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A novel, conserved cluster of genes promotes symbiotic colonization and σ^{54}-dependent biofilm formation by *Vibrio fischeri*

Emily S. Yip, Brian T. Grublesky, Elizabeth A. Hussa and Karen L. Visick*
Department of Microbiology and Immunology, Loyola University Chicago, 2160 S. First Ave. Bldg. 105, Maywood, IL 60153, USA.

Summary

Vibrio fischeri **is the exclusive symbiont residing in the light organ of the squid** ***Euprymna scolopes*****. To understand the genetic requirements for this association, we searched a library of** ***V. fischeri*** **transposon insertion mutants for those that failed to colonize** ***E. scolopes*****. We identified four mutants that exhibited severe defects in initiating colonization. Sequence analysis revealed that the strains contained insertions in four different members of a cluster of 21 genes oriented in the same direction. The predicted gene products are similar to proteins involved in capsule, exopolysaccharide or lipopolysaccharide biosynthesis, including six putative glycosyltransferases. We constructed mutations in five additional genes and found that they also were required for symbiosis. Therefore, we have termed this region** ***syp*****, for symbiosis polysaccharide. Homologous clusters also exist in** ***Vibrio parahaemolyticus*** **and** ***Vibrio vulnificus*****, and thus these genes may represent a common mechanism for promoting bacteria–host interactions. Using** ***lacZ*** **reporter fusions, we observed that transcription of the** ***syp*** **genes did not occur under standard laboratory conditions, but could be induced by multicopy expression of** ***sypG*****, which encodes a response regulator with a predicted σ^{54} interaction domain. This induction depended on σ^{54}, as a mutation in** ***rpoN*** **abolished** ***syp*** **transcription. Primer extension analysis supported the use of putative σ^{54} binding sites upstream of** ***sypA*****,** ***sypI*** **and** ***sypM*** **as promoters. Finally, we found that multicopy expression of** ***sypG*** **resulted in robust biofilm formation. This work thus reveals a novel group of genes that** ***V. fischeri*** **controls through a σ^{54}-dependent response regulator and uses to promote symbiotic colonization.**

Accepted 20 June, 2005. *For correspondence. E-mail kvisick@lumc.edu; Tel. (+1) 708 216 0869; Fax (+1) 708 216 9574.

Introduction

The symbiotic colonization of the Hawaiian bobtail squid *Euprymna scolopes* by the marine bioluminescent bacterium *Vibrio fischeri* serves as a model for studying bacteria–host interactions (Ruby, 1999; Nyholm and McFall-Ngai, 2004). Like well-studied pathogenic associations, the formation of this symbiosis requires the bacterium to express traits that allow it to enter and multiply within a host organ. Furthermore, colonization by *V. fischeri* promotes developmental changes within the host, including tissue remodelling (Foster and McFall-Ngai, 1998; Foster *et al*., 2000).

Factors involved in establishing the exclusive relationship between *V. fischeri* and its invertebrate host have been examined from the perspective of both organisms. The surface of the symbiotic organ, known as the light organ, includes ciliated epithelial appendages that project into the body cavity of the squid and help direct the flow of the seawater towards this organ (Montgomery and McFall-Ngai, 1993). *V. fischeri* cells in the seawater then aggregate in squid-secreted mucus on the surface of the light organ (Nyholm *et al*., 2000). In addition to *V. fischeri*, other Gram-negative bacteria, such as *Vibrio parahaemolyticus*, also exhibit the capability to aggregate on the light organ (Nyholm *et al*., 2000). However, if both *V. fischeri* and *V. parahaemolyticus* are introduced into the seawater, *V. fischeri* rapidly becomes the dominant species in the mixed bacterial aggregate (Nyholm and McFall-Ngai, 2003). These data suggest that *V. fischeri* cells contribute to the observed specificity of the interaction.

After 2–3 h of aggregation, during which bacteria–bacteria and bacteria–host signalling likely occurs (Nyholm *et al*., 2000; Lupp and Ruby, 2005), *V. fischeri* cells enter the light organ. First, they migrate into one of six openings known as pores (Nyholm *et al*., 2000). This migration requires bacterial motility: non-motile bacteria aggregate but fail to migrate into the light organ (Graf *et al*., 1994; Nyholm *et al*., 2000; Millikan and Ruby, 2003). Next, they passage through ducts that contain

nitric oxide, which may provide protection against colonization by non-symbionts (Davidson *et al*., 2004). Finally, they reach nutrient-filled crypts, where they multiply to high cell density and induce bioluminescence (Ruby and Asato, 1993; Graf and Ruby, 1998). Mutants defective for structural or regulatory genes required for light production (*luxA*, *luxR* and *luxI*) reach this stage of colonization, but subsequently fail to persist at wild-type levels within the light organ (Visick *et al*., 2000). Within the crypts, additional signalling occurs between the two organisms. Specifically, colonization by *V. fischeri* triggers apoptosis and subsequent regression of the surface ciliated appendages and a decrease in duct size (Foster and McFall-Ngai, 1998; Foster *et al*., 2000; Kimbell and McFall-Ngai, 2004). These changes presumably reduce the likelihood of colonization by other bacteria.

Besides motility and luminescence, other bacterial factors are required for various stages of symbiosis. Colonization requires the outer membrane protein OmpU for initiation, the metabolic enzyme Pgm for growth to high cell density, and the nitrogen and siderophore regulator GlnD for persistence (Graf and Ruby, 2000; Aeckersberg *et al*., 2001; DeLoney *et al*., 2002). In addition to these factors, a number of regulators have been identified as important for symbiotic colonization. These include quorum-sensing regulators (AinS, LuxS and LuxO), members of the class of two-component regulators (RscS, GacA and FlrA) and an alternative sigma factor (σ^{54}, encoded by *rpoN*). Other than RscS, for which targets have not been identified, these regulators control multiple factors known or predicted to play roles in symbiotic colonization (Visick and Skoufos, 2001; Lupp *et al*., 2003; Whistler and Ruby, 2003; Lupp and Ruby, 2004; 2005; Wolfe *et al*., 2004). Two of these regulators, FlrA and LuxO, are predicted to work with σ^{54} to control expression of flagella and bioluminescence respectively (Lupp *et al*., 2003; Millikan and Ruby, 2003). Indeed, *rpoN* mutants exhibit defects in motility and bioluminescence regulation, as well as in nitrogen metabolism and biofilm formation (Wolfe *et al*., 2004). Mutants defective for *rpoN* fail to initiate symbiotic colonization; however, whether this defect is due solely to the loss of motility exhibited by these strains or also to other σ^{54}-dependent phenotypes, such as biofilm formation, remains unclear.

Genes controlled by σ^{54}-containing RNA polymerase require transcription factors (such as FlrA and LuxO) to promote formation of an open, initiation-competent, complex by RNA polymerase (Reitzer and Schneider, 2001). Often, the transcription factor is a two-component response regulator activated by phosphorylation in response to an environmental signal received by a membrane-associated sensor kinase. The result is a tightly controlled, environment-specific regulation of gene expression.

In this article, we report the identification of a novel cluster of genes required for symbiotic initiation by *V. fischeri*. These genes are co-ordinately regulated by σ^{54} and a previously uncharacterized response regulator, SypG. Multicopy expression of *sypG* resulted in a dramatic, σ^{54}-dependent, enhancement of biofilm formation. This cluster, which includes putative genes for polysaccharide biosynthesis, is strongly conserved in the pathogens *V. parahaemolyticus* and *V. vulnificus* and thus may define a new paradigm for bacteria–host interactions in *Vibrio* species.

Results

Isolation of symbiosis-defective mutants

To identify novel bacterial genes required for establishing symbiosis, we screened a transposon (Tn)-mutagenized library of *V. fischeri* using newly hatched juveniles of *E. scolopes* as described previously (Visick and Skoufos, 2001; DeLoney *et al*., 2002). Briefly, we inoculated animals with individual mutant strains. We measured successful colonization by monitoring bacterial bioluminescence, produced when *V. fischeri* reaches high cell density in the symbiotic light organ. We then determined colonization levels by homogenizing the animals and counting the resulting number of colony-forming units (cfu). Mutants defective in symbiotic initiation were further confirmed using additional animals.

Using this assay, we identified four independent mutant strains, from among about 600 screened, that exhibited severe defects in initiating symbiotic colonization. None of the animals exposed to these strains achieved symbiotic luminescence above background levels within 17 h post inoculation (Fig. 1A). In contrast, 90% of the animals exposed to the parent strain, ESR1, began to emit light within 10 h, and all 10 animals inoculated with ESR1 produced detectable levels of light by 17 h (Fig. 1A). In culture, the mutant bacteria grew like their parent on a variety of media. They also exhibited normal kinetics of bioluminescence induction and similar overall levels of light relative to the parent strain (data not shown). These data suggested that the lack of symbiotic luminescence reflected either a delay in or an absence of colonization. In support of this prediction, we found that most animals exposed to the Tn mutants remained uncolonized while the few that were colonized contained less than 1% of the symbionts found in animals inoculated with ESR1 (Fig. 1B). Because the majority of the animals remained uncolonized, these data indicated that the defect in initiation was severe.

We next investigated whether these mutant strains were defective for known symbiotic factors. Non-motile strains of *V. fischeri* fail to colonize (Graf *et al*., 1994;

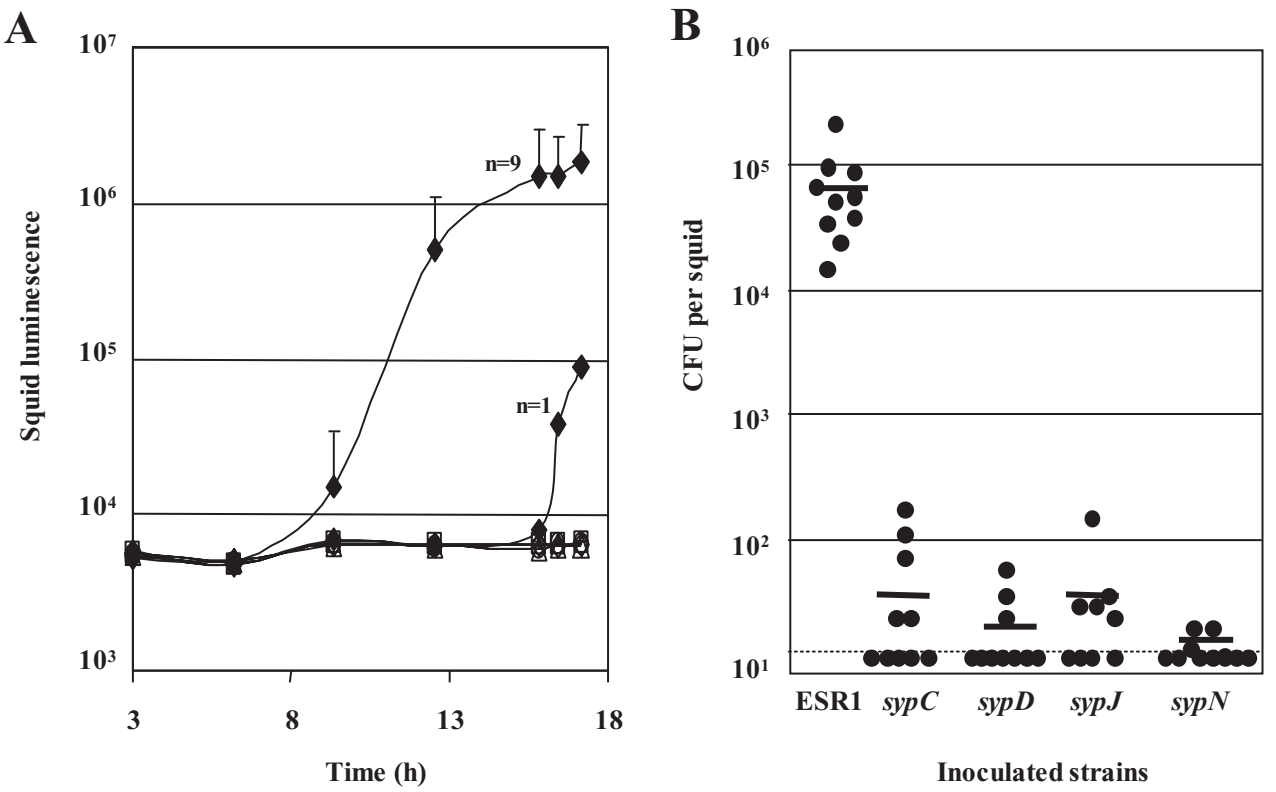

Fig. 1. Symbiotic colonization by transposon insertion mutants. Newly hatched juvenile squid were inoculated with either the parent strain, ESR1, or the transposon insertion mutants defective for *sypC*, *sypD*, *sypJ* or *sypN* (KV1637, KV1635, KV1636 and KV1601 respectively) with approximately 2300–3300 cfu ml^{-1} for 3 h.
A. Bioluminescence emission was monitored for 10 individual squid inoculated with ESR1 (solid diamonds) or the transposon insertion mutants *sypC* (open triangles), *sypD* (open squares), *sypJ* (open circles) or *sypN* (open diamonds). One ESR1-inoculated animal emitted bioluminescence after a significant delay, but achieved a high level of colony-forming units (cfu).
B. Within 1 h of the last time point shown in (A), the animals were homogenized and plated to determine the number of cfu in each squid. Each solid circle represents the number of cfu from an individual animal, while the bars represent the average of 10 (ESR1, *sypC*, *sypD* and *sypN*) or nine (*sypJ*) animals. The dotted line represents the limit of detection.

Millikan and Ruby, 2003; Wolfe *et al*., 2004), while hypermotile strains, if they colonize, do so with a significant delay (Millikan and Ruby, 2002). The four Tn mutants exhibited migration indistinguishable from that of their parent strain in a tryptone-based soft agar medium (data not shown). Symbiotic initiation also requires the outer membrane protein, OmpU (Aeckersberg *et al*., 2001); however, all four Tn mutants contained outer membrane protein profiles similar to that of the parent strain (data not shown). These data suggested that the Tns had inserted into genes not previously characterized as symbiosis determinants of *V. fischeri*.

Identification of a novel symbiotic gene cluster

We further characterized these novel colonization-defective mutants by examining the number and location of the Tn insertions by Southern analysis, cloning and sequencing. Each of the four mutant strains contained a single Tn insertion (data not shown; see *Experimental procedures*). Each insertion mapped to a distinct and previously uncharacterized gene within a cluster of 21 genes oriented in the same direction on chromosome 2 of *V. fischeri* (Fig. 2).

BLAST analyses (Altschul *et al*., 1990; 1997) and motif searches (Marchler-Bauer and Bryant, 2004) indicated that many of the genes in this cluster exhibited weak similarities to those involved in lipopolysaccharide (LPS), capsule or polysaccharide biosynthesis or export (Table 1). Six predicted proteins exhibited sequence similarities to glycosyltransferases, enzymes that transfer a sugar moiety from an activated donor (typically nucleotide sugar derivatives) to a specific acceptor (Kikuchi *et al*., 2003; Zhang *et al*., 2003). Target acceptors could be a growing carbohydrate chain of LPS, capsule or glycoproteins (Upreti *et al*., 2003). In addition, four genes were predicted to encode regulatory proteins such as two-component regulators. Based on these and other analyses (see below), we designated the genes in this locus as *syp*, for symbiosis polysaccharide.

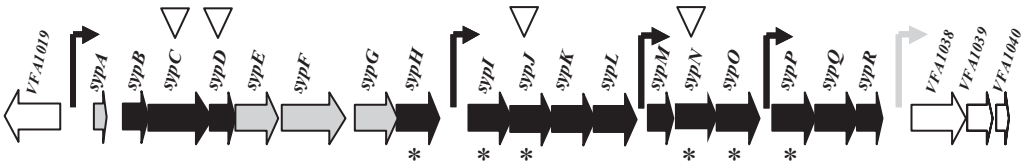

Fig. 2. Schematic representation of the *syp* cluster. Each gene in the *syp* cluster is represented by a block arrow. Grey arrows indicate genes with predicted regulatory function and solid arrows indicate genes with similarity to those involved in polysaccharide biosynthesis. Genes that encode putative glycosyltransferases are indicated by asterisks. Open arrows indicate genes flanking the *syp* cluster (*VFA1019* and *VFA1038–1040*). Bent black arrows indicate promoter sequences containing putative σ^{54} binding sites. The grey line arrow indicates a potential promoter region. Inverted triangles indicate transposon insertions.

To verify the importance of the *syp* cluster, we constructed derivatives of the wild-type strain, ES114, with mutations in this region. We cloned internal portions of specific genes into a suicide vector and introduced the constructs into *V. fischeri*. The resulting insertional mutants were confirmed by Southern analysis (see *Experimental procedures*). The ability of each mutant to initiate symbiotic colonization was compared with that of the wild-type strain. We used *sypN* as a control for this approach, as this gene was disrupted in one of the original Tn mutants and thus was not expected to colonize successfully. Indeed, like the original mutant, the insertional mutant of *sypN* colonized poorly (Fig. 3). Similarly, mutations in *sypL*, *sypO*, *sypP*, *sypQ* and *sypR* resulted in substantial defects (more than three orders of magnitude) in symbiotic colonization. In contrast, mutations in two downstream genes, *VFA1038* (Fig. 2; Table 1) and *VFA1043* (a putative ABC transporter), did not affect colonization of *V. fischeri* cells (Fig. 3). These data supported our hypothesis that this region functions in symbiotic colonization and suggested that the *syp* cluster does not extend to *VFA1038* or beyond.

Identification of Syp homologues in related Vibrio *species*

Significant similarities of the Syp proteins to others in the database were largely limited to predicted proteins found in two marine pathogens, *V. parahaemolyticus* (Makino *et al.*, 2003) and *V. vulnificus* (Chen *et al.*, 2003). Further analysis using the TIGR database (http://

Table 1. Putative functions of the *V. fischeri* Syp proteins and VFA1038–1040.

Syp	VFA[a]	RPS-BLAST[b]	E-value
A	1020	Sulphate transporter and anti-sigma factor antagonist	7e-11
B	1021	Outer membrane protein and related peptidoglycan-associated (lipo) proteins	2e-19
C	**1022**	**Wza, periplasmic protein involved in polysaccharide export**	4e-16
D	**1023**	**Mrp, ATPases involved in chromosome partitioning**	1e-11
E	1024	Response regulator receiver domain	3e-21
		Sigma factor PP2C-like phosphatases	4e-13
F	1025	Signal transduction histidine kinase	3e-41
		Response regulator receiver domain	1e-24
G	1026	Sigma-54 interaction domain	3e-84
		Response regulator receiver domain	1e-22
		HTH_8, bacterial regulatory protein, Fis family	1e-05
H	1027	Glycosyl transferases group 1	2e-22
I	1028	Glycosyl transferases group 1	5e-27
J	**1029**	**Glycosyl transferases group 1**	3e-09
K	1030	RfbX, membrane protein involved in the export of O-antigen and teichoic acid	5e-12
L	1031	RfaL, lipid A core-O-antigen ligase and related enzymes	1e-07
M	1032	WbbJ, acetyltransferase (isoleucine patch superfamily)	3e-19
N	**1033**	**RfaG, Glycosyltransferase**	1e-08
O	1034	GumC, uncharacterized protein involved in exopolysaccharide biosynthesis	3e-15
P	1035	RfaG, glycosyltransferase	4e-27
Q	1036	Glycosyltransferase, probably involved in cell wall biogenesis	2e-21
R	1037	WczJ, sugar transferase involved in lipopolysaccharide synthesis	1e-53
	1038	EAL domain	1e-61
		DUF1, domain of unknown function with GGDEF motif	3e-09
	1039	COG3310, uncharacterized protein conserved in bacteria	1e-67
	1040	Glutaredoxin	8e-06

a. The transposons inserted into *sypC*, *sypD*, *sypJ* and *sypN*, shown in bold.
b. Protein sequences were submitted to BLASTP. The most significant matches over the length of the protein are listed, along with the *e*-value to the motif. No conserved domains were noted for VFA1019. The alignments spanned over 70% of the protein sequence for all of the Syp proteins, except SypJ (51.7%).

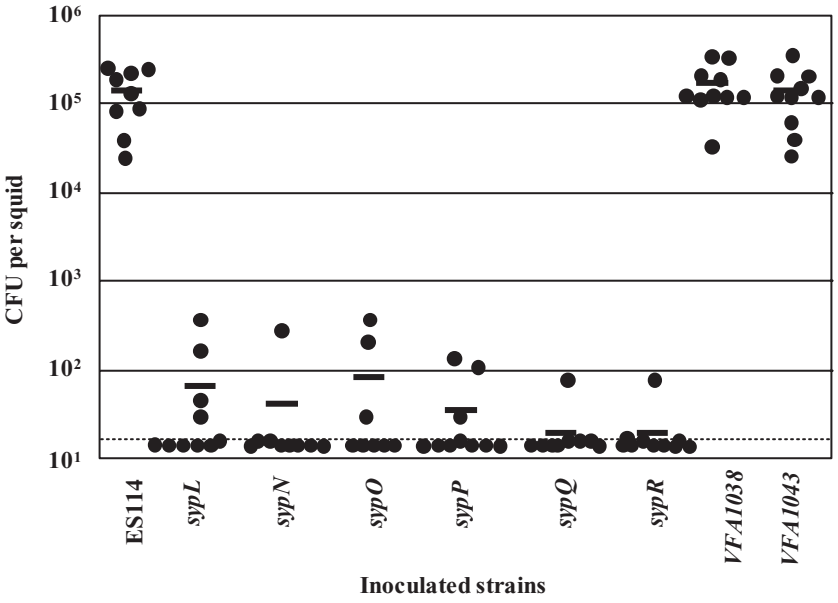

Fig. 3. Colonization by insertional mutants. Juvenile squid were inoculated for 3 h with approximately 2300–5000 cfu ml^{-1} of the wild-type strain, ES114, or insertional mutants defective for *syp* or downstream genes (*VFA1038* and *VFA1043*) (strains KV1816–18, KV1837–41). Animals were homogenized at ~18 h post inoculation for cfu determination. Each solid circle represents an individual animal and the bar represents the average of 10 (*sypL*, *sypP*, *sypQ*, *sypR*, *VFA1038* and *VFA1043*), nine (ES114 and *sypN*) or eight animals (*sypO*). The dotted line represents the limit of detection.

www.tigr.org/) revealed that homologues of almost all of the *syp* genes were present and similarly clustered in the above pathogens but not in *Vibrio cholerae* (Heidelberg *et al*., 2000) (Table S1). The *syp* cluster was located on the small chromosome of *V. fischeri*, but on the larger of two chromosomes in both *V. parahaemolyticus* and *V. vulnificus* (Chen *et al*., 2003; Makino *et al*., 2003; Ruby *et al*., 2005).

The three genes (*VFA1038–1040*) immediately downstream of the *syp* cluster, although apparently conserved in *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* (Table S1), were not found in proximity to the other 18 genes in these organisms. The lack of clustering of the *VFA1038–1040* homologues in *V. parahaemolyticus* and *V. vulnificus* suggests that these genes may not contribute to the function of this locus. This hypothesis is supported by our observation that a mutant defective for *VFA1038* is not deficient for colonization. Therefore, at this time, we designate only first 18 genes of the *V. fischeri* cluster as *syp*. Similarly, a homologue of *VFA1019*, the gene immediately upstream of and transcribed divergently to the cluster genes, exists in *V. parahaemolyticus* ($e = 1.2e^{-298}$) and potentially in *V. vulnificus* ($e = 1.9e^{-22}$); however, these genes were unlinked to the respective clusters. Our preliminary data suggested that this gene also is not required for colonization (B.T. Grublesky and K.L. Visick, unpublished data).

In *V. fischeri*, the G+C content of the *syp* cluster genes ranged from 34% to 42% and averaged 37.6%, a value similar to the overall G+C content of *V. fischeri* genome (38.85%). For *V. parahaemolyticus* and *V. vulnificus*, the values of the *syp* homologues also reflected that of the average G+C content. Thus, the *syp* cluster does not appear to represent an 'island'.

Transcriptional regulation of syp *genes by SypG and* σ^{54}

Nine of the open reading frames in the *syp* cluster appear to overlap their upstream neighbours, suggesting that these genes may be co-ordinately regulated. Close examination revealed larger gaps (~140 bp to 400 bp) between four sets of genes within the cluster as well as between *sypA* and the divergent gene *VFA1019* (Fig. 2). We examined these five intergenic regions and found four potential promoters that could be recognized by RNA polymerase containing the alternative sigma factor, σ^{54} (Fig. 4A). Unlike other sigma factors, σ^{54} recognizes sequences at positions –24 and –12 relative to the transcriptional start site. The consensus sequence of the σ^{54} binding site (mrNrYTGGCACG-N4-TTGCWNNw) is well conserved in a number of organisms (Barrios *et al*., 1999). The most important nucleotides in the consensus sequence (GG-N10-GC) (Barrios *et al*., 1999) are absolutely conserved at three of the four predicted promoters in the cluster (Fig. 4A).

Transcription by σ^{54}-containing RNA polymerase requires an interaction with an activator protein, often a response regulator whose ability to promote transcription is limited to specific environmental or cellular conditions. Sequence analysis of the *syp* cluster revealed a putative σ^{54}-dependent response regulator, SypG. This putative regulator was highly conserved in *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* (Table S1). Using Multiple Em for Motif Elicitation (MEME) analysis (see *Experimental procedures*), we identified a conserved 22 bp sequence in each of the four intergenic regions, upstream of the putative σ^{54} binding site (Fig. 4A). This sequence could potentially serve as a binding site for an activator. Both the putative σ^{54} binding sites and 22 bp sequences also

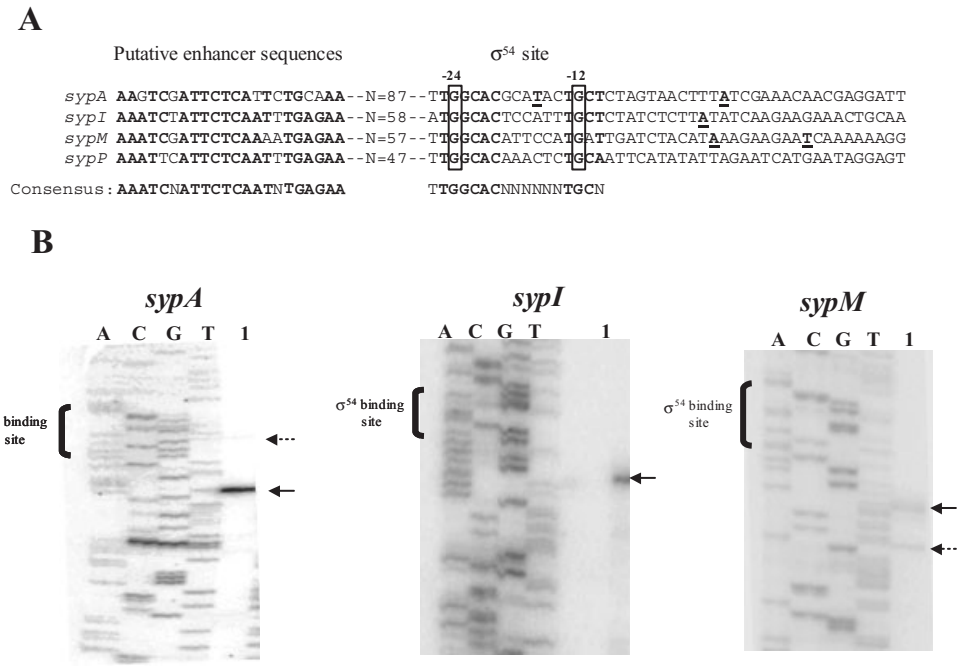

Fig. 4. Primer extension-based mapping of the transcriptional start sites of *sypA*, *sypI* and *sypM* genes.
A. Putative σ^{54} binding sites and potential enhancer binding sequences identified upstream of *sypA*, *sypI*, *sypM* and *sypP* are indicated. Conserved bases (three of four identical) are shown in bold. The –24 and –12 nucleotides of the predicted σ^{54} binding sites are boxed. The transcriptional start sites of each gene, based on primer extension experiments (B), are shown in bold and underlined.
B. Primer extension products obtained for transcripts initiating upstream of *sypA*, *sypI* and *sypM* are shown in lane 1 of the indicated panel. Bold line arrows indicate primer extension products predicted to be derived from the upstream σ^{54}-based promoter, while dashed line arrows indicate products from as yet undetermined sources. Lanes labelled A, C, G and T represent bands obtained from DNA sequencing reactions using the same primers.

are conserved in *V. parahaemolyticus* and *V. vulnificus* (data not shown).

To investigate the transcriptional control of the *syp* cluster, we used as reporter strains two of the Tn mutants, *sypD* and *sypN*, each of which contained a promoterless *lacZ* gene oriented correctly for *syp*-dependent transcription. Surprisingly, we observed no β-galactosidase activity from these strains under a variety of media and growth conditions (data not shown). We therefore hypothesized that SypG, in concert with σ^{54}, might control transcription of the *syp* genes in *V. fischeri*. Although response regulators typically depend on activation (by phosphorylation) from cognate sensor kinases, this requirement often can be overridden by multicopy expression of the response regulator. Thus, to investigate our hypothesis, we cloned a wild-type copy of *sypG* under the control of the *lac* promoter on a multicopy plasmid. We then introduced the resulting construct, pEAH40, into the Tn*10lacZ* reporter strains and observed the formation of blue colonies on X-gal-containing media, suggesting that SypG could induce *syp* transcription. We quantified this induction by measuring β-galactosidase activity, and found that multicopy expression of *sypG* increased the activity 37- to 70-fold over that of the vector control (Fig. 5). To determine whether the ability of SypG to induce *syp* transcription required the activity of σ^{54}, as predicted, we constructed double mutants (containing a mutation in *rpoN* and the *lacZ* reporter in either *sypD* or *sypN*). Indeed, a mutation in *rpoN* abolished the SypG-dependent activation of *syp* transcription (Fig. 5). These data suggest that SypG activates *syp* cluster expression in a σ^{54}-dependent manner.

Finally, to provide further support for the use of the identified σ^{54} binding sites, we determined the transcriptional start sites for *sypA*, *sypI* and *sypM* using primer extension experiments. Briefly, mRNA, isolated from wild-type cells containing multicopy *sypG*, was hybridized to a radiolabelled primer complementary to the gene of interest and subjected to reverse transcription. The resulting cDNA product was compared with a sequencing ladder (Fig. 4B). This analysis revealed two *sypA*-specific transcripts, with the primary band located 14 bp downstream from the putative σ^{54} binding site. Similarly, a *sypI* transcript initiated 12 bp downstream of the putative σ^{54} binding site. Finally, one of the *sypM* transcripts initiated at 13 bp downstream of the binding site. As these transcripts initiated within the range of start sites (8–16 bp) typical for σ^{54}-dependent transcription (Barrios *et al.*, 1999), these

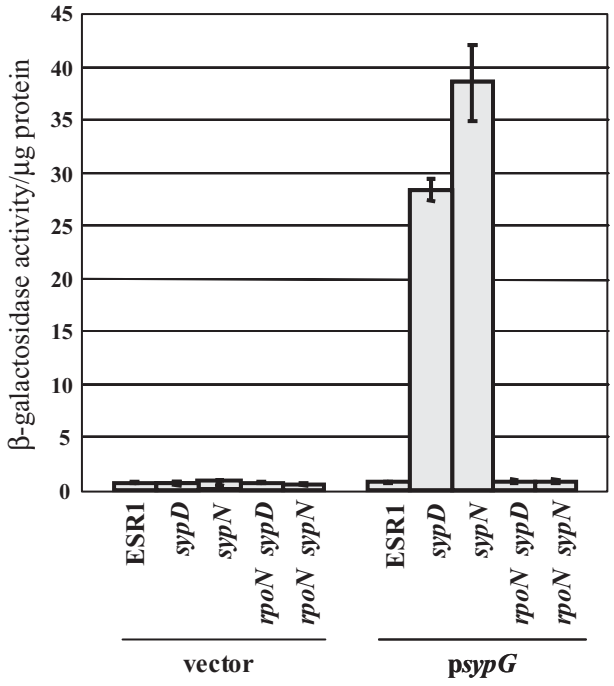

Fig. 5. Transcription of the *syp* genes. All strains were cultured in MM with shaking for 24 h before assaying activity. β-Galactosidase activity was measured in parent or *rpoN* mutant strains that contained a promoterless *lacZ* fusion to *sypD* or *sypN*. These strains also carried either the vector control or the *sypG*-expressing multicopy plasmid, pEAH40. As a control, β-galactosidase activity in parent strain, ESR1, which lacks a *lacZ* fusion, also was measured.

data further support a role for σ^{54} in promoting transcription of the *syp* genes.

Formation of biofilms under syp*-inducing conditions*

In nature, bacteria often exist in biofilms in which bacterial cells are embedded within a matrix of extracellular polysaccharides. Due to the putative functions of the *syp* genes in polysaccharide biosynthesis, we hypothesized that the *syp* cluster may function to alter the surface properties of *V. fischeri* to enhance biofilm formation. We therefore asked whether *syp* cluster members contributed to biofilm formation by *V. fischeri*. Not surprisingly, given the lack of *syp* transcription in culture, we noticed little difference in biofilm formation between the *syp* mutant strains and their parent when grown in static culture (data not shown). However, the addition of the *sypG* multicopy plasmid caused a significant, 3.5-fold, increase ($P = 0.0041$) in the ability of the parent strain, ESR1, to produce a biofilm in static culture (Fig. 6A and C). The *sypG*-expressing plasmid caused a similar increase in biofilm formation in all of the *syp* mutants except *sypC*, suggesting that *sypC* plays role in enhancing biofilm formation. Finally, consistent with our β-galactosidase results, a mutation in *rpoN* eliminated the *sypG*-dependent induction of biofilm formation, further supporting the roles of these two regulators in controlling the *syp* cluster.

During the course of our β-galactosidase experiments, we observed that cells containing multicopy *sypG*, when grown with shaking, produced a substantial ring of cells around the test tube. This large biofilm ring seemed inconsistent with the relatively modest increase in biofilm production we observed using the static culture assay. We therefore examined biofilms formed by these strains during growth with shaking. We found that these conditions dramatically increased the magnitude of the *sypG*- and *rpoN*-dependent biofilm formation (Fig. 6B and D). The parent strain containing multicopy *sypG* exhibited a >30-fold increase relative to the vector control and a > 12-fold increase relative to that achieved under static conditions. This increase in stainable material stemmed from an increase in adherent cells: we observed a 7.8-fold increase of adherent cells when they contained the *sypG* plasmid, relative to the vector control. Among *syp* mutant strains, biofilms of the *sypC*, and to a lesser extent, *sypJ*, mutants were substantially reduced relative to their parent strain. In contrast, mutants defective for *sypD* or *sypN* exhibited biofilm formation similar to that of the parent strain. Finally, the formation of biofilms under these conditions depended on a functional copy of *rpoN*. These data further demonstrate the dependence of *syp* biofilm formation on SypC, SypG and σ^{54}, and provide an *in vitro* function for the *syp* cluster members.

Discussion

Identification of a novel cluster that is required for symbiotic colonization

In this study, we identified four Tn mutants of *V. fischeri* that exhibited severe defects in initiating symbiotic colonization of *E. scolopes*. The Tns in the four strains mapped to four genes within a cluster of genes, termed *syp*, that encodes proteins with putative functions in LPS or capsule biosynthesis. Our investigations of these mutants in culture revealed no defects in traits known to be important for symbiotic colonization, including motility and luminescence. Insertional mutations in five other genes within this locus similarly caused severe initiation defects, supporting the hypothesis that this locus represents a novel gene cluster that functions in symbiotic initiation.

Transcriptional control of the syp *genes*

Two of the *syp* mutants contained insertions in which a promoterless *lacZ* reporter was oriented correctly for *syp*-dependent transcription. Surprisingly, we observed no β-galactosidase activity under any of a variety of media and growth conditions. Through a bioinformatics approach, we

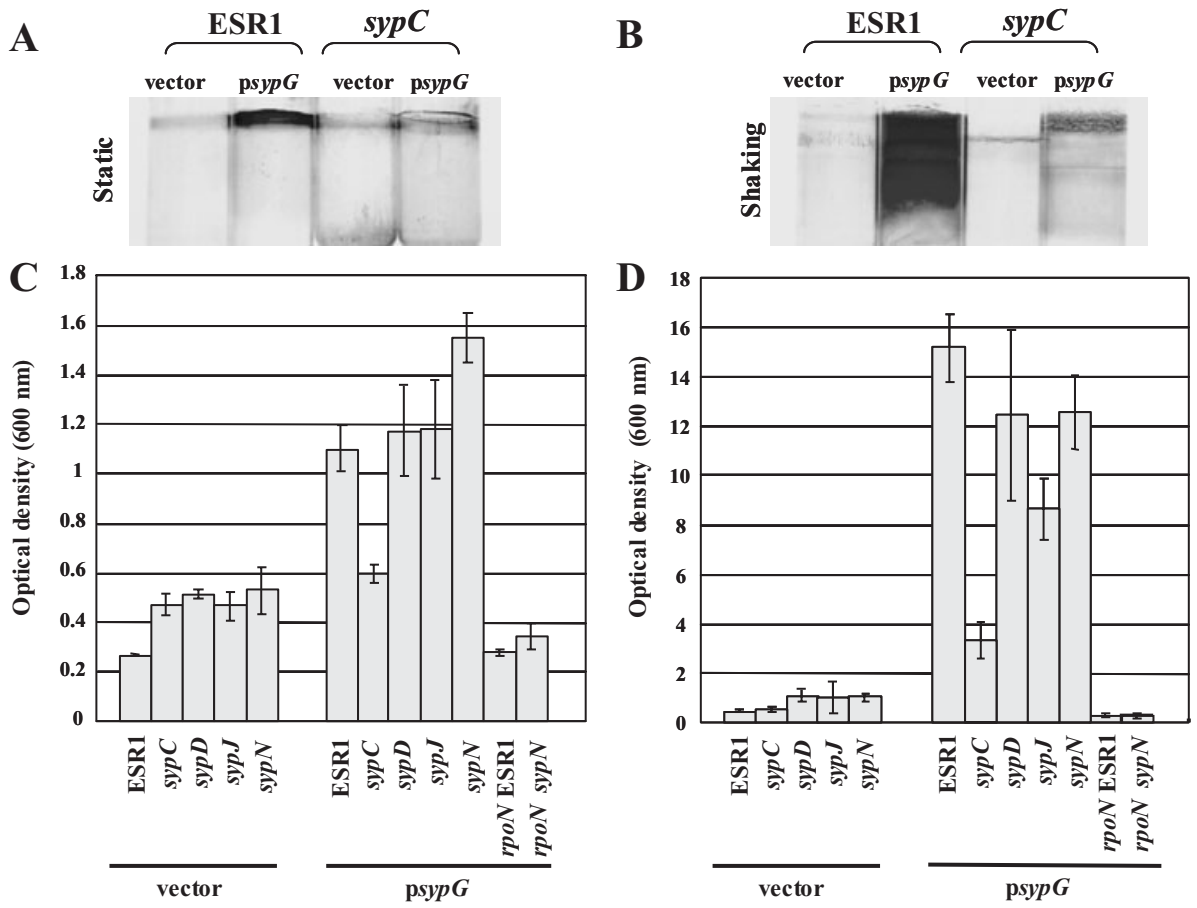

Fig. 6. Biofilm formation by *syp* mutants and their parent. Biofilm formation by parent strain ESR1, the transposon mutants and *rpoN* mutants was determined by growing strains in MM either statically for 30 h (A and C) or with shaking for 24 h (B and D). Biofilms on the surface of the test tubes were visualized by crystal violet staining (A and B), and quantified by measuring the absorbance at 600 nm of ethanol-solubilized crystal violet (C and D). Note that scale of *y*-axis in (D) is different from that of (C).

identified both SypG, a putative σ^{54}-dependent transcriptional activator encoded by the *syp* cluster, and potential binding sites for RNA polymerase carrying σ^{54}. Multicopy expression of *sypG* induced transcription of the two *lacZ* reporters (in *sypD* and *sypN*) by 37- to 70-fold. This induction depended on σ^{54}, as mutations in *rpoN* abolished β-galactosidase activity by these strains. Similarly, multicopy expression of *sypG* in a wild-type strain resulted in *sypA*, *sypI* and *sypM* transcripts that initiated between 12 and 14 based downstream of putative σ^{54} consensus sequences, a common range of initiation sites for such promoters (Barrios *et al.*, 1999). In addition, in preliminary experiments, we have fused the putative *sypP* promoter region to the *lacZ* reporter, introduced it in single copy in the chromosome, and similarly observed a *sypG*-dependent induction of transcription (E.A. Hussa and K.L. Visick, unpubl. data). Together, these data support the existence of multiple operons within *syp* that depend on both SypG and σ^{54}.

Control by σ^{54}-containing RNA polymerase is an effective method for keeping transcription turned off: because the σ^{54}-bound RNA polymerase fails to promote transcription without the aid of a regulatory protein, transcription of target genes is largely off until the appropriate, activated transcription factor binds and interacts with the polymerase. For *syp*, this presumably occurs through the binding of the two-component response regulator, SypG, to its target, perhaps the conserved 22 bp sequence we identified in four intergenic regions. We postulate that a colonization signal transduced by a sensor kinase activates SypG and turns on *syp* transcription to permit symbiotic initiation. A sensitive assay for gene expression in the host, such as recombination-based *in vivo* expression technology (RIVET) (Camilli and Mekalanos, 1995; Angelichio and Camilli, 2002), will be needed to determine whether the *syp* genes are induced early in symbiotic colonization. Consistent with the predicted importance of *sypG* in colonization, our preliminary results suggest that a *sypG* mutant initiates colonization poorly (E.A. Hussa, T. O'Shea and K.L. Visick, unpubl. data).

If SypG functions as a response regulator that is activated during symbiotic colonization, then what is the identity of its cognate sensor kinase? Sensor kinases typically detect a particular environmental signal and respond by autophosphorylating and subsequently serving as a phospho-donor to a response regulator. Just upstream of *sypG* are two additional putative two-component regulator genes: *sypE*, which likely encodes a response regulator, and *sypF*, which encodes a putative sensor kinase. The SypF sensor could potentially function with SypG, or, alternatively, with both SypE and SypG in a complex phosphorelay such as that which controls capsule biosynthesis in *Escherichia coli* (the *rcs* system) (Takeda *et al*., 2001). Another possibility is the sensor kinase, RscS, that we have previously identified as essential for symbiotic initiation (Visick and Skoufos, 2001). The *rscS* gene is not located within the *syp* cluster, and to date, we have not identified its cognate response regulator nor any members of its regulon. Clear identification of the cognate sensor kinase for SypG may require simulation of the host conditions that induce *syp* transcription, or the construction of constitutively active sensor kinase genes.

Role for SypG in promoting biofilm formation

We found that multicopy expression of *sypG* substantially enhanced biofilm formation by *V. fischeri*. Biofilms, dynamic mixtures of cells encased within an extracellular matrix, are being intensively studied in part due to their importance in medicine (Parsek and Singh, 2003; Parsek and Fuqua, 2004). Work in other organisms has shown that the biofilm structures formed by a single organism may vary substantially depending on the exact environmental condition, perhaps due to the environment-specific expression of factors that contribute to the extracellular matrix. For example, experiments in *Pseudomonas aeruginosa* indicate that decreased oxygen availability is a factor in robust biofilm formation (Yoon *et al*., 2002). Our studies in *V. fischeri* revealed that multicopy *sypG* expression enhanced biofilm formation to a greater extent when the cells were grown with shaking, relative to static growth. We thus hypothesize that aeration plays a role in biofilm formation under these conditions. Alternatively, it is possible that another difference between the two conditions, such as fluid distribution, which plays a role in *Bordetella* biofilms (Mishra *et al*., 2005), or the relative amounts of cell growth, accounts for the differences observed. Future work will address these possibilities.

Multicopy *sypG* expression also substantially increased biofilm formation by mutants defective for *sypD*, *sypJ* and *sypN*, but not *sypC*. Multiple explanations are possible. For example, SypG could control a locus, in addition to *syp*, that promotes biofilm formation. Alternatively, SypG-mediated overexpression of *sypC*, which encodes a protein with similarity to those involved in capsule export (KpsD, OtnA and Wza) (Bik *et al*., 1996; Arrecubieta *et al*., 2001; Nesper *et al*., 2003), could result in inappropriate export of a biofilm-promoting factor. It is also possible that the *syp* genes encode redundant functions with respect to biofilm formation, and thus multiple mutations are necessary to counteract the effect of multicopy *sypG*. Regardless of the explanation, these data make it clear that, in contrast to what has been assumed from this and previous studies of biofilm formation by wild-type strains (Wolfe *et al*., 2004), *V. fischeri* is not a poor biofilm former; rather, like pathogenic bacteria, this symbiont can produce substantial biofilms, under the appropriate conditions.

Function of the syp *gene cluster*

Our biofilm results suggest that, under conditions in which the *syp* genes are expressed, *V. fischeri* cells are producing an extracellular matrix that enables them to adhere to a glass surface and, perhaps, to a host tissue. The tentative functional assignments for Syp proteins in capsule, exopolysaccharide or LPS synthesis, macromolecules that contribute to biofilm formation by other bacteria, are consistent with that hypothesis. Our studies to date have yielded no differences in the overall carbohydrate levels produced by multicopy *sypG* or vector containing cells (E.S. Yip and K.L. Visick, unpubl. data). This result is similar to recent studies of *P. aeruginosa psl* (polysaccharide synthesis locus) – a locus that, like *syp*, contains multiple putative glycosyltransferase genes (Frideman and Kolter, 2004; Jackson *et al*., 2004; Matsukawa and Greenberg, 2004): a comparison of matrix carbohydrate levels in a *psl* mutant relative to the wild-type strain revealed similar overall yields (Matsukawa and Greenberg, 2004). Mutations in the *psl* locus result in an altered carbohydrate composition; this could potentially be true for *syp* as well. Recently, we have identified another phenotype consistent with potential exopolysaccharide production: prolonged static growth of *V. fischeri* with multicopy *sypG* results in the formation of a pellicle at the air/liquid interface, a phenotype that we do not observe in cultures of wild-type or vector-control cells (E.S. Yip and K.L. Visick, unpublished). We anticipate that investigation of this pellicle will provide insights into the nature of the product of the *syp* cluster.

Homologues of syp *genes in related* Vibrio *species*

Our analysis of the *syp* gene cluster of *V. fischeri* revealed similar clusters, with the same gene order, in the related human pathogens, *V. parahaemolyticus* and *V. vulnificus*. Neither of these two species is competent to colonize *E. scolopes*, although at least one strain of *V. parahaemolyticus* can adhere to the surface of the squid light organ

(Nyholm *et al*., 2000). We speculate that the *syp* cluster may be used by all of these *Vibrio* species in the early stages of colonization with their hosts, either marine invertebrates, or, potentially, human hosts. If so, then comparative studies of the three loci will aid our understanding of such bacteria–host interactions. Although there is high overall conservation, a number of individual genes exhibit significant sequence divergence; these differences could potentially account for the observed differences in host range. Further study of the role and regulation of the *syp* cluster may enhance our knowledge not only of the communication between *V. fischeri* and its symbiotic host but also of the signals and responses leading to initiation of pathogenic associations.

Experimental procedures

Bacterial strains and media

Vibrio fischeri strains used in this study are listed in Table 2. Strain ES114 (Boettcher and Ruby, 1990) was used as the wild-type strain, and ESR1, a rifampin-resistant (Rif^R) derivative of ES114 (Graf *et al*., 1994), was used as the parent

Table 2. Strains and plasmids used or constructed in this study.

	Genotype or characteristics	Reference
Strains		
E. coli		
DH5α	*endA1 hsdR17* (r_K^- m_K^+) *glnV44 thi-1 recA1 gyrA* (Nal^r) *relA* Δ(*lacIZYA-argF*)*U169 deoR* [φ80 *dlac*Δ(*lacZ*)M15]	Woodcock *et al*. (1989)
CC118λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE* (Am) *recA1* λ*pir*	Herrero *et al*. (1990)
V. fischeri		
ES114	Wild type	Boettcher and Ruby (1990)
ESR1	Rif^R derivative of ES114	Graf *et al*. (1994)
KV1601	ESR1 *sypN*::Tn*10lacZ*	This study
KV1635	ESR1 *sypD*::Tn*10lacZ*	This study
KV1636	ESR1 *sypJ*::Tn*10lacZ*	This study
KV1637	ESR1 *sypC*::Tn*10lacZ*	This study
KV1816	ES114 *sypP*::pESY9	This study
KV1817	ES114 *VFA1038*::pESY10	This study
KV1818	ES114 *VFA1043*::pESY11	This study
KV1837	ES114 *sypL*::pTMB53	This study
KV1838	ES114 *sypN*::pTMB54	This study
KV1839	ES114 *sypO*::pTMB55	This study
KV1840	ES114 *sypQ*::pTMB56	This study
KV1841	ES114 *sypR*::pTMB57	This study
KV2080	ESR1 *rpoN*::pESY17	This study
KV2082	KV1601 *rpoN*::pESY17	This study
KV2083	KV1635 *rpoN*::pESY17	This study
Plasmids	Characteristics or construction	
pCR2.1-TOPO	commercial cloning vector; Amp^R, Kan^R	Invitrogen
pEAH40	pKV69 (BamHI/SphI) + 3.3 kb *sypG*$^+$ fragment; Tet^R	This study
pESY9	pKV194 (EcoRI) + 389 bp internal fragment of *sypP*; Cm^R	This study
pESY10	pKV194 (EcoRI) + 307 bp internal fragment of *VFA1038*; Cm^R	This study
pESY11	pKV194 (EcoRI) + 254 bp internal fragment of *VFA1043*; Cm^R	This study
pESY16	pKV194 (SmaI) + 252 bp XbaI/HindIII fragment from pLD1 (Wolfe *et al*., 2004) containing internal fragment of *rpoN*; Cm^R	This study
pESY17	pESY16 (MluI) + 1.2 kb fragment encoding Em^R from pKV168	This study
pEVS104	Conjugal helper plasmid (*tra trb*); Kan^R	Stabb and Ruby (2002)
pEVS122	R6Kγ *oriV*, *oriT*RP4, Em^R, *lacZα*, *cosN, loxP, incD*	Dunn *et al*. (2005)
pKV69	Mobilizable vector; Tet^R, Cm^R	Visick and Skoufos (2001)
pKV124	Mini-Tn*10lacZ* delivery plasmid	Visick and Skoufos (2001)
pKV168	Vector containing a 1.2 kb fragment encoding Em^R	Visick and Ruby (1998)
pKV189	pCR2.1-TOPO + 1.8 kb fragment of *VFA1019-sypA* intergenic DNA	This study
pKV190	pCR2.1-TOPO + 294 bp fragment of *sypH-sypI* intergenic DNA	This study
pKV191	pCR2.1-TOPO + 398 bp fragment of *sypL-sypM* intergenic DNA	This study
pKV194	1.4 kb BamHI/SacI fragment from pKV124 + 1.6 kb SspI/XmnI fragment from pEVS122; Cm^R, *oriR6K, oriT, lacZ*	This study
pTMB53	pEVS122 (EcoRI) + 386 bp internal fragment of *sypL*; Em^R	This study
pTMB54	pEVS122 (EcoRI) + 406 bp internal fragment of *sypN*; Em^R	This study
pTMB55	pEVS122 (EcoRI) + 375 bp internal fragment of *sypO*; Em^R	This study
pTMB56	pEVS122 (EcoRI) + 364 bp internal fragment of *sypQ*; Em^R	This study
pTMB57	pEVS122 (EcoRI) + 341 bp internal fragment of *sypR*; Em^R	This study

Amp^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Em^R, erythromycin resistance; Kan^R, kanamycin resistance; Tet^R, tetracycline resistance.

strain in Tn mutagenesis and mutant analyses. *E. coli* strains DH5α and CC118*λpir* (Herrero *et al*., 1990) were used as hosts for cloning and conjugation. *V. fischeri* strains were grown on SWT medium (0.5% tryptone, 0.3% yeast extract, 210 mM NaCl, 35 mM $MgSO_4$, 7 mM $CaCl_2$ and 7 mM KCl) for all colonization experiments and Hepes-minimal medium (MM) (Ruby and Nealson, 1977) supplemented with 0.2% glucose and 0.3% Casamino acids for β-galactosidase and biofilm experiments. For motility assays, *V. fischeri* cells were spotted on tryptone-based soft agar medium containing 0.25% Bacto-Agar (Difco, Detroit, MI). Antibiotics were added to media, where appropriate, to the following final concentrations: ampicillin (Amp), 100 μg ml^{-1} for *E. coli*; chloramphenicol (Cm), 25 μg ml^{-1} for *E. coli* and 2.5 μg ml^{-1} for *V. fischeri*; erythromycin (Em), 150 μg ml^{-1} for *E. coli* and 5 μg ml^{-1} for *V. fischeri*; tetracycline (Tet), 15 μg ml^{-1} for *E. coli* and 30 μg ml^{-1} for *V. fischeri*.

Plasmid and mutant constructions

Transposon mutagenesis was performed using the mini-Tn*10lacZ* delivery plasmid pKV124 (Visick and Skoufos, 2001). Cloning of the Tn and flanking DNA was carried out as described previously (Visick and Skoufos, 2001), using the origin of replication and Cm-resistance gene contained within the Tn. All plasmids were constructed using standard molecular biology techniques, with restriction and modifying enzymes from New England Biolabs (Beverly, MA) or Promega (Madison, WI). To construct *syp* and *rpoN* mutants of *V. fischeri*, we first amplified, by polymerase chain reaction (PCR), an internal fragment of specific genes using DNA oligonucleotides (Table S2) from MWG Biotech (High Point, NC). The PCR fragments were then cloned into cloning vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and subcloned into one of two 'suicide' plasmids [pEVS122 (Dunn *et al*., 2005) or pKV194], which do not replicate but integrate into genomic DNA via homologous recombination between the inserted *V. fischeri* sequence and the chromosome. Triparental matings were performed and potential insertional mutants were selected on the appropriate antibiotic-containing plates.

Colonization assays

To screen for Tn mutants defective in colonizing the light organ, *V. fischeri* mutant strains were inoculated into artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH) containing individual newly hatched juvenile squid. After 3 h incubation, juvenile squid were washed and transferred to symbiont-free seawater. Colonization of the light organ was monitored by measuring luminescence over the course of 18 h in a scintillation counter as described previously (Ruby, 1996). To quantify the number of bacteria present in the light organ, juvenile squid were homogenized, serially diluted and plated on SWT agar. The limit of detection is 14 bacterial cells per squid.

Luminescence assays

ESR1 and the Tn mutants were diluted 1:100 from overnight cultures and grown in SWT/IO medium [0.5% tryptone, 0.3% yeast extract, 0.3% glycerol and 43 g l^{-1} Instant Ocean (Aquarium Systems) (Stabb *et al*., 2004)]. Samples were taken for luminescence and optical density measurements over the course of 4 h. A TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) was used to determine the level of bioluminescence.

Protein analysis

To examine the outer membrane protein (OMP) profiles of the mutant strains, cellular protein extracts were obtained as described previously (Aeckersberg *et al*., 2001). Upon extraction, OMP-enriched fractions were separated by electrophoresis on a 8% SDS-polyacrylamide gel and visualized by staining with either Coomassie brilliant blue (Sigma, St Louis, MO) or SYPRO Red protein gel stain (Molecular Probes, Eugene, OR).

Southern blot analysis

We analysed both the original Tn mutants and our constructed insertional mutants by Southern blot experiments. Chromosomal DNA was extracted, digested and separated by a 0.6% agarose gel, transferred onto a nylon membrane (Hybond XL; Amersham-Pharmacia Biotech, Piscataway, NJ) and cross-linked using UV light. Detection of DNA fragments was performed by using the Boehringer Mannheim DIG DNA labelling kit (Roche Molecular Biochemicals, Indianapolis, IN) as previously described (Visick and Skoufos, 2001) using either Tn or vector probes. We found that all Tn mutant strains carried a single Tn insertion. The insertion in *sypC* also contained the delivery vector integrated into the chromosome at the site of the insertion. Similarly, for the ES114-derived *syp* insertional mutants, the resulting banding pattern showed that each contained a single insertion of suicide plasmid vector at the appropriate location in the chromosome.

Bioinformatics

In this work, we examined potential Syp function and searched for *syp* homologues using the sequence-based similarity searching methods, BLAST and RPS-BLAST (Altschul *et al*., 1990; 1997). To search for homologues in *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*, we submitted Syp protein sequences to the TIGR website (http://www.tigr.org/) which uses WU BLAST analysis (W. Gish, 1996–2003; available at: http://blast.wustl.edu). To search for σ^{54}-dependent promoter sequences and a potential enhancer consensus sequence, we used the PROMSCAN program (http://www.promscan.uklinux.net/home.html) and MEME (http://meme.sdsc.edu/meme/website/meme.html) respectively.

Primer extension

Twenty-five millilitres of cultures of strain ES114 containing either pEAH40 or pKV69 were grown in MM for 24 h at 28°C. The cells were lysed in GITCN lysis buffer (Totten and Lory, 1990), and mRNA was isolated by ultracentrifugation through

a cesium chloride gradient. Oligonucleotide primers (VFA1020PER, VFA1028PER and VFA1032PER; see Table S2) complementary to *sypA*, *sypI* and *sypM* sequences were radiolabelled via T4 polynucleotide kinase (USB) and [γ^{32}-P]-ATP (Amersham-Pharmacia Biotech, Piscataway, NJ). The labelled primers were hybridized to 32 μg of mRNA and incubated with Moloney murine leukaemia virus (MMLV) reverse transcriptase (Stratagene, LaJolla, CA) and nucleotides per the manufacturer's instructions. Primer extension products were visualized upon separation on a 6% polyacrylamide gel. To determine the start of transcription, the extension product was compared with a sequencing ladder generated from pKV189 (*sypA*), pKV190 (*sypI*) or pKV191 (*sypM*) using Sequenase Version 2.0 DNA Sequencing Kit (USB) with the same primer used for the primer extension.

β-Galactosidase measurements

To measure the expression of *syp* genes, Tn reporter strains which carried either pKV69 or pEAH40 were grown in MM with shaking for 24 h; β-galactosidase activity was measured as described (Miller, 1972). Total protein concentrations were determined by the method of Lowry *et al*. (1951). All experiments were performed in triplicate.

Biofilm assays

To quantify biofilm formation, we used methods modified from Djordjevic *et al*. (2002). Briefly, all *V. fischeri* strains were grown in triplicate in MM. Overnight cultures were diluted to an optical density at 600 nm (OD_{600}) ~0.1 and then incubated in test tubes with and without shaking for 24 and 30 h respectively. We quantified biofilm by adding 1 ml of 1% crystal violet to each culture. After 30 min of staining, all liquid was removed from the tubes and each was rinsed 10 times with dH_2O. Tubes were then dried by aspiration and 4 ml of 100% ethanol was added to each for destaining. Samples were destained for 1 h with vortexing every 15 min. Each crystal violet-containing sample was diluted, if necessary, and quantified by measuring its absorbance at OD_{600}. In parallel, samples were grown over the same time-course, vortexed and the optical density was determined to assess growth of the strains. Under these experimental conditions, strains containing multicopy *sypG* exhibited a decreased growth yield (and increased biofilm formation) relative to vector controls. Statistical analysis was performed using the Student's *t*-test.

To quantify viable cells within biofilms we inoculated cells as described above to generate biofilms in triplicate. After incubation, we removed the liquid from the culture without staining and rinsed tubes with 70% artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH) 10 times each. We then added 3 ml of 70% artificial seawater to each tube and 1 mm glass beads. After 15 min of incubation with vortexing every 5 min, we plated dilutions of biofilm material and calculated viable cell recovery.

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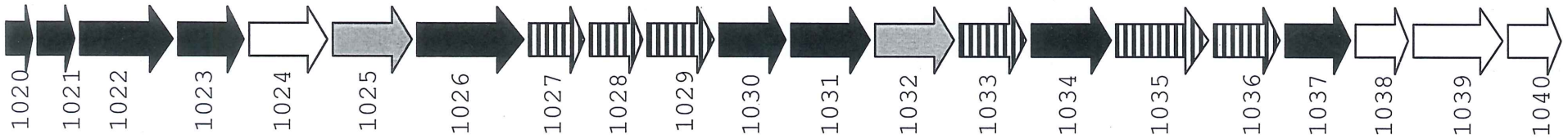
Supplementary material

The following supplementary material is available for this article online:

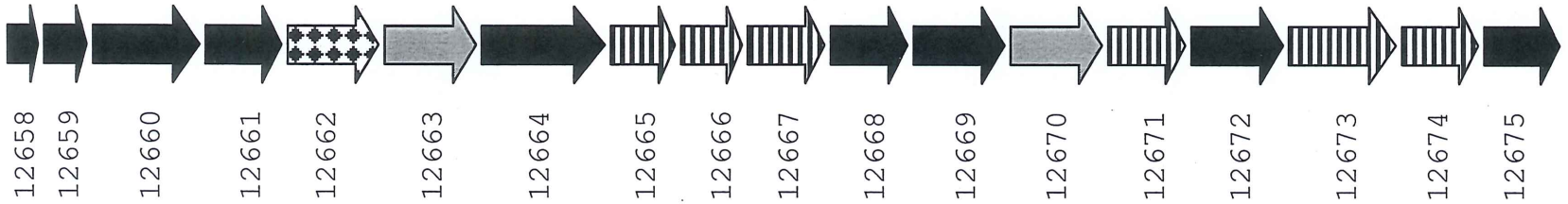
Table S1. Conservation of the *syp* cluster and flanking genes among sequenced *Vibrio* strains.

Table S2. Oligonucleotides used in this study.

V. fischeri ES114 chromosome 2



V. vulnificus CMCP6 chromosome 1



V. parahaemolyticus RIMD 2210633 chromosome 1

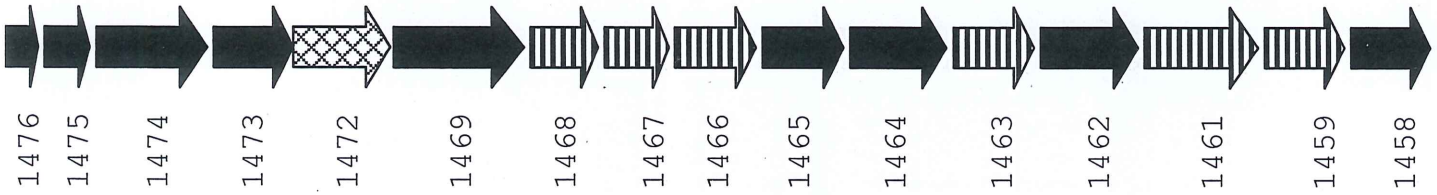


Table S1. Conservation of the *syp* cluster and flanking genes among sequenced *Vibrio* strains.*

syp	*V. fischeri*	*V. parahaemolyticus*	e-value	*V. vulnificus*	e-value	*V. cholerae*	e-value
	VFA1019	VPA1172	1.2e-198	VV13143	1.9e-22	VC2612	0.28
A	VFA1020	**VP1476**	2.3e-30	**VV12658**	2.1e-27	VCA1087	3.6e-06
B	VFA1021	**VP1475**	2.9e-71	**VV12659**	8.7e-70	VC1835	5.7e-11
C	VFA1022	**VP1474**	8.5e-246	**VV12660**	2.0e-262	**VC0936**	1.1e-06
D	VFA1023	**VP1473**	2.3e-39	**VV12661**	4.5e-34	**VC0937**	0.088
E	VFA1024	VPA0148	2.4e-12	VV12140	3.7e-12	VC1719	8.1e-14
F	VFA1025	VPA1130	2.3e-93	**VV12663**	5.0e-154	VC2453	3.4e-64
G	VFA1026	**VP1469**	6.3e-179	**VV12664**	5.4e-180	VC1021	1.7e-126
H	VFA1027	**VP1468**	5.8e-73	**VV12665**	1.1e-64	**VC0925**	8.7e-09
I	VFA1028	**VP1467**	2.5e-79	**VV12666**	1.3e-66	VC0259	2.5e-18
J	VFA1029	**VP1466**	6.3e-60	**VV12667**	5.4e-70	VC0511	0.098
K	VFA1030	**VP1465**	3.1e-67	**VV12668**	3.1e-65	**VC0921**	1.2e-07
L	VFA1031	**VP1464**	1.1e-126	**VV12669**	6.2e-124	VC1244	6.2e-124
M	VFA1032	VP1758	1.2e-15	**VV12670**	1.2e-51	VCA0473	2.0e-13
N	VFA1033	**VP1463**	1.6e-102	**VV12671**	1.4e-91	VC0862	0.7
O	VFA1034	**VP1462**	1.2e-120	**VV12672**	1.4e-117	VC0057	0.0068
P	VFA1035	**VP1461**	2.7e-91	**VV12673**	2.1e-84	**VC0925**	6.1e-13
Q	VFA1036	**VP1459**	1.5e-99	**VV12674**	2.1e-100	VC1052	0.56
R	VFA1037	**VP1458**	8.5e-72	**VV12675**	7.6e-71	**VC0934**	1.9e-26
	VFA1038	VPA0869	1.4e-149	VV20264	1.0e-155	VCA0785	2.7e-143
	VFA1039	VP1314	2.3e-78	VV12779	4.9e-76	VC1619	1.8e-75
	VFA1040	VP1317	7.6e-32	*VV12776*	7.5e-32	VC1616	3.1e-32

*We used *V. fischeri* protein sequences to search the TIGR Web site (http://www.tigr.org/) for homologs in *V. parahaemolyticus* (VP) , *V. vulnificus* (VV) , and *V. cholerae* (VC). Gene numbers shown in bold represent genes that are closely linked to one another in that organism (not in bold-face for *V. fischeri*). Note that *V. cholerae* gene *VC0925* is listed twice. This gene exhibits weak sequence similarity to both *sypH* and *sypP*. For each organism, the letter A in the gene name represents genes found on chromosome 2, while its absence reflects genes found on chromosome 1.

Table S2. Oligonucleotides used in this study.

Primer	Gene	Sequence
VFA1019 intR	*VFA1019*	TTTTTCGTACGTGATGGGAAATGACGTTGTG
VFA1020PER	*sypA*	CCGATGGCGTCCATATCAC
VFA1028PER	*sypI*	GGTATTTCTGATGGTTGATGC
5147 (SacI)	*sypJ*	AAAAAGAGCTCGTCTGCTTATCAAATTTAATTTG
ORF1031F	*sypL*	AAACACGACAATGGGTTACAGC
ORF1031R	*sypL*	TGAAATACTCAATGGCTAAAGGATG
VFA1031-RTF	*sypL*	GTTAATGGGAACCATAGTATCG
VFA1032PER	*sypM*	GGCGAGGAGTTGGCAACTC
ORF1033F	*sypN*	TTACGCCAACCTCGCTTCTC
ORF1033R	*sypN*	GTAACTCTTTACTGGCGGCTAGG
ORF1034F	*sypO*	TGCCTTTTATTGGACTGACTGTTG
ORF1034R	*sypO*	TGTTCAGTAAAATGACGGCTCAC
3642F	*sypP*	GTCCAACACCTTTCACCTG
3642R	*sypP*	GACAGAGAAAGAGCAAATCG
VFA1035PER	*sypP*	CTCCAACATATCTAATACCATAC
ORF1036F	*sypQ*	GCTGGTTTGCTTCTCGTCAC
ORF1036R	*sypQ*	CAGAGCAATGACATCACTTGACTG
ORF1037F	*sypR*	GCGATTAAGTTAGATTCAAAAGGC
ORF1037R	*sypR*	CGGTAATACTCCATAAGTTCTTTCAC
3645F	*VFA1038*	GCCATCGACATTCCTGAAC
3645R	*VFA1038*	GCGGATAACACCCAATGG
3650F	*VFA1043*	GACCTTGCTTGCAGGATTGG
3650R	*VFA1043*	CTCGTCCAGACAGCCCAAC