LETTERS

A single regulatory gene is sufficient to alter bacterial host range

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Microbial symbioses are essential for the normal development and growth of animals[1–3]. Often, symbionts must be acquired from the environment during each generation, and identification of the relevant symbiotic partner against a myriad of unwanted relationships is a formidable task[4]. Although examples of this specificity are well-documented, the genetic mechanisms governing it are poorly characterized[5]. Here we show that the two-component sensor kinase RscS is necessary and sufficient for conferring efficient colonization of *Euprymna scolopes* squid by bioluminescent *Vibrio fischeri* from the North Pacific Ocean. In the squid symbiont *V. fischeri* ES114, RscS controls light-organ colonization by inducing the Syp exopolysaccharide, a mediator of biofilm formation during initial infection. A genome-level comparison revealed that *rscS*, although present in squid symbionts, is absent from the fish symbiont *V. fischeri* MJ11. We found that heterologous expression of RscS in strain MJ11 conferred the ability to colonize *E. scolopes* in a manner comparable to that of natural squid isolates. Furthermore, phylogenetic analyses support an important role for *rscS* in the evolution of the squid symbiosis. Our results demonstrate that a regulatory gene can alter the host range of animal-associated bacteria. We show that, by encoding a regulator and not an effector that interacts directly with the host, a single gene can contribute to the evolution of host specificity by switching 'on' pre-existing capabilities for interaction with animal tissue.

Genomic technologies are facilitating major advances in understanding the relationships between metazoans and their bacterial symbionts. The analysis of unculturable endosymbionts has revealed complex genetic interdependence between host and bacteria amid patterns of genome reduction in endosymbiotic lineages[3]. Similarly, members of the human microbiota are being identified through metagenomic analysis[1,2], and the molecular communication between host and microbe has begun to be interpreted through transcriptional profiling[6]. Despite these advances, the mechanisms by which host–symbiont specificity develops in animal–bacterial interactions are not clear. Many animals, including humans, are born devoid of symbionts and must recruit their microbiota from the environment[7]. The process by which hosts and symbionts find each other to initiate a mutualism must be sensitive enough to identify the correct partner even when the symbiont is a minority constituent of the microbial community, and specific enough to exclude interlopers from gaining access to the host. The basis of species specificity is also poorly understood for pathogenic interactions, as similar congeneric bacteria often have distinct host ranges[8–10].

In this study we used a comparative genomics approach to reveal how bacteria–host specificity is established in the *E. scolopes*–*V. fischeri* mutualism. We took advantage of the fact that *V. fischeri* strain MJ11, which was isolated from the Japanese pinecone fish, *Monocentris japonica*[11], is unable to colonize *E. scolopes* efficiently. As such, comparison of MJ11 with natural squid symbionts provided a valuable system for examining the genomic basis of host specificity in an animal symbiont.

Although the genome sequence of squid-symbiotic *V. fischeri* strain ES114 is known[12,13], we determined here the sequence of the fish symbiont MJ11. Genome assembly of MJ11 was based on the ES114 model using a combination of PCR- and fosmid-based approaches. Genome sequencing also revealed a 179-kilobase (kb) circular plasmid in MJ11 that we term pMJ100, in which 82% of the open reading frames are annotated as hypothetical proteins, and which is distinct from the plasmid carried by ES114. Alignment of the assembled chromosomes revealed two circular MJ11 chromosomes that are co-linear to those in ES114 (Fig. 1a). Over 90% of ES114 open reading frames are shared by MJ11, and the orthologues have a median amino-acid identity of 98.8%. One exception to the high level of conservation was significant divergence observed specifically in the LuxR quorum-sensing system (Supplementary Fig. 1 and Supplementary Discussion).

Examination of ES114 genes for those that could facilitate specific recognition identified *rscS* as a promising candidate because its product acts during symbiotic initiation[14,15], and we discovered it to be absent in the MJ11 genome (Fig. 1b). RscS is a membrane-bound two-component sensor kinase that acts upstream of the response-regulator SypG[16]. SypG, a σ^{54}-dependent transcriptional activator, facilitates transcription of the 18-gene exopolysaccharide locus *sypA-R*[17]. Production of the Syp exopolysaccharide enables *V. fischeri* aggregation in squid-derived mucus during colonization of *E. scolopes*. During growth in culture, *syp* genes are expressed at low levels but can be induced by the plasmid-borne *rscS1* overexpression allele[15,17,18], leading to the production of robust biofilms. Because the MJ11 genome revealed an intact *syp* locus, we asked whether signal transduction downstream of *rscS* was maintained in MJ11 by introducing *rscS1*. As shown in Fig. 1c, d, *rscS1* in MJ11 induced multiple biofilm phenotypes, suggesting that the *syp* locus of MJ11 was functional. We therefore examined whether *rscS* was sufficient to allow MJ11 to colonize *E. scolopes* efficiently.

We tested ES114 and MJ11 for their ability to colonize aposymbiotic *E. scolopes* hatchlings in a 3-h inoculation assay. ES114 colonized successfully, whereas MJ11 failed to initiate colonization, even if present at a tenfold higher inoculum concentration (Fig. 1e). However, when provided with *rscS*$^+$ *in trans*, MJ11 was competent to colonize *E. scolopes* to levels comparable to those seen with the natural symbiont ES114 (Fig. 1e). Furthermore, the luminescence emitted by MJ11/*rscS*$^+$-colonized animals was 100-fold greater than that from animals colonized by ES114. The increased luminescence is consistent with that of the brighter fish symbiont[19], and was not influenced by plasmid carriage (Supplementary Fig. 2). This result argues that the carrying capacity of the juvenile squid light organ is specified by symbiont cell

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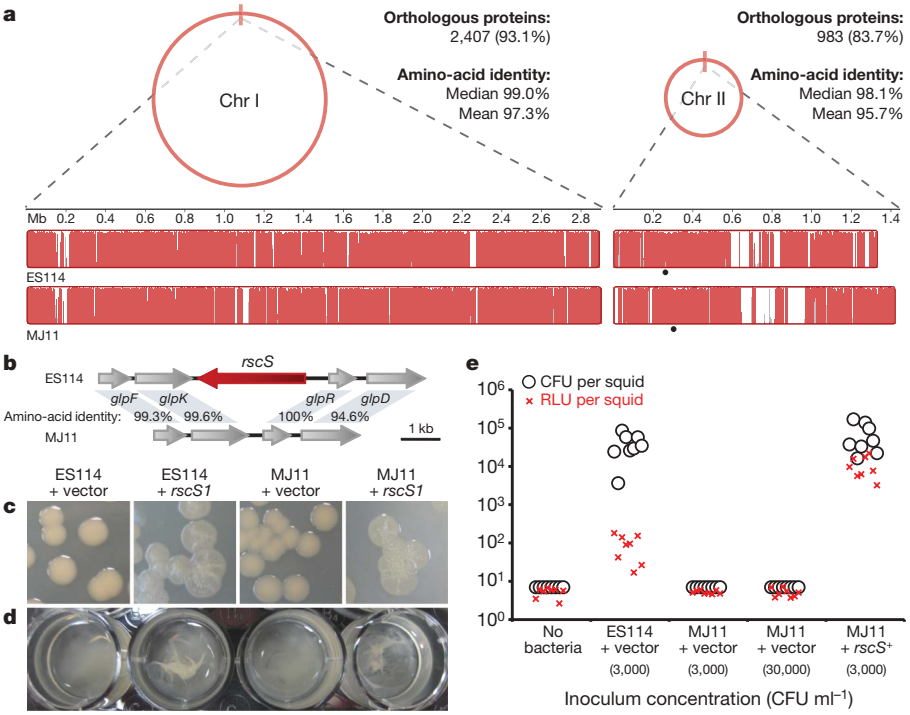

Figure 1 | *rscS* is sufficient to confer efficient colonization of *E. scolopes*. **a**, Mauve output shows each chromosome (chr) as one locally co-linear block. **b**, *rscS* is absent in MJ11 despite a high level of conservation in the surrounding locus. **c**, **d**, RscS-controlled biofilm phenotypes, including colony wrinkling (**c**) and pellicle formation (**d**) of strains harbouring either the vector control (pKV69) or the vector carrying *rscS1* (pKG11). **e**, Squid-colonization assay of strains harbouring either the vector control or the vector carrying $rscS^+$ (pLMS33), after 3 h inoculation and assayed at 48 h post-inoculation. Data points are individual animals. CFU, colony-forming units; RLU, relative light units.

number and not by the amount of luminescence emitted, provided that a minimum threshold of light production is achieved[20,21].

We next asked whether *rscS* was present in a collection of *V. fischeri* squid and fish isolates from the North Pacific Ocean to determine whether the gene's host distribution was consistent with a functional role in nature. All *V. fischeri* in the analysis revealed the presence of three representative *syp* genes (Fig. 2a). In contrast, although all of the squid isolates encoded *rscS*, regardless of geography, only five of the ten fish isolates encoded *rscS*; in four of these five the allele was significantly divergent. PCR amplification of the divergent alleles produced a band that was distinctively larger than the allele in ES114 and the rest of the squid isolates, whereas MJ12 was the only fish isolate that had this smaller squid–symbiont band (Fig. 2a).

We term the allele encoded by the smaller band $rscS_A$, and that encoded by the larger band $rscS_B$. $RscS_A$ was found in all assayed North Pacific squid isolates, and fish isolate MJ12, whereas $RscS_B$ was identified only in four fish isolates. Sequencing revealed that, within each type, the alleles for RscS are highly conserved (amino-acid identity at least 96%), but that divergence between the types was greater (84–86%; Supplementary Fig. 3a). The presence of an identical domain structure in all *V. fischeri* RscS proteins (Supplementary Fig. 3b) led us to ask whether there was detectable functional significance to this level of divergence.

All of the $rscS_A$ strains were competent to colonize *E. scolopes* squid efficiently (Fig. 2b). In contrast, strains lacking *rscS* or encoding the divergent $rscS_B$ were unable to colonize consistently. The defect appeared to be due to RscS function and not to the *syp* locus or other differences; introduction of $rscS^+$ from ES114 (A-type) into $rscS_B$-containing *mjapo*.8.1 conferred 100% colonization efficiency (Fig. 2b). The only fish-symbiotic strain that was competent to colonize *E. scolopes* reproducibly (MJ12) was also the only one with the conserved $rscS_A$ allele. Interruption of *rscS* in MJ12 abolished its ability to colonize *E. scolopes* (Fig. 2b), confirming that $rscS_A$ is both sufficient and necessary to colonize the squid host in these populations.

To understand the evolution of *rscS* and its role in determining specificity in nature, we reconstructed the phylogeny of *V. fischeri* strains (Fig. 3 and Supplementary Fig. 4) using three well-characterized loci. Strains encoding *rscS* formed a monophyletic group within *V. fischeri* that was statistically well supported. Parametric bootstrapping rejected the alternative hypothesis of non-monophyletic origin for the *rscS*-encoding strains, at a significance level of $P < 0.01$.

We propose a model for *rscS* evolution in the symbioses of North Pacific Ocean squids and fish. This model represents a parsimonious synthesis of the colonization and genomics data, within the phylogenetic framework. Specifically, we hypothesize that an acquisition event introduced *rscS* into the *V. fischeri* lineage before the expansion of this species into squid hosts in the North Pacific Ocean (Figs 3 and 4). An initial acquisition, followed by vertical transmission of *rscS* among *V. fischeri*, would predict both a similar guanine–cytosine (GC) content among all *rscS* alleles in the species, and a single conserved genomic location for the gene in all extant *V. fischeri* genomes. We confirmed these predictions, as the *rscS* alleles from *V. fischeri* have similar GC-content (Supplementary Fig. 3a) and are present in the same genomic position (Fig. 2a).

Because the fish isolates that contain *rscS* fall within the same clade as squid isolates (Fig. 3), we argue that the fish- and squid-symbiotic populations in Japan are, indeed, sympatric and that the *rscS*-containing fish isolates are descendents of squid symbionts. We hypothesize that $rscS_A$ diverged significantly in the fish host to generate the $rscS_B$ allele, which is not sufficient to allow these strains to colonize the squid host niche. Further, because $RscS_B$ maintained its reading frame and domain structure—despite significant amino-acid divergence and loss of function for squid colonization—we hypothesize that $RscS_B$ is fish-adapted, and may play a role in activating *syp*, and/or other targets, under fish-specific conditions. The identification of a fish isolate encoding $RscS_A$ provides strong evidence that the $rscS_A$ locus does not preclude successful fish colonization by *V. fischeri*, despite the low frequency of this allele among the fish isolates examined. Unfortunately, *M. japonica* eggs do not fully develop in the laboratory, so we are unable to test this aspect of our model by investigating *V. fischeri* colonization of fish[22].

There are two formal possibilities for how *rscS* first entered the *V. fischeri* lineage. Either a gene duplication/translocation event within *V. fischeri* led to the initial generation of *rscS*, or *rscS* was generated outside *V. fischeri* and then acquired by horizontal gene transfer. We have found no DNA sequences paralogous to either of the *rscS* alleles in the full genomes of ES114 or MJ11. These data are also the most compelling, if indirect, evidence supporting the proposal of horizontal gene transfer. Nonetheless, it is difficult to reconstruct the event that introduced *rscS* to *V. fischeri* based on the usual criteria that define larger genomic islands (for example, direct repeats or insertion elements in flanking DNA, or aberrant codon usage or GC content within

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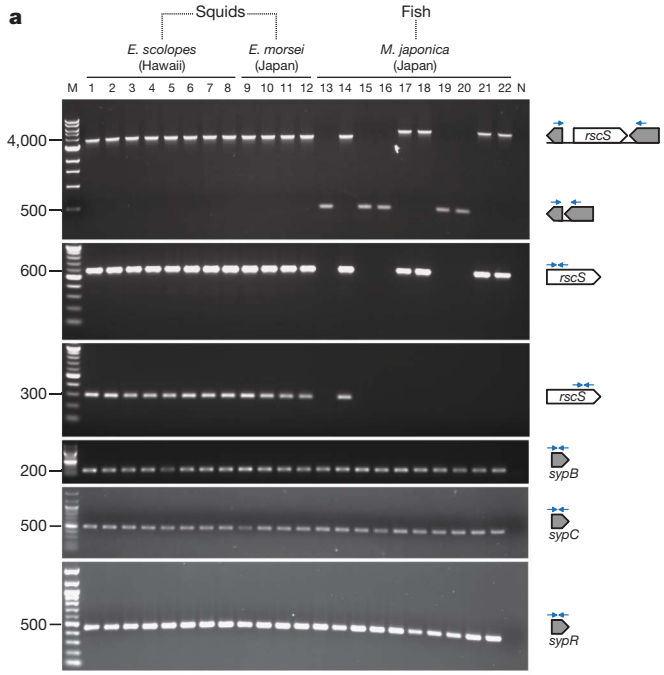

b

Lane	Strain	Host	*rscS* type	Number of *E. scolopes* colonized/attempted
1	ES114	*Es*	A	29/29 0/8 ...[*rscS*$^-$]
2	KB1A97	*Es*	A	16/16 0/8 ...[*rscS*$^-$]
3	KB2B1	*Es*	A	8/8
4	KB5A1	*Es*	A	8/8
5	ES213	*Es*	A	8/8
6	MB11B1	*Es*	A	8/8
7	MB13B1	*Es*	A	8/8
8	MB14A1	*Es*	A	8/8
9	EM17	*Em*	A	8/8
10	EM18	*Em*	A	8/8
11	EM24	*Em*	A	8/8
12	EM30	*Em*	A	8/8
13	MJ11	*Mj*	–	0/21
14	MJ12	*Mj*	A	20/20 0/8 ...[*rscS*$^-$]
15	*mjapo*.2.1	*Mj*	–	1/8
16	*mjapo*.3.1	*Mj*	–	0/8
17	*mjapo*.4.1	*Mj*	B	0/8
18	*mjapo*.5.1	*Mj*	B	0/8
19	*mjapo*.6.1	*Mj*	–	1/8
20	*mjapo*.7.1	*Mj*	–	0/8
21	*mjapo*.8.1	*Mj*	B	0/8 4/4 ...[+*rscS*$_A$]
22	*mjapo*.9.1	*Mj*	B	1/8

Figure 2 | The presence of *rscS* is correlated to a natural association with squid and to the ability to colonize *E. scolopes* experimentally. **a**, PCR assay for the presence of *rscS* and *syp* genes with primer sets (top to bottom) *rscS*-flank, *rscS*-internal1, *rscS*-internal2, *sypB*-internal, *sypC*-internal or *sypR*-internal. M, markers; N, negative control. **b**, Colonization competence of strains in **a**, colonized as described in Fig. 1, at a concentration of approximately 5×10^3 colony-forming units per millilitre. Entries in red indicate *rscS*$^-$ derivatives; entry in blue indicates a strain carrying a plasmid (pLMS33) encoding *rscS*$^+$ from ES114. *Em*, *Euprymna morsei*; *Es*, *E. scolopes*; *Mj*, *M. japonica*.

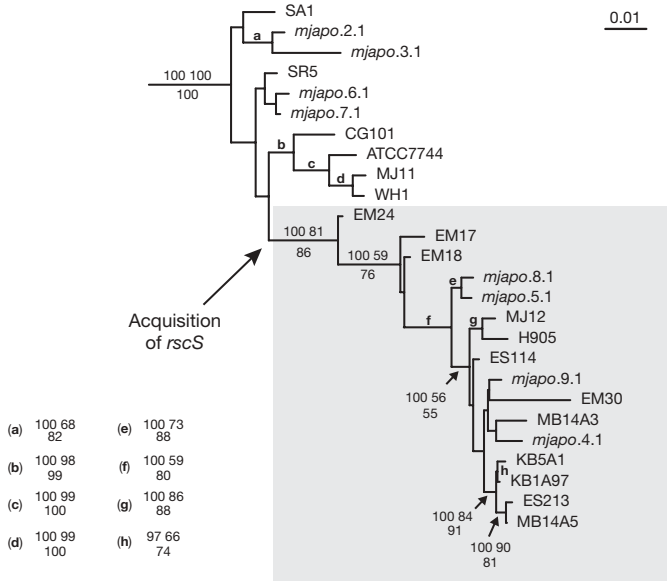

Figure 3 | *rscS* entered the *V. fischeri* lineage before colonization of squid in the North Pacific Ocean. Bayesian phylogeny of 26 *V. fischeri* strains using the concatenation of three loci (*recA*, *mdh*, *katA*). The tree was rooted with *Aeromonas salmonicida* subsp. *salmonicida* A449. Support for branches is indicated in cases where support is greater than 50% for all three: Bayesian posterior probabilities (top left), maximum likelihood bootstrap (top right), and maximum parsimony bootstrap (bottom). *rscS* DNA was detected in all strains in the grey box and in none of the strains outside the box. Bar: 0.01 expected changes per site.

rscS)[23]. Furthermore, the only convincing orthologue of *rscS* outside *V. fischeri* is *V. shiloi* AK1 VSAK1_16757. If a horizontal transfer event were responsible for *rscS* transmission into *V. fischeri*, the *V. shiloi* orthologue is unlikely to be the source: the GC content of ES114 *rscS* is 31.7%, or 6.6% below the ES114 genome average (38.3%). The *V. shiloi* orthologue has a GC content (41.0%) that is even higher than this average, and 9.3% higher than that of the ES114 *rscS* allele.

Attempts to understand the molecular basis of host specificity have been unsuccessful in many pathogen–host animal interactions. *Salmonella enterica* serovar Typhi can infect only humans, whereas serovar Typhimurium has a broad host range that includes mice[9]. Although the conserved regions of the genomes of these two strains are over 97% identical, efforts to account for this differential host specificity have not succeeded. Similarly, different *Brucella* species share over 98% identity across 90% of their genes, yet exhibit strict host specificity; the molecular basis of this specificity remains unclear[10]. In contrast, the study of mutualisms is providing insight into how specificity develops. In plant-associated bacteria, work from many laboratories has established nitrogen-fixing, nodulating rhizobia as the best-understood system for the development and evolution of host specificity[24]. Bacteria secrete Nod factors—lipo-chitooligosaccharide signals—to the plant host, and host-strain-specific backbone modifications encoded by the bacteria lead to relationship specificity. Recently, in an animal–bacterial mutualism, the *nilABC* genes of *Xenorhabdus nematophila* were characterized as sufficient for colonization of *Steinernema carpocapsae* worms by congeneric *Xenorhabdus* bacteria[25].

In contrast to rhizobia and *Xenorhabdus*, in which specificity comes either from the modification of a secreted signal or from structural proteins in the cell envelope, respectively, *rscS*-mediated specificity in *V. fischeri* is novel because the immediate effect of cytoplasmic RscS is on bacterial gene expression, which only subsequently has an effect on the interaction with the host. Because RscS is a signal transduction protein, the evolutionary consequence of the introduction of *rscS* appears to be a re-programming of inherent *V. fischeri* capabilities to expand the host range into squid populations that *V. fischeri* could not previously colonize, or could colonize only inefficiently. It remains a mystery as to why the *syp* genes are conserved in *V. fischeri* strains that are naive to *rscS* (for example, MJ11). That such *syp* clusters are functional, and ancestral to *rscS* in *V. fischeri*, strongly suggests that regulation of *syp* in these strains may be achieved in a manner independent of *rscS*. In support of this, there are *V. fischeri* isolated from the Mediterranean Sea that lack *rscS*, yet have *syp* genes and colonize squid hosts of a different genus through morphological structures that are conserved with those of *E. scolopes*[26].

Our study indicates that a regulatory gene is sufficient to alter host range in an animal–bacterial mutualism. The fundamental biological question of how animal–bacterial partnerships are established has been difficult to access through investigations of pathogenic interactions. In

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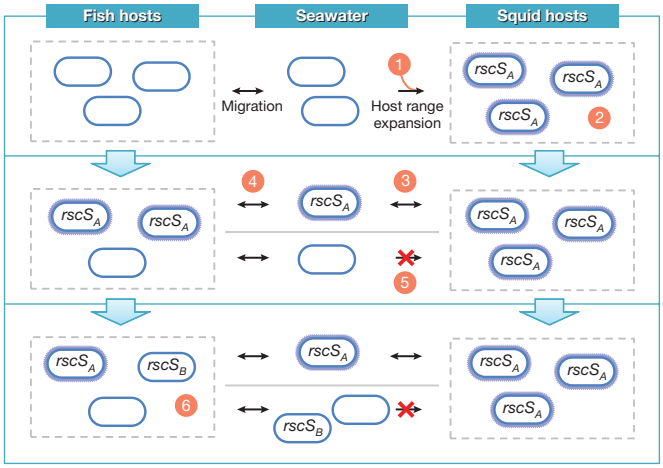

Figure 4 | Reconstruction of the evolution of *V. fischeri* symbioses in the North Pacific Ocean. All strains are genotypically *syp*$^+$; strains that are phenotypically Syp$^+$ in squid are illustrated with the grey mottled outline. The model reconstructs host range expansion into squid by (1) acquisition of *rscS*$_A$, and (2) subsequent developmentally appropriate expression of the ancestral *syp* polysaccharide genes. (3) Gene flow into planktonic strains and (4) into fish symbionts accounts for the presence of *rscS*$_A$ in fish isolates, but (5) *rscS*$^-$ strains maintain a colonization incompatibility for squid. (6) We postulate that *rscS*$_B$ evolved from *rscS*$_A$ in fish.

contrast, mutualism evolves to confer joint benefits to its partners, and relies on a strict specificity for this outcome: that is, entry of only a few appropriate symbionts and exclusion of the many non-specific interlopers. The evolution of developmental mechanisms to winnow the appropriate partner(s) is a hallmark of all horizontally acquired mutualisms. The binary squid–*Vibrio* system thus represents a valuable model in which to interrogate the mechanisms that underlie the development of bacteria–host specificity.

METHODS SUMMARY

The previously deposited draft genome of *V. fischeri* MJ11 was assembled into the final scaffold by comparing contigs to the ES114 assembly using Mauve[27]. Hypotheses were tested by PCR across the contig gaps or by sequencing of fosmids spanning the gap. In a few cases, no PCR product was produced, and the model was refined by rearranging contigs and retesting. In this manner, all of the contigs were arranged relative to ES114, and contig gaps that could be spanned by PCR were sequenced to complete the gap sequence. Three gaps on chromosome I contained tandem (at least two) *rrn* operons; in these cases, the sequence flanking the gap was PCR-amplified through the first ribosomal RNA (rRNA) gene at each end of the *rrn* array so that the completed genome was expected to contain all predicted open reading frames in *V. fischeri* MJ11.

We identified and corrected frameshift and nonsense mutations in the genome model[12], and the final sequences were annotated by the J. Craig Venter Institute (JCVI). To identify ES114–MJ11 orthologues, we performed reciprocal BLASTP searches between the predicted proteomes. Percentage identity was used to score results, and at least 60% coverage of each protein was demanded.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 18 August; accepted 20 November 2008.
Published online 1 February 2009.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank: P. Dunlap for sharing bacterial strains; N. Perna, J. Glasner, K. Geszvain, D. Baum, J. Johnson, M. Sarmiento and S. Ferriera for technical assistance; A. Wier, N. Bekiares, R. Gates and the Hawaii Institute of Marine Biology for animal facilities and care; J. McCosker and the Steinhart Aquarium for access to fish specimens; M. McFall-Ngai, H. Goodrich-Blair, C. Brennan and J. Troll for discussions; and L. Proctor for project support. MJ11 genome sequencing was funded by the Gordon and Betty Moore Foundation Marine Microbial Genome Sequencing Project; E.G.R. and co-workers are funded by the National Institutes of Health–National Center for Research Resources and the National Science Foundation Division of Integrative Organismal Systems; E.V.S. is funded by a National Science Foundation CAREER Award; K.L.V. is funded by the National Institute of General Medical Sciences; M.J.M. is funded by a National Institute of General Medical Sciences National Research Service Award Postdoctoral Fellowship; M.S.W. is funded by a National Science Foundation Predoctoral Fellowship and a National Institutes of Health Molecular Biosciences Training Grant to the University of Wisconsin.

Author Contributions M.J.M. designed the experiments, performed all work not described below, and wrote the paper. M.S.W. conducted the phylogenetic studies. K.L.V. constructed plasmids and strains, and imaged biofilm phenotypes. M.J.M. planned and performed the genome assembly and analytics, and M.J.M., E.V.S. and E.G.R. analysed the bioinformatics results.

Author Information The *recA*, *mdh*, *katA* and *rscS* sequence data from the additional strains described in the article are deposited in GenBank under accession numbers EU907941–EU908017; MJ11 genome data are deposited under accession numbers CP001133, CP001134 and CP001139. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to M.J.M. (mmandel@wisc.edu).

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METHODS

Bacterial growth, strains and plasmids. Standard microbial techniques were used to construct strains and plasmids[28]. Growth of *V. fischeri* was at 20–28 °C with aeration. Media for growth of *V. fischeri* was LBS[29], and for *E. coli* was LB[30] or brain heart infusion (Bacto, Becton Dickinson). Antibiotics used included: chloramphenicol (2.5 μg ml^{-1} for *V. fischeri*, 25 μg ml^{-1} for *E. coli*), erythromycin (5 μg ml^{-1} for *V. fischeri*, 150 μg ml^{-1} for *E. coli*), and tetracycline (5 μg ml^{-1} for *V. fischeri*).

Plasmids pKV69, pLMS33 and pKG11 were introduced into *V. fischeri* by triparental conjugation as described previously[28] with *E. coli* carrying the pEVS104 helper plasmid. Briefly, overnight cultures of the following strains were used for the reaction. One hundred microlitres from each of donor and helper *E. coli* strains were pelleted at 16,000*g* for 2 min in a microfuge tube, and the supernatant was aspirated. One hundred microlitres of recipient *V. fischeri* was added to the same tube, and pelleted as above. After aspiration of the supernatant, the pellet was re-suspended in 10 μl LBS, and the entire 10 μl was spotted onto an LBS agar plate, and incubated at 28 °C overnight. The spot was re-suspended in 500 μl LBS, and 50 μl were plated onto selective media (LBS–chloramphenicol) to select for plasmid transfer.

The *rscS* mutagenesis plasmid pKV188 was constructed by subcloning an approximately 700-base-pair (bp) internal *rscS* fragment from ES114, terminating in the internal *Pst*I site, into the *Kpn*I/*Pst*I sites of pEVS122 (ref. 28). Mutagenesis of *rscS* in the strains noted (Supplementary Table 1) was as described previously[31]; briefly, after triparental conjugation as above, integration of the suicide vector was identified by selection of the entire mating spot on LBS–erythromycin.

V. fischeri strain MJ11 (alias MJ101) was isolated by sterile expression of the light-organ sample from a live *M. japonica* at the Steinhart Aquarium, in February 1991. *M. japonica* symbiont strains denoted '*mjapo.#.#*' were shared by P. Dunlap.

Sources of other strains were as noted in Supplementary Table 1.

MJ11 genome assembly. The *V. fischeri* MJ11 draft genome was sequenced by the JCVI as part of the Gordon and Betty Moore Foundation Marine Microbial Genome Sequencing Project. Cloning and shotgun sequencing was performed at JCVI; draft coverage was obtained at 8.57-fold coverage, and the contigs were previously deposited into GenBank as a whole genome shotgun sequencing project, accession number ABIH00000000, project version 01.

Step 1: sealing contig gaps. *V. fischeri* MJ11 contigs from the above project were aligned to the ES114 genome using Mauve[27] and Projector2 (ref. 32). Alignment of all contigs that were from multiple reads (greater than 2 kb), excluding repetitive rDNA sequences, identified strong matches to the ES114 chromosome I and chromsome II, with the exception of 179-kb contig number 1101159000798. Owing to the high level of conservation between the strains, the corresponding ES114 sequences at all contig gaps were used as estimates of the gap lengths—and as guides for sequencing primers across long gaps—and primers were designed to amplify across each contig-gap, including at least an additional 200 bp of overlap with each adjacent contig, and extending beyond regions of repetitive DNA at either side of the gap boundary. PCR primers were designed with Primer3Plus[33] and sequencing primers were designed at the SGD website (http://seq.yeastgenome.org/cgi-bin/web-primer). In some cases, custom software was used to assist in the identification of probable-unique regions for primer-binding sites within extended repeat regions.

Contig number 1101159000798 was unique in bearing no homology to ES114 or to any extended sequence in GenBank at the start of this project (autumn 2006). We postulated that this represented a large (circular) plasmid in MJ11, and consistent with this hypothesis primers pointing outward from both ends of the contig together amplified a small fragment (less than 1 kb).

Step 2: PCR-walking into tandem rRNA operons. Three of the contig gaps contained tandem rRNA operons, and PCR across the entire gap was unsuccessful. We initiated PCR-walking into each gap by amplifying from one end of the gap to a conserved region that was distal to the 16S–23S spacer on that end of the gap, using primers listed in Supplementary Table 2. Using this approach, we identified the six unique spacer sequences that comprised the terminal rRNA spacers for the three gaps, leaving only additional rRNA (and potentially transfer RNA (tRNA)) sequences remaining to be sealed within each of these three regions. These contigs were still assembled into a scaffold and submitted for deposition so that information about the relative positions of DNA sequences within the molecule (chromosome I) was preserved.

Step 3: re-sequencing of select targets. We previously published methodologies to identify and correct sequencing errors in microbial genomes[12]. We applied this technology to the MJ11 genome and identified 15 high-priority re-sequencing targets. Nine of these sites were in fact in error, and we corrected these errors for inclusion in the final genome release. The PCR/sequencing primers used to target these regions are listed in Supplementary Table 2.

After assembly, the sequence was resubmitted to the JCVI Annotation Service and post-processing at JCVI and the National Center for Biotechnology Information, and deposited into GenBank as listed in Supplementary Table 3.

Orthologue comparisons between ES114 and MJ11. We compared the predicted proteomes from both ES114 chromosomes against both MJ11 chromosomes. Reciprocal exhaustive BLASTP[34] searches were performed with an expect cutoff of 10. Results were filtered to demand that the query length and subject length each be a minimum of 60% of their respective total lengths. Among the remaining results for each query protein, best-hits were scored by percentage amino-acid identity, and additional results were included for analysis if they scored at least 70% of the maximum score for that query. ES114–MJ11 protein pairs included on reciprocal lists were candidate orthologues, and for pairs in which there was a duplicate of query or subject protein, manual assignment of orthology was curated using the parameters of percentage amino-acid identity, percentage of each protein aligned and the local genomic context (synteny) of the two proteins.

Biofilm phenotypes. The two biofilm phenotypes evaluated were colony morphology and pellicle formation. To assay for the ability to form wrinkled colonies, cells were streaked onto LBS–tetracycline agar and the plates were incubated at room temperature for two days. To assay for the ability to form pellicles, cells were inoculated into HEPES minimal medium[35] containing 0.3% casamino acids, 0.2% glucose and tetracycline at a final concentration of 30 μg ml^{-1}. After overnight growth at 28 °C with shaking, cells were diluted to an $OD_{600\,nm}$ of 0.1 in fresh HEPES minimal medium. Three millilitres of cell suspensions were introduced into the wells of a 12-well microtitre dish, and the cells were incubated statically at room temperature for five days. To facilitate visualization and imaging of the pellicles, the media surface was disturbed with a pipette tip, resulting in clumps of cells if a pellicle had formed.

Squid colonization assays. Juvenile *E. scolopes* hatchlings were collected aposymbiotically, and washed in Instant Ocean (Aquarium Systems) that was filter-sterilized through a 22-μm pore-sized Nalgene filter (FSIO: filter-sterilized Instant Ocean). Overnight cultures of bacteria in LBS were subcultured 1:40, grown for 70 min, assayed for $OD_{600\,nm}$, and then inoculated into 40 ml FSIO-squid at a volume equivalent to 1.25 μl per $OD_{600\,nm}$. The inoculum was plated onto LBS plates to confirm that the bacterial concentration was 2×10^3 to 10×10^3 colony-forming units per millilitre. Squid were washed with fresh FSIO at 3 and 24 h post-inoculation. Individual animal luminescence was recorded at 48 h post-inoculation before they were euthanized at 48 h by freezing at −80 °C. Symbiont colony-forming units per squid were determined by homogenizing thawed animals, and plating the homogenates onto LBS. For experiments involving pKV69-series plasmids, squid hatchlings were maintained in FSIO containing chloramphenicol at a final concentration of 2.5 μg ml^{-1}.

Luminescence of animals is reported as RLU (1 RLU $\approx 1.98 \times 10^4$ quanta per second) $= 24 \times lum$, where *lum* is the recorded luminescence of a single animal in a TD20/20 luminometer, with recordings performed in 4 ml FSIO in glass scintillation vials at 51.9% sensitivity with integration for 6 s. Animals with RLU > 25 were scored as colonized. Because the strains that failed to colonize *E. scolopes* were significantly brighter than ES114, this metric served as a conservative measure of colonization competency for the set of isolates examined in this study.

PCR amplification for MJ11 genome closure. PCR amplification was conducted using Platinum Taq DNA Polymerase High-Fidelity (Invitrogen). Fifty-microlitre reactions contained: 50 ng MJ11 genomic DNA, 1× reaction buffer, 0.2 mM of each dNTP, 2 mM $MgSO_4$, 0.25 μM of each primer, and 1 U DNA polymerase. At least three independent PCR reactions were combined for sequencing to minimize the effect of PCR error. Thermal cycling was conducted in a PTC-200 thermal cycler (MJ Research): 95 °C for 2 min; then 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 68 °C for 30 s to 1 min per kilobase amplified; then 68 °C for 5 min.

Products greater than 5 kb were amplified with Platinum Pfx DNA Polymerase (Invitrogen) before sequencing. Fifty-microlitre reactions contained: 50 ng MJ11 genomic DNA, 2× reaction buffer, 0.3 mM of each dNTP, 1 mM $MgSO_4$, 0.30 μM of each primer, and 1 U DNA polymerase. Thermal cycling was conducted as above.

Primers for MJ11 genome closure are listed in Supplementary Table 2.

Diagnostic PCR amplification of rscS and syp genes. Conditions were as described above (MJ11 genome closure) with the following alterations. Template preparation consisted of bacterial strains grown overnight in LBS, diluted 1:100 in dH_2O, and then used as template in the PCR reactions at a dilution of 1:10. Annealing temperature for the *sypR*-internal primer set was 50 °C. Primers for diagnostic amplification of *rscS* and *syp* locus genes are listed in Supplementary Table 2.

PCR amplification for phylogenetic analyses. PCR amplification was conducted using GoTaq (Promega). Bacterial strains were grown overnight in

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LBS, diluted 1:100 in dH_2O, and then used as template in the PCR reactions at a dilution of 1:10. Twenty-five-microlitre reactions contained: template preparation (2.5 μl), 1 × colourless reaction buffer, 0.2 mM of each dNTP, 0.9 μM of each primer and 1 U DNA polymerase. Thermal cycling was conducted in a PTC-200 thermal cycler (MJ Research): 95 °C for 3 min; then 26 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; then 72 °C for 10 min. Primers for phylogenetic analyses are listed in Supplementary Table 2.

DNA sequencing. Sanger-type sequencing of PCR products for MJ11 genome assembly, and for phylogenetic analyses, was performed at the University of Washington High-Throughput Genomics Unit (Seattle, Washington) and the University of Wisconsin Biotechnology Center DNA Sequencing Facility (Madison, Wisconsin), with the primers listed in Supplementary Table 2.

Sequence data for *rscS* from *V. fischeri* ES114 and from *V. shiloi* AK1 are from GenBank accession numbers AF319618 and EDL55668, respectively. Sequence data for phylogenetic analysis of the following strains are as noted: *A. salmonicida* subsp. *salmonicida* (GenBank accession number CP000644), *Vibrio harveyi* BB120 (CP000789, CP000790), *V. parahaemolyticus* RIMD2210633 (BA000031, BA000032), *Photobacterium profundum* 3TCK (AAPH00000000) and *V. fischeri* ES114 (CP000020, CP000021).

Phylogenetic analyses. Sequences from the three loci (*recA*, *mdh*, *katA*) were aligned using ClustalX 1.83 (ref. 36), and trimmed and concatenated using custom Perl scripts (https://mywebspace.wisc.edu/wollenberg/web/science/scripts/scripts.html) and MEGA4 (ref. 37). The best-fit model of DNA substitution and parameter estimates used for tree reconstruction was chosen by performing hierarchical likelihood ratio tests on these data, as implemented in PAUP* 4.0b10 (ref. 38) and MODELTEST 3.7 (ref. 39); this model was SYM + I + G, a submodel under the GTR + I + G (general time reversible with gamma-rate distribution across sites and a proportion of invariant sites). Phylogenetic trees' clade topology and confidence were studied using three approaches. (1) MrBayes 3.1 (ref. 40), implementing the Markov chain Monte Carlo method with an evolutionary model set to GTR with gamma-distributed rate variation across sites and a proportion of invariable sites, was run for 5,000,000 generations using the CIPRES project portal (http://www.phylo.org/). Sample frequency was 1,000, creating a posterior probability distribution of 5,000 trees; when summarizing the substitution model parameters and trees, 1,250 trees were discarded as burn-in to address potential chain instability. (2) Maximum likelihood analyses were performed using the genetic algorithm approach of GARLI[41] as implemented in the CIPRES portal, with an evolutionary model set to GTR with gamma-distributed rate variation across sites, and a proportion of invariable sites. Bootstrap analysis of 1,000 replications was used to assess the support for internal nodes. (3) Unweighted maximum parsimony analysis and bootstrap were performed by PAUP* (1,000 replications) using heuristic searches implementing tree bisection and reconnection branch-swapping to find the shortest trees and assess the support for internal nodes. For maximum likelihood and Bayesian approaches, the process was independently repeated three times to ensure arrival at a similar, most-likely tree topology. Resulting trees were rooted with *A. salmonicida* subsp. *salmonicida* A449 as the outgroup (Supplementary Fig. 4).

Parametric bootstrap analysis. From our original data, the difference in likelihood scores between an unconstrained phylogeny and a constrained phylogeny with a non-monophyletic, *rscS*-containing clade was calculated. One hundred simulations of the data set were created using the constrained topology; likelihood scores were produced from these 100 simulated data sets both with and without the constraint of the non-monophyly of the *rscS*-containing clade using the software PAUP*. Our null hypothesis of the significance of the constraint of non-monophyly of the *rscS*-containing clade within our initial phylogeny was rejected based on analysis of the resulting likelihood ratio distribution ($P < 0.01$).

Codon usage. Whole genome codon usage for ES114 was analysed by the methods of Karlin and Mrázek[42], against a set of reference sequences from ES114, which included ribosomal proteins, chaperones and transcription/translation factors. Calculations were performed on the Computational Microbiology Laboratory server (http://www.cmbl.uga.edu/software/phxpa.html), and the codon usage of *rscS* was predicted to be neither highly expressed (PHX) nor alien (PA). All comparisons of codon bias performed placed *rscS* in the 95% confidence interval for the ES114 genome.

Bioinformatic notes and software used. In addition to the software noted above, Lasergene Seqbuilder and Seqman (DNASTAR) were used for sequencing and genome assembly. Mauve[27] was used extensively during the dynamic process of contig assembly and orientation. Analysis of RscS domain structure was assisted by PFAM[43] and Phobius[44]. Primer design was aided by Primer3Plus[33]. TreeView 1.6.6 (ref. 45) was used to view phylogenetic trees.

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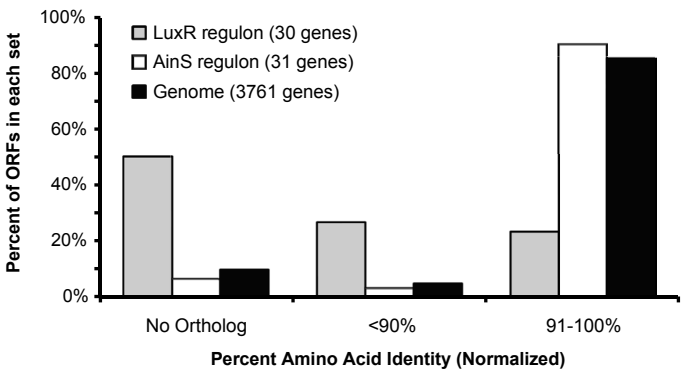
SUPPLEMENTARY INFORMATION

a

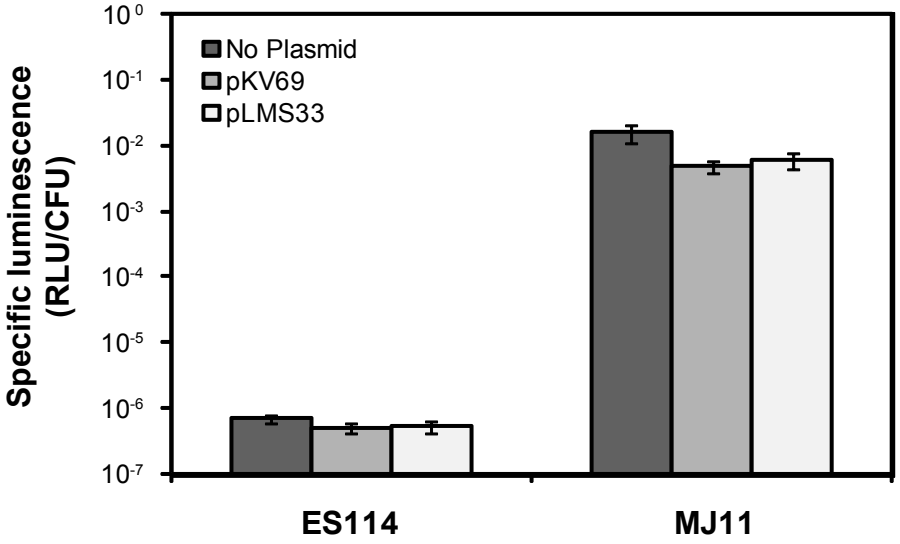
Regulator	QS Pathway	Amino Acid Identity
LuxU	Core	99.2 %
LuxO	Core	99.8
LitR	Core	99.5
CsrA	Core	100.0
Hfq	Core	100.0
Qrr1	Core	100.0 [1]
LuxS	AI-2	99.4
LuxP	AI-2	98.1
LuxQ	AI-2	98.2
AinS	AinSR	83.1
AinR	AinSR	91.7
LuxI	LuxIR	88.8
LuxR	LuxIR	75.0

[1] Percent RNA identity

b



Supplementary Figure 1. The LuxR regulon specifically has diverged between MJ11 and ES114. (a) Conservation of quorum sensing circuitry between the two strains, in the core pathway and in the 3 specific pathways. Qrr1 is a small regulatory RNA (noncoding). (b) The relative fraction of ORFs in three groupings of ES114 genes—all genes, genes regulated by AinS, or genes regulated by LuxR—and their relative level of conservation in MJ11.

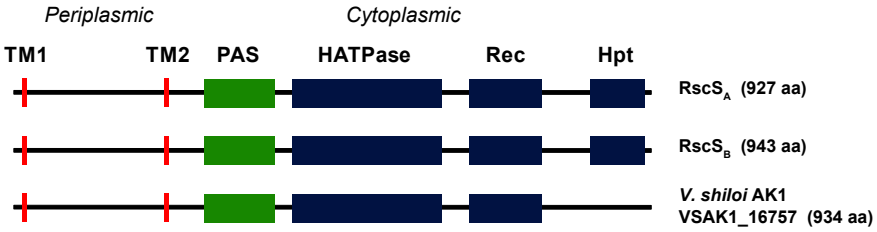


Supplementary Figure 2. Carriage of *rscS*⁺ on a plasmid does not affect luminescence in the strains under study. Strains ES114 and MJ11, and their derivatives carrying the vector control (pKV69) or the vector containing *rscS*⁺ (pLMS33) were examined in culture for viable counts and luminescence. Error bars display the standard error. Wild-type strains were examined from LBS plates, whereas plasmid-carrying strains were examined from LBS-chloramphenicol plates (n=4 for each). Colonies of each strain were resuspended into 1 ml of sterile, 70% Instant Ocean, vortexed, and the relative luminescence (RLU) and viable counts (CFU) of each sample were determined. All plasmid-carrying strains had slightly-diminished luminescence under these conditions, but the presence of *rscS* on the plasmid did not affect specific luminescence.

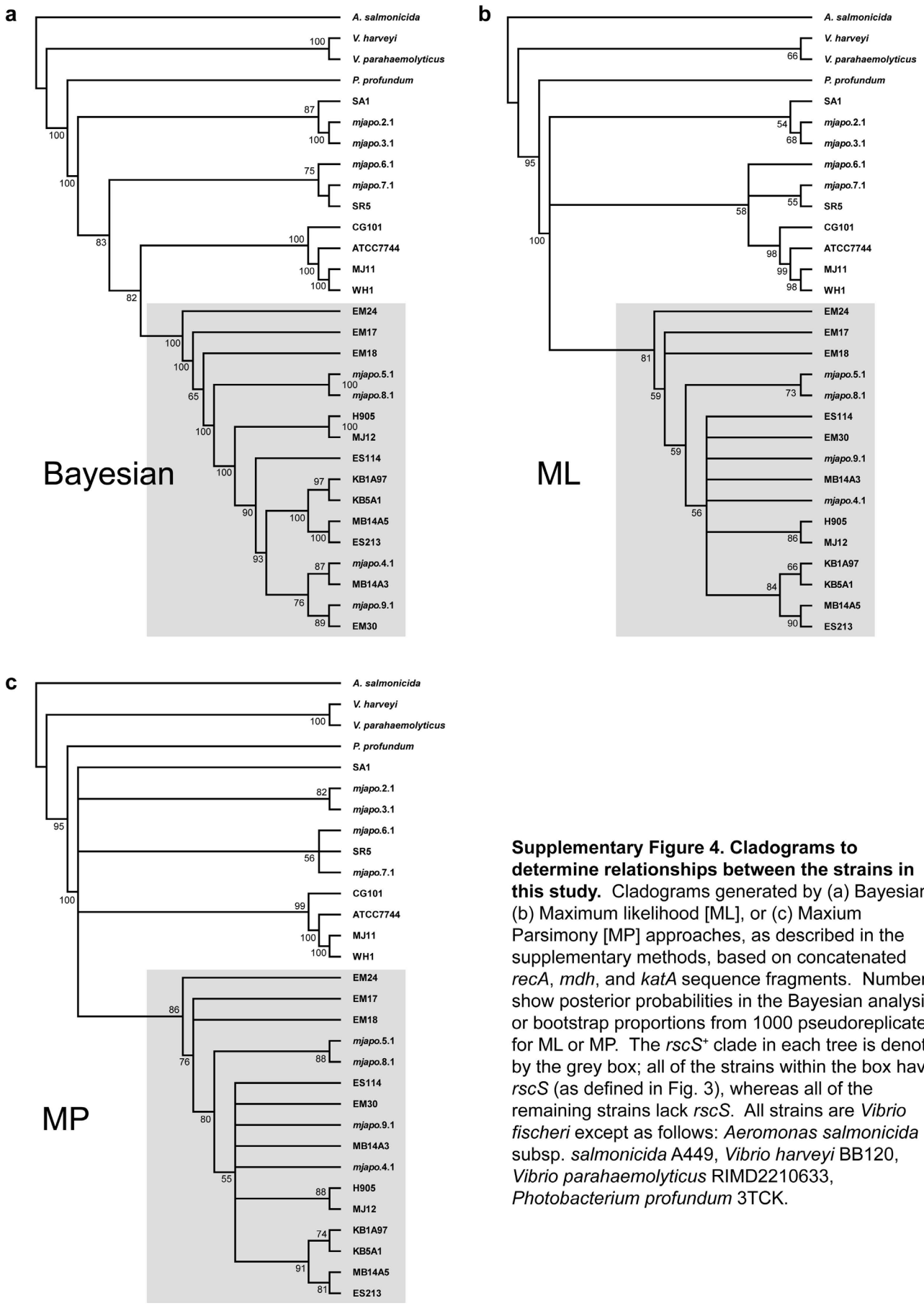
a

--	1	7	9	14	17	18	21	22	LANE	STRAIN/LOCUS (*rscS* TYPE)	GC%
-	38	38	38	38	38	38	38	38	--	VSAK1_16757	41.0 (genome 44%)
	-	96	96	99	86	86	86	86	1	ES114 (A)	31.7 (genome 38%)
		-	98	96	84	84	85	84	7	MB13B1 (A)	31.8
			-	96	84	84	85	84	9	EM17 (A)	31.9
				-	86	86	86	86	14	MJ12 (A)	31.7
					-	97	98	99	17	*mjapo*.4.1 (B)	32.0
						-	98	97	18	*mjapo*.5.1 (B)	31.7
							-	99	21	*mjapo*.8.1 (B)	31.8
								-	22	*mjapo*.9.1 (B)	31.9

b



Supplementary Figure 3. RscS conservation across homologs. (a) Distance matrix of the amino acid identity between RscS proteins encoded by the strains listed (the lane in Fig. 2 that corresponds to each strain is also listed) . VSAK1_16757 is the *rscS* ortholog from *V. shiloi* AK1. The GC-content for the corresponding *rscS* genes (and genome, where known) is listed. (b) Domain conservation of RscS proteins. TM, transmembrane region; PAS, PAS domain; HATPase, histidine kinase/ATPase domain; Rec, receiver domain; Hpt, histidine phosphotransferase domain.



Supplementary Figure 4. Cladograms to determine relationships between the strains in this study. Cladograms generated by (a) Bayesian, (b) Maximum likelihood [ML], or (c) Maxium Parsimony [MP] approaches, as described in the supplementary methods, based on concatenated *recA*, *mdh*, and *katA* sequence fragments. Numbers show posterior probabilities in the Bayesian analysis, or bootstrap proportions from 1000 pseudoreplicates for ML or MP. The *rscS*$^{+}$ clade in each tree is denoted by the grey box; all of the strains within the box have *rscS* (as defined in Fig. 3), whereas all of the remaining strains lack *rscS*. All strains are *Vibrio fischeri* except as follows: *Aeromonas salmonicida* subsp. *salmonicida* A449, *Vibrio harveyi* BB120, *Vibrio parahaemolyticus* RIMD2210633, *Photobacterium profundum* 3TCK.

Supplementary Table 1. Strains and plasmids used in this study.

Strain Name	Isolate No.	Genotype	Parent	Reference/Source
Strains: Natural isolates				
ES114	MJM1100, KV2291	*Euprymna scolopes* light-organ isolate		50
KB1A97	MJM1127	*E. scolopes* light-organ isolate		51
KB2B1	MJM1128	*E. scolopes* light-organ isolate		51
KB5A1	MJM1129	*E. scolopes* light-organ isolate		51
ES213	MJM1117	*E. scolopes* light-organ isolate		52
MB11B1	MJM1130	*E. scolopes* light-organ isolate		51
MB13B1	MJM1131	*E. scolopes* light-organ isolate		51
MB14A1	MJM1132	*E. scolopes* light-organ isolate		51
EM17	MJM1136	*Euprymna morsei* light-organ isolate		53, 54
EM18	MJM1119	*E. morsei* light-organ isolate		53, 55
EM24	MJM1120	*E. morsei* light-organ isolate		53, 54
EM30	MJM1121	*E. morsei* light-organ isolate		53
MJ11	MJM1059	*Monocentris japonica* light-organ isolate		53, 55
MJ12	MJM1114	*M. japonica* light-organ isolate		53
mjapo .2.1	MJM1137	*M. japonica* light-organ isolate		P.V. Dunlap
mjapo .3.1	MJM1139	*M. japonica* light-organ isolate		P.V. Dunlap
mjapo .4.1	MJM1141	*M. japonica* light-organ isolate		P.V. Dunlap
mjapo .5.1	MJM1143	*M. japonica* light-organ isolate		P.V. Dunlap
mjapo .6.1	MJM1147	*M. japonica* light-organ isolate		P.V. Dunlap
mjapo .7.1	MJM1149	*M. japonica* light-organ isolate		56
mjapo .8.1	MJM1151	*M. japonica* light-organ isolate		P.V. Dunlap
mjapo .9.1	MJM1153	*M. japonica* light-organ isolate		P.V. Dunlap
H905	MJM1124	Seawater isolate, Oahu		57
WH1	MJM1122	Seawater isolate, Woods Hole		58
ATCC7744	MJM1224	*Vibrio fischeri* type strain		Ruby Lab
CG101	MJM1115	*Cleidopus gloriamaris* light-organ isolate		55
SA1	MJM1126	*Sepiola affinis* light-organ isolate		59
SR5	MJM1125	*Sepiola robusta* light-organ isolate		59
Strains: Constructed				
KV4251	MJM1201	ES114 *rscS* ::pKV188	KV2291	This study
KV4252	MJM1205	KB1A(97) *rscS* ::pKV188	MJM1127	This study
KV4253	MJM1206	MB11B(1) *rscS* ::pKV188	MJM1130	This study
MJM1104	MJM1104	ES114/pKV69	MJM1100	This study
MJM1105	MJM1105	ES114/pLMS33	MJM1100	This study
MJM1106	MJM1106	ES114/pKG11	MJM1100	This study
MJM1109	MJM1109	MJ11/pKV69	MJM1059	This study
MJM1110	MJM1110	MJ11/pLMS33	MJM1059	This study
MJM1111	MJM1111	MJ11/pKG11	MJM1059	This study
MJM1219	MJM1219	*mjapo* .8.1/pKV69	MJM1151	This study
MJM1220	MJM1220	*mjapo* .8.1/pLMS33	MJM1151	This study
Plasmids				
pKV69		Mobilizable vector (Tet^R, Cam^R)		14
pLMS33		pKV69 *rscS*$^+$ (Tet^R, Cam^R)		14
pKG11		pLMS33 with silent mutations that lead to overproduction of RscS (Tet^R, Cam^R)		15
pKV188		pEVS122 KpnI-*'rscS'*-PstI (Erm^R)		This study
pEVS104		Conjugation helper plasmid (Kan^R)		28

Supplementary Table 2. DNA oligonucleotides used in this study. The reference for all primers is this study.

Primer Name	Primer DNA Sequence (5'-3')	Notes (Seq = for DNA Sequencing)
MJ11 GENOME CLOSURE		
…STEP 1		
MJ11 Gap 1 [Chromosome II; spans contigs 1101159000791 - 1101159000794]		
808rc-F	tatcgcaatggataatgataaagc	PCR/Seq
808rc-R	aagatccaatgcgtgtgattc	PCR/Seq
1_F1	gttcccttccgttacctcaac	Seq
1_R1	gcgaaagaagcagcagcggct	Seq
MJ11 Gap 2 [Chromosome II; spans contigs 1101159000794 - 1101159000793]		
808rc-1F	aattcagaagctttttcattttcg	PCR/Seq
808rc-1R	gtgatcatcttggtgatacggtta	PCR/Seq
2_F1	cctagaagggctattaaccct	Seq
MJ11 Gap 3 [Chromosome II; spans contigs 1101159000793 - 1101159000792]		
808rc-2F	tgccatcgagattgaaagtattta	PCR/Seq
808rc-2R	gtaccactttaaatggcgaacag	PCR/Seq
3_F1	gcggaaacgacttaactg	Seq
3_F2	tcagccacactggaactg	Seq
3_F3	ggaattactgggcgtaaa	Seq
3_F4	acgccgtaaacgatgtct	Seq
3_F5	ctgtcgtcagctcgtgtt	Seq
3_F6	attggagtctgcaactcg	Seq
3_F7	ggtttatgagtgttcaca	Seq
3_F9	gtaaaaaccatttgagtc	Seq
3_F10	gacatctcatcatcagtg	Seq
3_F11	tggcaaggttaaccgttt	Seq
3_F12	tactgggggtagagcact	Seq
3_F13	cctgcgcggaagatgtaa	Seq
3_F14	ccgaaaggcgtagtcgat	Seq
3_F15	gtaccccaaaccgacaca	Seq
3_F16	aattgatggggttagact	Seq
3_F17	accctacatgtgtaggat	Seq
3_F18	gagaatgacaattcgagc	Seq
3_R2	acctttatccgttgagc	Seq
3_R3	agcatacaagggtggtat	Seq
3_R4	tttcgctaccttaggacc	Seq
3_R5	acggtaccattttgccta	Seq
3_R6	tccccccatcgcaattgt	Seq
3_R7	ctacccaatacagtaaac	Seq
3_R8	cggatagtacttactggt	Seq
3_R9	atcacctggttcgggtct	Seq
3_R10	agtatttagccttggagg	Seq
3_R11	cactacgctatgtattca	Seq
3_R14	aaggcccgggaacgtatt	Seq
3_R15	caccattacgtgctggca	Seq
3_R16	cccaggcggtctacttaa	Seq
3_R17	agtttcaaatgcggttcc	Seq
3_R18	catcaggctttcgcccat	Seq
3_R19	cacactaaggcatattcc	Seq
MJ11 Gap 4 [Chromosome II; spans contigs 1101159000792 - 1101159000791]		
808rc-3F	caacaggaataacttgatcagcac	PCR/Seq
808rc-3R	gaagtgattcataacggtggtaaa	PCR/Seq
4_F1	ctgttgttctgctctatttgt	Seq
4_R1	tttaactggtcgtgttcgtga	Seq
MJ11 Gap 5 [Chromosome I; spans contigs 1101159000788 - 1101159000757]		
807rc-F	ctgcaacagatggtccaatg	PCR
803-R	gcgatgcattatacagatcacact	PCR

Supplementary Table 2. DNA oligonucleotides used in this study. The reference for all primers is this study.

Primer Name	Primer DNA Sequence (5'-3')	Notes (Seq = for DNA Sequencing)
no new sequence required, aligned from sequence present in trace archive		
MJ11 Gap 8 [Chromosome I; spans contigs 1101159000787 - 1101159000731]		
806-F	tattaacgattgtagaaggcacga	PCR/Seq
800-R	cagatatgacaaaagccttgaatg	PCR/Seq
8_F1	cttacgagcgtctttcttaga	Seq
8_R1	attctgagtcaaaattgcact	Seq
MJ11 Gap 10 [Chromosome I; spans contigs 1101159000773 - 1101159000796]		
801-F	tctgtacgacgataaatcacacct	PCR/Seq
810rc-R	tattaaaagttcttggccgtggta	PCR/Seq
10_F1	aatcgtttgatgattaattag	Seq
10_R1	aacaagcagcagaagaagcga	Seq
MJ11 Gap 11 [Chromosome I; spans contigs 1101159000796 - 1101159000797]		
v1-E3	cagctaaatcacgtactgcttgtt	PCR/Seq
v1-F3	attcggccatataaactggataaa	PCR/Seq
11_F1	aagtcctgcatagcttgg	Seq
11_F2	ttcattgtattatctccg	Seq
11_F3	atggtattgaaggtttag	Seq
11_F4	taccaagatattgaactc	Seq
11_R1	gcgagtatttaccttaaa	Seq
11_R2	cttggtatttgatactac	Seq
11_R3	gcttctattacgttatag	Seq
MJ11 Gap 12 [Chromosome I; spans contigs 1101159000797 - 1101159000785]		
v1-G3	ccaaacaacctttctaacttccat	PCR/Seq
v1-H3	gagcttgcagaagcactagattc	PCR/Seq
12_F1	gctgtgctgtgtattttc	Seq
12_F2	cggtaacctttgaagaaa	Seq
12_R1	agaaccagagcgtgctgt	Seq
12_R2	cccacacactaaatttga	Seq
12_R3	ctagtaaaaagggcatca	Seq
MJ11 Gap 16 [Chromosome I; spans contigs 1101159000789 - 1101159000788]		
807rc-3F	aagtaaaaagggacgaaagtgaga	PCR/Seq
807rc-3R	acactcgataaccatacgctacaa	PCR/Seq
16_F1	gaacctgaagttgaagcagca	Seq
16_R1	aaaattgtaaggtaacttatg	Seq
MJ11 Gap 17 [Chromosome I; spans contigs 1101159000757 - 1101159000781]		
803-1F	tagtgcggaagtagcggagta	PCR/Seq
803-1R	cgagctgtagcgttggtacttat	PCR/Seq
17_F1	actgcggaagtagttcagcac	Seq
17_R1	cagggctttagaattaactta	Seq
MJ11 Gap 18 [Chromosome I; spans contigs 1101159000781 - 1101159000774]		
803-2F	ttcagacatacagtctctctttcttt	PCR/Seq
803-2R	gaggctgtttgagttggtactttt	PCR/Seq
18_F1	ttcaataagaggcccacactt	Seq
18_R1	aggaactaaaatgcgtaatgc	Seq
MJ11 Gap 21 [Chromosome I; spans contigs 1101159000740 - 1101159000782]		
803-4F/v1-E5	ggtggtggtaagctcataaagact	PCR/Seq
803-4R/v1-F5	tctggagtctctccatcatcaata	PCR/Seq
21_F1	tttgacctaatttggatc	Seq
21_F3	ccgatctgtgaaaaaatc	Seq
21_R1	ccacgaatgctgtccgta	Seq
21_R2	aatgagatataagccaca	Seq
21_R3	taaggctatctgattaag	Seq
MJ11 Gap 23 [Chromosome I; spans contigs 1101159000778 - 1101159000777]		
799rc-1F	gtggcctgataggttgtgataaa	PCR/Seq
799rc-1R	gcattcggttcttttgaagaattt	PCR/Seq
23_F1	acagatcttcagttacttt	Seq

Supplementary Table 2. DNA oligonucleotides used in this study. The reference for all primers is this study.

Primer Name	Primer DNA Sequence (5'-3')	Notes (Seq = for DNA Sequencing)
23_R1	acatcgatttccgtaatgttc	Seq
MJ11 Gap 24 [Chromosome I; spans contigs 1101159000777 - 1101159000776]		
799rc-2F	ctagcagcatttccaattatgatg	PCR/Seq
799rc-2R	tagatcaacagcagacggtaattc	PCR/Seq
24_F1	ttacgttcatggcagcaagg	Seq
24_R1	tttgaagctttcgtctgctt	Seq
MJ11 Gap 25 [Chromosome I; spans contigs 1101159000776 - 1101159000775]		
799rc-3F	ggcgccattctttatattaacttg	PCR/Seq
799rc-3R	gtgttgataagccgattactgatg	PCR/Seq
25_F1	ctctaccagctgagctagtgt	Seq
25_R1	agtcaagctgagatggaccag	Seq
MJ11 Gap 30 [Plasmid; spans contig 1101159000798 ends]		
812rc-F	ttcagaagttgttggtttttatcg	PCR/Seq
812rc-R	ttgagcaacaaagtcgaacg	PCR/Seq
MJ11 Gap 32 [Chromosome I; spans contigs 1101159000732 - 1101159000789]		
v1-D4	gcacatcagttgtgattgtgat	PCR
v1-F4	tcaaacgtatcgttcgtgaaagt	PCR/Seq
32_F1	cagaatattacgttgggtat	Seq
32_R1	cgctgtcgatttactccaac	Seq
MJ11 Gap 33 [Chromosome I; spans contigs 1101159000731 - 1101159000737]		
v1-A3	atcacctttggtctttcattttc	PCR/Seq
v1-A7	accattacttgctgaatgctatga	PCR/Seq
33_F1	acaatcgatgagcagagaaa	Seq
33_R1	cgatgaagctgaagatggta	Seq
MJ11 Gap 34 [Chromosome I; spans contigs 1101159000737 - 1101159000779]		
v1-B3	ctggacgtacatcaacaagagaac	PCR/Seq
v1-B7	aatattgaagaagttgtcgtgcaa	PCR/Seq
34_F1	gagcaacgtctacttcagaa	Seq
34_R1	gtactgtagtagctatcgag	Seq
MJ11 Gap 35 [Chromosome I; spans contigs 1101159000782 - 1101159000783]		
v1-G5	gaaagctaagggttggagaagtaa	PCR/Seq
v1-B8	gccttctttaatatggcagaggta	PCR/Seq
35_F1	agctaattgcgactcaaa	Seq
35_F2	gaaacctattaatctgtg	Seq
35_F3	atgcctgggaatatgcct	Seq
35_F4	ttcgggttgtaaagtact	Seq
35_F5	cccctggacagacactga	Seq
35_F6	ttagtgccttcgggagct	Seq
35_F7	actcgactccatgaagtc	Seq
35_F8	ccgattattgattaaagc	Seq
35_F9	gtacacggtggatgcctt	Seq
35_F10	tgatagccccgtaaccga	Seq
35_F11	ggaaaccgagtcttaact	Seq
35_F12	tactatccgggagacaca	Seq
35_F13	gctggacgtatcagaagt	Seq
35_F14	ctactacggtagtgaagc	Seq
35_F15	cccggtgcttgaaggtta	Seq
35_F16	tttgaagcacgtacgcca	Seq
35_R1	cattgcactaacctcacg	Seq
35_R2	tcacagcgatttcaattt	Seq
35_R3	gcaagtctcatcaccgct	Seq
35_R4	tccccccatcgcaattgt	Seq
35_R5	gactcgaccagtgagcta	Seq
35_R6	taaatagatttcggggag	Seq
35_R7	gtacgtacacggtttcag	Seq
35_R8	tcttcctcggggtactta	Seq

Supplementary Table 2. DNA oligonucleotides used in this study. The reference for all primers is this study.

Primer Name	Primer DNA Sequence (5'-3')	Notes (Seq = for DNA Sequencing)
35_R9	tcattgcgagttctggtt	Seq
35_R10	cctagggctaccttgtta	Seq
35_R11	tactcgtaagggccatga	Seq
35_R12	ttgagttttaatcttgcg	Seq
35_R13	ccgggctttcacatctga	Seq
35_R14	tccagtgtggctgatcat	Seq
35_R15	aatctgagccatgatcaa	Seq
35_R16	aaatttctaaccgccaaa	Seq
MJ11 Gap 36 [Chromosome I; spans contigs 1101159000779 - 1101159000780]		
v1-C8	tgtccaacttcacttattcctgtt	PCR
v1-D7	gcagaacctgtattaagaacgaca	PCR
v1-C7	gcttagatgctttcagcgtttatc	Seq
36_F1	gaacttagctaccgggca	Seq
36_F2	gatatgaactcttgggcg	Seq
36_F3	tgtccgcaaccccgataa	Seq
36_F4	cattacgccattcgtgca	Seq
36_F5	cggcagcttagagagcaa	Seq
36_F6	tccccccatcgcaattgt	Seq
36_F7	agtgagctattacgcttt	Seq
36_F8	acccctagccacaagtca	Seq
36_F9	tcactatcggtcagtcag	Seq
36_F10	cagaaatcccagactcaa	Seq
36_F11	gccttgagctgttttgtt	Seq
36_F12	ttacgacttcaccccagt	Seq
36_F13	cggtttatcaccggcagt	Seq
36_F14	cactcctcaagggaacaa	Seq
36_F15	gagttagccggtgcttct	Seq
36_F16	cctctttggtccgaagac	Seq
36_R2	ttgttcggttgattgttt	Seq
36_R3	gcaggcctaacacatgca	Seq
36_R4	aggcagcagtggggaata	Seq
36_R5	ggtagaatttcaggtgta	Seq
36_R6	catccagagaattcgcta	Seq
36_R7	actcgactccatgaagtc	Seq
36_R8	ggtaacaggggttcgact	Seq
36_R9	ctgttttgtcttcacttt	Seq
36_R10	ttagcccttaagctttta	Seq
36_R11	tggcaaggttaaccgttt	Seq
36_R12	agtaagtactatccggga	Seq
36_R13	cgaatgctgacatgagta	Seq
36_R14	cctctaagcttcagattg	Seq
36_R15	aaacggcggccgtaacta	Seq
36_R16	agtgcctggtgggtagtt	Seq
36_R17	ggctgttcgccatttaaa	Seq
MJ11 Gap 37 [Chromosome I; spans contigs 1101159000780 - 1101159000773]		
v1-D8	gcagtgtctattcagcataattgc	PCR
v1-F7	taccacttaagagtgggattttcg	PCR/Seq
v1-E7	gttctatttcactcccctcacag	Seq
37_F1	gggttcttttcgcctttc	Seq
37_F3	acaaaggatattaagaac	Seq
37_F4	gagtggatttgaaccacc	Seq
37_F5	cgattactagcgattccg	Seq
37_F6	aggcactaagctatctct	Seq
37_F7	tctacgcatttcaccgct	Seq
37_F8	gcccattgtgcaatattc	Seq
37_F9	cgacttgcatgtgttagg	Seq

Supplementary Table 2. DNA oligonucleotides used in this study. The reference for all primers is this study.

Primer Name	Primer DNA Sequence (5'-3')	Notes (Seq = for DNA Sequencing)
37_F10	aaaaaccatctagaccaa	Seq
37_R1	ggtatttatgattgagag	Seq
37_R2	taaccaaatcaatctgtg	Seq
37_R3	aactattggaaacgatag	Seq
37_R4	catttgacgttacctaca	Seq
37_R5	tagtccacgccgtaaacg	Seq
37_R6	tgccagcacgtaatggtg	Seq
37_R7	gaggacgctcaccacttt	Seq
37_R8	gttcgatcccgcatagct	Seq
37_R9	ttcaaccttggttgctgt	Seq
37_R10	cgagcgaaattgggttag	Seq
MJ11 Gap 41 [Chromosome I; spans contigs 1101159000786 - 1101159000787]		
v1-C9	agttaatcgatatggacagccatc	PCR/Seq
v1-D9	aatgaattgaaaagcgaacttagg	PCR/Seq
26_F2	atttgcaatccgctgcatag	Seq
26_R2	tgaattgaaaagcgaacttagg	Seq
MJ11 Gap 42 [Chromosome I; spans contigs 1101159000775 - 1101159000786]		
v1-E9	cgttgcttctacattcataccaac	PCR/Seq
v1-F9	accttgtggaaagtaaccattcat	PCR/Seq
v1-E2	aatcaataaaaccaaccgtaccag	PCR/Seq
v1-A9	ccacgtacatttttgttgacctaa	PCR/Seq
7_F1	ctttaatagctgcaatcgcct	Seq
42_F1	agaaaaacgaagtttgct	Seq
42_F2	aaggtcgttgggaaaact	Seq
42_R4	ttatacagttattttggc	Seq
42_R5	taggtattacaggcacaa	Seq
MJ11 Gap 43 [Chromosome I; spans contigs 1101159000783 - 1101159000778]		
v1-C10	ggcagggtgttaatttaggtttta	PCR/Seq
v1-D10	caaacttacttcactgctggtgtt	PCR/Seq
43_F1	caacgaattgttttacta	Seq
43_F2	gttaatacgttaagtagaaatt	Seq
43_F3	gctaagtagccaaattgtc	Seq
43_F4	atctccagtttcactat	Seq
43_F6	gatcaccaatctccaa	Seq
43_F7	tctccagtttcactac	Seq
43_F8	caatatttgtgcggtc	Seq
43_R1	gcaagacaatttggctac	Seq
43_R4	caattaaaaaatggtgg	Seq
43_R5	gttttactaaacaatt	Seq
v1-H9	gttcatttccgcttcaacgtat	Seq

Supplementary Table 2. DNA oligonucleotides used in this study. The reference for all primers is this study.

Primer Name	Primer DNA Sequence (5'-3')	Notes (Seq = for DNA Sequencing)
…STEP 2		
General rDNA amplification primers		
16S-amp	aaacgacttaactgaaccttcggg	
23S-amp	gtatggttaagcctcacgggca	
rDNA PCR-walk first-round primers		
31L1	tagcaccgatttgtatggacttta	With 23S-amp; 1101159000790-1101159000732 gap.
31R1	cattagggtgtgggataggaatag	With 16S-amp; 1101159000790-1101159000732 gap.
13L1	ccatgagaacaatgagttttagga	With 23S-amp; 1101159000785-1101159000790 gap.
13R1	aattaaacgaatcaaagcagatca	With 16S-amp; 1101159000785-1101159000790 gap.
19L1	cagatgtggataactttgtgggta	With 23S-amp; 1101159000774-1101159000740 gap.
19R1a	tacaagggtcaacaatcactccta	With 16S-amp; 1101159000774-1101159000740 gap.
rDNA sequencing primers		
MJrrnaF01	aacgctaagtggttgaaa	upstream of 16S
MJrrnaF02	ggacggtgagtaatgcct	16S
MJrrnaF03	aaggccttcgggttgtaa	16S
MJrrnaF04	tctgaaggaataccagtg	16S
MJrrnaF05	tcgctagagatagcttag	16S
MJrrnaF06	attggagtctgcaactcg	16S
MJrrnaF07	ggctggatcacctcctta	16S
MJrrnaF08	caacttttcaattattttg	5S spacer
MJrrnaF09	gcaacttcaaatccgttc	generic spacer
MJrrnaF10	aatttggaaagctgactg	tRNA spacer
MJrrnaF11	aatccggcgaaaaaccag	tRNA spacer
MJrrnaF12	atggttaagtgactaagc	23S
MJrrnaF13	aaagtagcggcgagcgaa	23S
MJrrnaF14	tggtgtgactgcgtacct	23S
MJrrnaF15	gggtagagcactgttaag	23S
MJrrnaF16	accgaagctgcggcaata	23S
MJrrnaF17	acggtcgtccaagttcaa	23S
MJrrnaF18	agataccaggtggctgca	23S
MJrrnaF19	tagacggaaagaccccgt	23S
MJrrnaF20	gagaatgacaattcgagc	23S
MJrrnaF21	tagtacgagaggaccgga	23S
MJrrnaF22	tgaggcttaaccatacaa	23S
MJrrnaR01	caatcatccaacgcccac	23S
MJrrnaR02	gcttatgccattgcacta	23S
MJrrnaR03	ctgcatcttcacagcgat	23S
MJrrnaR04	cttagagagcaagtctca	23S
MJrrnaR05	tccccccatcgcaattgt	23S
MJrrnaR06	cgttacatcttccgcgca	23S
MJrrnaR07	gctctacctaaatagatt	23S
MJrrnaR08	gtacgtacacggtttcag	23S
MJrrnaR09	tgatttctcttcctcggg	23S
MJrrnaR10	aaggggttttgaacggat	generic spacer
MJrrnaR11	gccaagtcaacataacgt	5S spacer
MJrrnaR12	caagaacacttgaatgtg	tRNA spacer
MJrrnaR13	agtcgaacccctgttacc	tRNA spacer
MJrrnaR14	gcacttttgggattcgc	16S
MJrrnaR15	tcaagagtaggtaaggtt	16S
MJrrnaR16	tcaccgctacacctgaaa	16S
MJrrnaR17	catcaggctttcgcccat	16S
MJrrnaR18	cgctcgacgcccttaacgt	16S

Supplementary Table 2. DNA oligonucleotides used in this study. The reference for all primers is this study.

Primer Name	Primer DNA Sequence (5'-3')	Notes (Seq = for DNA Sequencing)
…STEP 3		
Primer pairs for PCR. PCR primers used for sequencing.		
Target 201	F:ctcagtacaccaactgggacttta, R:gtaaaatagacgggccagataaac	MJ11 draft sequence changed
Target 202	F:cgtagaatttatttctggcggtag, R:attaacaataaaagagccgtttcc	MJ11 draft sequence changed
Target 203	F:ttgtttattgaacctaaaattgagca, R:taaacttcgattgtgacttctgc	MJ11 draft sequence changed
Target 204	F:gcttcaagagatcaatcagctttc, R:gttacttgggttggcttgataaac	MJ11 draft sequence changed
Target 205	F:tggtattttaacgatttcaatgct, R:actctcacctttccaaagtacagc	MJ11 draft sequence changed
Target 206	F:caacatcgctgtctttttatcact, R:acttgagccaagagtaagccaata	No change in MJ11 sequence
Target 207	F:gtaaaacctttatggttcctgtcg, R:aagagataattggaacaaaacgag	No change in MJ11 sequence
Target 208	F:gttgtgcaagctataggtaactgg, R:tactttcattcattgcaccttctg	No change in MJ11 sequence
Target 209	F:ggcaaaagtatctctggaaaaaga, R:aatgcctaattgtgtaccgatatg	MJ11 draft sequence changed
Target 210	F:ggagtcaatcaacaaaagaaaaga, R:aagcctccaataaagttgctaaga	MJ11 draft sequence changed
Target 211	F:gtgtgtgaaagagttcgacgag, R:agtcatgtcgccttttgaataaac	MJ11 draft sequence changed
Target 212	F:ttgaaagaaaagtgcttgattcac, R:ttcgccatagataccgtaattttt	No change in MJ11 sequence
Target 213	F:gcagaaggatctcaatcaaaaatta, R:gttcatcaagagtaagctcgatcc	No change in MJ11 sequence
Target 214	F:aaaaagcagtagaccctgaaacag , R:agagtttataagtagcagcgtttcg	No change in MJ11 sequence
Target 215	F:aatggctaaacagttttcagatcc, R:aatggtagaaccacgttttgaatta	MJ11 draft sequence changed

Supplementary Table 2. DNA oligonucleotides used in this study. The reference for all primers is this study.

Primer Name	Primer DNA Sequence (5'-3')	Notes (Seq = for DNA Sequencing)
DIAGNOSTIC AMPLIFICATION OF *RSCS* AND *SYP* LOCUS GENES		
MJM-145F	tgtcttgataattgcttcacgaat	145F/146R = primer set "*rscS*-flank"
MJM-146R	ttactgaagtaacggctcttggt	145F/146R = primer set "*rscS*-flank"
MJM-307F	caaataacgtacaaaatgttgagga	307F/308R = primer set "*rscS*-internal1"
MJM-308R	ggatgttcctgtttctaaggattg	307F/308R = primer set "*rscS*-internal1"
MJM-147F	tacgagaaaaccgactaaaacaca	147F/148R = primer set "*rscS*-internal2"
MJM-148R	tcttcagcgattaatatggacaaa	147F/148R = primer set "*rscS*-internal2"
MJM-160F	tgagcatggacttagatttgattg	160F/161R = primer set "*sypB*-internal"
MJM-161R	atcgttttagcaagttacgttgaa	160F/161R = primer set "*sypB*-internal"
MJM-390F	actttacgcagcaggtggttt	309F/391R = primer set "*sypC*-internal"
MJM-391R	ccatattagagcctgatttaatttctt	309F/391R = primer set "*sypC*-internal"
MJM-387F	aatcgaccattaaagccaaacg	387F/388R = primer set "*sypR*-internal"
MJM-388R	accttggttaatttgagctaatcc	387F/388R = primer set "*sypR*-internal"
SEQUENCING DIVERGENT *RSCS* ALLELES FROM MJM-145F/MJM-146R PCR PRODUCT		
MJM-153F	taacgtacaaaatgttgagg	
MJM-154F	taaaaagggaattaatccgc	
MJM-159F	tcgatacatcagaagaaaac	
MJM-306R	aactctaaccaagaagca	
MJM-310R	ggttgttataaataattgag	
MJM-311F	actttaatgatgttatcg	
MJM-312R	ataggcatggtttgttct	
MJM-330F	atcacggaaaagtacaaa	
MJM-331F	atcttgtagagcaatatc	
MJM-332F	cttattccaatatcgttg	
MJM-333F	ttatggataaccatatgc	
MJM-334F	atcatagaattgaaacgc	
MJM-335R	tctaatgtattgccagat	
MJM-336R	atgttgttgttgaaagag	
MJM-337R	gttctatttttgaaaagtcc	
MJM-338R	tgttagagtatggctaaa	
MJM-339R	tgatttggtgatttcaag	
MJM-340R	accgtccattacaggcat	
MJM-341R	atatttagaagggcgttt	
MJM-342R	tgaacatcctctagcata	
MJM-343R	ttgatcgttcgtttgaac	
PHYLOGENETIC ANALYSES		
recA_whole_fw	gacgataacaagaaaaaagcactgg	PCR
recA_whole_rv	cgttttcttcaatttcwggagc	PCR
recA610Wolfwd	ctagnhttdawygsngcngc	Seq
recA610Wolrev	cttcacggttaaaaccttgg	Seq
mdh_whole_fw	aagtagctgttattggtgc	PCR
mdh_whole_rv	cttcgccaattttgatatcg	PCR
mdh_1_fw	ggcattggacaagcgttagc	Seq
mdh_2_rv	cgcctcttagcgtatctagc	Seq
katA_whole_fw	tgtcctgttgcacataacc	PCR
katA_whole_rv	cgcttacatcaatatcaag	PCR
katA_2_fw	cgtggtattcctgcaacatac	Seq
katA_3_rv	ccgataccttcaccataagc	Seq

Supplementary Table 3. GenBank accession numbers submitted in this study.

MJ11 Genome Chromosome I	CP001139
MJ11 Genome Chromosome II	CP001133
MJ11 Plasmid pMJ100	CP001134

	recA	*mdh*	*katA*	*rscS*
ATCC7744	EU907941	EU907965	EU907989	
CG101	EU907942	EU907966	EU907990	
EM17	EU907943	EU907967	EU907991	
EM18	EU907944	EU907968	EU907992	
EM24	EU907945	EU907969	EU907993	
EM30	EU907946	EU907970	EU907994	
ES213	EU907947	EU907971	EU907995	
H905	EU907948	EU907972	EU907996	
KB1A97	EU907949	EU907973	EU907997	
KB5A1	EU907950	EU907974	EU907998	
MB14A3	EU907951	EU907975	EU907999	
MB14A5	EU907952	EU907976	EU908000	
MJ12	EU907953	EU907977	EU908001	EU908013
***mjapo*.2.1**	EU907954	EU907978	EU908002	
***mjapo*.3.1**	EU907955	EU907979	EU908003	
***mjapo*.4.1**	EU907956	EU907980	EU908004	EU908014
***mjapo*.5.1**	EU907957	EU907981	EU908005	EU908015
***mjapo*.6.1**	EU907958	EU907982	EU908006	
***mjapo*.7.1**	EU907959	EU907983	EU908007	
***mjapo*.8.1**	EU907960	EU907984	EU908008	EU908016
***mjapo*.9.1**	EU907961	EU907985	EU908009	EU908017
SA1	EU907962	EU907986	EU908010	
SR5	EU907963	EU907987	EU908011	
WH1	EU907964	EU907988	EU908012	

SUPPLEMENTARY DISCUSSION

This high level of conservation across both chromosomes was surprising in light of the divergence discovered when the sequences of the MJ11 quorum-sensing genes, *luxIR*, were first reported[58]. Quorum-sensing (QS) is the density-dependent cell-cell communication system that facilitates coordinate expression of certain behaviors[60]. LuxI and LuxR are the signal synthase and receptor, respectively, for the QS system that controls both luminescence and extracellular proteases[19,61,62,63]. Comparison of the sequences of other QS regulators revealed that the core pathway[19] in the two strains is highly-conserved (Supplementary Fig. 1a). In contrast, not only are the LuxIR regulators divergent, but also half of the LuxR-regulated genes in ES114[61] are absent from MJ11 (Supplementary Fig. 1b). The genes that are most highly-activated in the ES114 microarray analysis are among those not present in the MJ11 genome. Regulon members lacking orthologs in MJ11 include VF_1161 through VF_1165 (encoding an efflux system), VF_1246 (OmpT omptin protease), VF_1725 (secretory tripeptidyl aminopeptidase), VF_A0090 (astacin-like metalloendopeptidase), VF_A0894 (intimin-like protein), and VF_A1058 (periplasmic protein QsrP). Furthermore, in all of the above cases, the flanking genes are intact and well-conserved, whereas only the LuxR-regulated genes show evidence of host specificity. This is not a general QS pattern, but is specific to the LuxR pathway: targets of the AinS QS system[63,64] are present in both strains (Supplementary Fig. 1b). This comparative transcriptomic approach has revealed that, while QS circuitry is generally conserved, the distinct LuxR pathways in squid and fish symbiotic *V. fischeri* extend beyond differences in luminescence regulation[19] to broad gene acquisition and/or loss in regulon membership.

Because LuxR regulon members are coregulated with luminescence and show genomic signatures of host specificity, they are likely to play important roles in the long-term association (persistence) between symbiont and host.

SUPPLEMENTARY NOTES

Supplementary Acknowledgments. Our work built on the draft MJ11 genome sequence previously deposited in GenBank, and we acknowledge the high-quality product generated by the teams at JCVI and supported by the Gordon and Betty Moore Foundation (GBMF) as part of its Marine Microbial Sequencing Project which made this study possible. We thank Jason Miller and Granger Sutton and their team for the ongoing development and maintenance of the Celera Assembler and related tools. The JCVI software team, under the leadership of Saul A. Kravitz, manages annotation, and web delivery of data for the GBMF-funded project. We thank Robert Friedman for his leadership of the GBMF-funded project. We acknowledge the J. Craig Venter Institute (JCVI) Joint Technology Center, under the leadership of Yu-Hui Rogers, for producing the genomic libraries and the sequence data.

Specific funding sources are as follows: E.G.R. and coworkers are funded by the NIH-NCRR (RR12294) and the NSF-IOS (IOB 0517007), E.V.S. is funded by an NSF CAREER Award (MCB-0347317), K.L.V. is funded by the NIGMS (GM59690), M.J.M. is funded by an NIGMS NRSA Postdoctoral Fellowship (GM078760), M.S.W is funded by an NSF Predoctoral Fellowship and an NIH Molecular Biosciences Training Grant (GM007215) to the UW.

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