated lectins in the biotinylated lectin kit I, which contains ConA, DBA, PNA, RCA$_{120}$, SBA, UEA I and WGA (Vector Laboratory, Burlingame, CA). Then, the membrane was washed with TBST three times for 10 min each and subsequently incubated with horseradish peroxidase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) for 30 min. Reactive carbohydrates were detected by incubating the membrane with an equal volume of solution 1 (2.5 mM luminal, 0.4 mM p-coumaric acid and 100 mM Tris pH 8.5) and solution 2 (0.02% H$_2$O$_2$, 100 mM Tris pH 8.5).

Colonization assays

Both single-strain and competition assays were performed as described (Lee and Ruby, 1994; Ruby, 1996; Visick and Skoufos, 2001). For the competition assays, vector and *rscS1* were both introduced into *V. fischeri* strain KV1421, which is marked with an Em^R cassette inserted at the Tn*7* attachment site (DeLoney *et al.*, 2002). Strains used in the colonization experiments were grown in SWT with Tet for about 4 h before inoculation; these conditions did not promote significant aggregation or growth defects in culture. These strains also exhibited no motility defect when tested for their ability to migrate through complex soft agar media (DeLoney-Marino *et al.*, 2003). To test for a competitive defect, we inoculated 12 juveniles with a mixture of the Em^R vector strain (KV2435, 1750 cfu ml^{-1}) and the Em^S *rscS1* strain (KV1956, 750 cfu ml^{-1}) (a ratio of 2.3:1). In a control experiment, 11 juveniles were inoculated with a mixture of cells with vector in the Em^R strain background (KV2435, 2477 cfu ml^{-1}) and vector in the Em^S strain (KV1844, 3283 cfu ml^{-1}) (a ratio of 0.75:1). Approximately 20 h after inoculation, the individual squid were monitored for luminescence, rinsed with bacteria-free artificial seawater and frozen at −80°C. The following day, the squid were homogenized and plated on SWT. In these experiments, all animals luminesced and were colonized to at least 10^4 cfu per squid. Between 100 and 200 colonies from each squid homogenate

were patched onto LBS with Em to determine the ratio of Em^S to Em^R bacteria within the light organ.

Squid aggregation assays

Log-phase cells (OD$_{600}$ 0.3–0.6) were grown in 2 ml SWT medium at 28°C. Bacterial cells were then inoculated into unfiltered natural seawater or filtered artificial seawater at concentrations of between 10^6 and 10^8 cells per ml. Juvenile squid were then placed into inoculated seawater and the two organisms were allowed to associate at RT for between 2 and 6 h prior to dissection. At various times, the juvenile squid were removed to vials containing 2 ml filter-sterilized seawater and 5 µM of the counter-stain, Cell Tracker Orange (Molecular Probes, Eugene, OR). The animals were then anaesthetized in seawater or filtered artificial seawater containing 2% ethanol. Each squid was placed ventral side up on a depression well slide and dissected to remove the mantle and funnel and expose the light organ. Fluorescently labelled light organs (red) and GFP-labelled (green) bacteria were viewed using a Zeiss LSM 510 confocal microscope.

References

Araujo, R.S., Robleto, E.A., and Handelsman, J. (1994) A hydrophobic mutant of *Rhizobium etli* altered in nodulation competitiveness and growth in the rhizosphere. *Appl Environ Microbiol* **60:** 1430–1436.

Boettcher, K.J., and Ruby, E.G. (1990) Depressed light emission by symbiotic *Vibrio fischeri* of the sepiolid squid *Euprymna scolopes*. *J Bacteriol* **172:** 3701–3706.

Branda, S.S., Vik, S., Friedman, L., and Kolter, R. (2005) Biofilms: the matrix revisited. *Trends Microbiol* **13:** 20–26.

Del Re, B., Sgorbati, B., Miglioli, M., and Palenzona, D. (2000) Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Lett Appl Microbiol* **31:** 438–442.

DeLoney, C.R., Bartley, T.M., and Visick, K.L. (2002) Role for phosphoglucomutase in *Vibrio fischeri-Euprymna scolopes* symbiosis. *J Bacteriol* **184:** 5121–5129.

DeLoney-Marino, C.R., Wolfe, A.J., and Visick, K.L. (2003) Chemoattraction of *Vibrio fischeri* to serine, nucleosides,

and N-acetylneuraminic acid, a component of squid light-organ mucus. *Appl Environ Microbiol* **69:** 7527–7530.

Doyle, R.J. (2000) Contribution of the hydrophobic effect to microbial infection. *Microbes Infect* **2:** 391–400.

Dunn, A.K., Millikan, D.S., Adin, D.M., Bose, J.L., and Stabb, E.V. (2006) New rfp- and pES213-derived tools for analyzing symbiotic *Vibrio fischeri* reveal patterns of infection and lux expression in situ. *Appl Environ Microbiol* **72:** 802–810.

Enos-Berlage, J.L., and McCarter, L.L. (2000) Relation of capsular polysaccharide production and colonial cell organization to colony morphology in *Vibrio parahaemolyticus*. *J Bacteriol* **182:** 5513–5520.

Friedman, L., and Kolter, R. (2004a) Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. *J Bacteriol* **186:** 4457–4465.

Friedman, L., and Kolter, R. (2004b) Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. *Mol Microbiol* **51:** 675–690.

Graf, J., Dunlap, P.V., and Ruby, E.G. (1994) Effect of transposon-induced motility mutations on colonization of the host light organ by *Vibrio fischeri. J Bacteriol* **176:** 6986–6991.

Herrero, M., DeLorenzo, V., and Timmis, K.N. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J Bacteriol* **172:** 6557–6567.

Hung, D.T., Zhu, J., Sturtevant, D., and Mekalanos, J.J. (2006) Bile acids stimulate biofilm formation in *Vibrio cholerae. Mol Microbiol* **59:** 193–201.

Jackson, K.D., Starkey, M., Kremer, S., Parsek, M.R., and Wozniak, D.J. (2004) Identification of *psl*, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. *J Bacteriol* **186:** 4466–4475.

Kelley, J.T., and Parker, C.D. (1981) Identification and preliminary characterization of *Vibrio cholerae* outer membrane proteins. *J Bacteriol* **145:** 1018–1024.

Kirn, T.J., Lafferty, M.J., Sandoe, C.M., and Taylor, R.K. (2000) Delineation of pilin domains required for bacterial association into microcolonies and intestinal colonization by *Vibrio cholerae. Mol Microbiol* **35:** 896–910.

Kulasakara, H., Lee, V., Brencic, A., Liberati, N., Urbach, J., Miyata, S., *et al.* (2006) Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3′-5′)-cyclic-GMP in virulence. *Proc Natl Acad Sci USA* **103:** 2839–2844.

Lee, K.H., and Ruby, E.G. (1994) Competition between *Vibrio fischeri* strains during initiation and maintenance of a light organ symbiosis. *J Bacteriol* **176:** 1985–1991.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* **193:** 265–275.

Luft, J.H. (1971) Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanism of action. *Anat Rec* **171:** 347–368.

McCann, J., Stabb, E.V., Millikan, D.S., and Ruby, E.G. (2003) Population dynamics of *Vibrio fischeri* during infection of *Euprymna scolopes. Appl Environ Microbiol* **69:** 5928–5934.

McFall-Ngai, M., Brennan, C., and Lamarcq, L. (1998) *Mannose Adhesin–Glycan Interactions in the Euprymna Scolopes-Vibrio Fischeri Symbiosis*. New York: Plenum Press.

McNab, R., Forbes, H., Handley, P.S., Loach, D.M., Tannock, G.W., and Jenkinson, H.F. (1999) Cell wall-anchored CshA polypeptide (259 kilodaltons) in *Streptococcus gordonii* forms surface fibrils that confer hydrophobic and adhesive properties. *J Bacteriol* **181:** 3087–3095.

Manoil, C., and Beckwith, J. (1985) Tn phoA: a transposon probe for protein export signals. *Proc Natl Acad Sci USA* **82:** 8129–8133.

Matsukawa, M., and Greenberg, E.P. (2004) Putative exopolysaccharide synthesis genes influence *Pseudomonas aeruginosa* biofilm development. *J Bacteriol* **186:** 4449–4456.

Miller, J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Millikan, D.S., and Ruby, E.G. (2002) Alterations in *Vibrio fischeri* motility correlate with a delay in symbiosis initiation and are associated with additional symbiotic colonization defects. *Appl Environ Microbiol* **68:** 2519–2528.

Millikan, D.S., and Ruby, E.G. (2003) FlrA, a sigma54-dependent transcriptional activator in *Vibrio fischeri*, is required for motility and symbiotic light-organ colonization. *J Bacteriol* **185:** 3547–3557.

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

Nyholm, S.V., and McFall-Ngai, M. (2004) The winnowing establishing the squid *Vibrio* symbiosis. *Nat Rev Microbiol* **2:** 632–642.

Nyholm, S.V., Stabb, E.V., Ruby, E.G., and McFall-Ngai, M.J. (2000) Establishment of an animal–bacterial association: recruiting symbiotic *Vibrios* from the environment. *Proc Natl Acad Sci USA* **97:** 10231–10235.

O'Shea, T.M., Klein, A.H., Geszvain, K., Wolfe, A.J., and Visick, K.L. (2006) Diguanylate cyclases control magnesium-dependent motility of *Vibrio fischeri. J Bacteriol* **188:** 23.

Paranjpye, R.N., and Strom, M.S. (2005) A *Vibrio vulnificus* type IV pilin contributes to biofilm formation, adherence to epithelial cells, and virulence. *Infect Immun* **73:** 1411–1422.

Patterson, H., Irvin, R., Costerton, J.W., and Cheng, K.J. (1975) Ultrastructure and adhesion properties of *Ruminococcus albus. J Bacteriol* **122:** 278–287.

Romling, U., Sierralta, W.D., Eriksson, K., and Normark, S. (1998) Multicellular and aggregative behaviour of *Salmonella typhimurium* strains is controlled by mutations in the *agfD* promoter. *Mol Microbiol* **28:** 249–264.

Rosenberg, M., Gutnick, D., and Rosenberg, E. (1980) Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett* **9:** 29–33.

Ross, P., Mayer, R., and Benziman, M. (1991) Cellulose biosynthesis and function in bacteria. *Microbiol Rev* **55:** 35–58.

Ruby, E.G. (1996) Lessons from a cooperative, bacterial–animal association: the *Vibrio fischeri-Euprymna scolopes* light organ symbiosis. *Annu Rev Microbiol* **50:** 591–624.

Ruby, E.G., and Nealson, K.H. (1977) Pyruvate production and excretion by the luminous marine bacteria. *Appl Environ Microbiol* **34:** 164–169.

Ruby, E.G., Urbanowski, M., Campbell, J., Dunn, A., Faini, M., Gunsalus, R., *et al.* (2005) Complete genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. *Proc Natl Acad Sci USA* **102:** 3004–3009.

Schaaper, R.M. (1988) Mechanisms of mutagenesis in the *Escherichia coli* mutator mutD5: role of DNA mismatch repair. *Proc Natl Acad Sci USA* **85:** 8126–8130.

Singh, P.K., Schaefer, A.L., Parsek, M.R., Moninger, T.O., Welsh, M.J., and Greenberg, E.P. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* **407:** 762–764.

Solano, C., Garcia, B., Valle, J., Berasain, C., Ghigo, J.M., Gamazo, C., and Lasa, I. (2002) Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose. *Mol Microbiol* **43:** 793–808.

Sonnenburg, J.L., Angenent, L.T., and Gordon, J.I. (2004) Getting a grip on things: how do communities of bacterial symbionts become established in our intestine? *Nat Immunol* **5:** 569–573.

Stabb, E.V., and Ruby, E.G. (2002) New RP4-based plasmids for conjugation between *Escherichia coli* and members of the *Vibrionaceae*. *Methods Enzymol* **358:** 413–426.

Stabb, E.V., Reich, K.A., and Ruby, E.G. (2001) *Vibrio fischeri* genes *hvnA* and *hvnB* encode secreted NAD(+)-glycohydrolases. *J Bacteriol* **183:** 309–317.

Stock, A.M., Robinson, V.L., and Goudreau, P.N. (2000) Two-component signal transduction. *Annu Rev Biochem* **69:** 183–215.

Visick, K.L., and McFall-Ngai, M.J. (2000) An exclusive contract: specificity in the *Vibrio fischeri-Euprymna scolopes* partnership. *J Bacteriol* **182:** 1779–1787.

Visick, K.L., and Skoufos, L.M. (2001) Two-component sensor required for normal symbiotic colonization of *Euprymna scolopes* by *Vibrio fischeri*. *J Bacteriol* **183:** 835–842.

Wadstrom, T., Andersson, K., Sydow, M., Axelsson, L., Lindgren, S., and Gullmar, B. (1987) Surface properties of *lactobacilli* isolated from the small intestine of pigs. *J Appl Bacteriol* **62:** 513–520.

Whiteley, M., Bangera, M.G., Bumgarner, R.E., Parsek, M.R., Teitzel, G.M., Lory, S., and Greenberg, E.P. (2001) Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* **413:** 860–864.

Woodcock, D.M., Crowther, P.J., Doherty, J., Jefferson, S., DeCruz, E., Noyer-Weidner, M., *et al.* (1989) Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acids Res* **17:** 3469–3478.

Yap, M.N., Yang, C.H., Barak, J.D., Jahn, C.E., and Charkowski, A.O. (2005) The *Erwinia chrysanthemi* type III secretion system is required for multicellular behavior. *J Bacteriol* **187:** 639–648.

Yildiz, F.H., and Schoolnik, G.K. (1999) *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proc Natl Acad Sci USA* **96:** 4028–4033.

Yildiz, F.H., Dolganov, N.A., and Schoolnik, G.K. (2001) VpsR, a member of the response regulators of the two-component regulatory systems, is required for expression of *vps* biosynthesis genes and EPS (ETr)-associated phenotypes in *Vibrio cholerae* O1 El Tor. *J Bacteriol* **183:** 1716–1726.

Yildiz, F.H., Liu, X.S., Heydorn, A., and Schoolnik, G.K. (2004) Molecular analysis of rugosity in a *Vibrio cholerae* O1 El Tor phase variant. *Mol Microbiol* **53:** 497–515.

Yip, E.S., Grublesky, B.T., Hussa, E.A., and Visick, K.L. (2005) A novel, conserved cluster of genes promotes symbiotic colonization and sigma-dependent biofilm formation by *Vibrio fischeri*. *Mol Microbiol* **57:** 1485–1498.

Zogaj, X., Nimtz, M., Rohde, M., Bokranz, W., and Romling, U. (2001) The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. *Mol Microbiol* **39:** 1452–1463.