

Global discovery of colonization determinants in the squid symbiont *Vibrio fischeri*

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Animal epithelial tissue becomes reproducibly colonized by specific environmental bacteria. The bacteria (microbiota) perform critical functions for the host's tissue development, immune system development, and nutrition; yet the processes by which bacterial diversity in the environment is selected to assemble the correct communities in the host are unclear. To understand the molecular determinants of microbiota selection, we examined colonization of a simplified model in which the light organ of *Euprymna scolopes* squid is colonized exclusively by *Vibrio fischeri* bacteria. We applied high-throughput insertion sequencing to identify which bacterial genes are required during host colonization. A library of over 41,000 unique transposon insertions was analyzed before and after colonization of 1,500 squid hatchlings. Mutants that were reproducibly depleted following squid colonization represented 380 genes, including 37 that encode known colonization factors. Validation of select mutants in defined competitions against the wild-type strain identified nine mutants that exhibited a reproducible colonization defect. Some of the colonization factors identified included genes predicted to influence copper regulation and secretion. Other mutants exhibited defects in biofilm development, which is required for aggregation in host mucus and initiation of colonization. Biofilm formation in culture and in vivo was abolished in a strain lacking the cytoplasmic chaperone DnaJ, suggesting an important role for protein quality control during the elaboration of bacterial biofilm in the context of an intact host immune system. Overall these data suggest that cellular stress responses and biofilm regulation are critical processes underlying the reproducible colonization of animal hosts by specific microbial symbionts.

bacterial colonization | symbiosis | biofilm | chaperone | functional genomics

Humans and other animals are often sterile before birth, from which point they immediately proceed to acquire environmental bacteria (1). The bacteria that reproducibly colonize animal hosts are critical for host tissue development, immune system development, and nutrient acquisition. The selection process by which the functional symbionts take residence in the animal from among the great diversity of environmental microbes is poorly understood, so model systems have been especially valuable to examine how specific patterns of colonization are shaped by the genetic makeup of the bacteria and the host environment (2).

The light organ of the Hawaiian bobtail squid, *Euprymna scolopes*, is colonized exclusively by the Gram-negative luminous bacterium *Vibrio fischeri*. The host inhabits seawater containing 10⁶ bacteria per milliliter, with *V. fischeri* comprising at most 0.02% of the environmental population (3). *E. scolopes* hatch without symbionts, but then rapidly acquire environmental bacteria and proceed to select for *V. fischeri* in a “winnowing” process that ensures colonization by only the specific symbiont (4). The squid-*Vibrio* system thus presents an opportunity to investigate the processes that underlie acquisition of specific environmental microbes. Forward genetic studies in *V. fischeri* have proven

fruitful for identification of relevant colonization factors. Previous work included identification of Tn10 transposon mutants that individually failed to colonize the squid host; that work identified the RscS (regulator of symbiotic colonization-sensor) biofilm regulator and Syp (symbiosis polysaccharide) biofilm effectors that are required for robust colonization (5, 6). Global genetic approaches have been applied in culture to identify regulators of the hallmark luminescence phenotype, of flagellar motility, and in other signaling pathways (7, 8).

We sought to advance the utility of this model system by applying a global forward genetic approach to identify animal colonization factors. Signature-tagged mutagenesis was initially developed to identify factors that are specifically defective for growth in an animal host, and the original approach has been recently updated to include global analysis using Illumina deep sequencing (9–11). We applied such a technology, insertion sequencing (INSeq), to mutagenize *V. fischeri* MJM1100 (strain ES114), an *Euprymna scolopes* squid isolate (Table S1). The global data generated from this analysis allows for the first identification to our knowledge of essential bacterial genes in this organism and a genome-wide identification of genes that are conditionally required for colonization of the squid host. The previously undiscovered constituents in known pathways and the pathways suggested by this analysis identify key processes required for reproducible host colonization.

Significance

Animals form associations with bacteria that play important roles in host development and fitness. The mechanisms by which animals horizontally acquire their bacterial partners from the environment are poorly understood. To address this question, we take advantage of a natural symbiosis between the luminous Gram-negative bacterium *Vibrio fischeri* and its squid host, *Euprymna scolopes*. We applied the insertion sequencing global approach and identified 380 colonization determinants in *V. fischeri*. Characterization of the factors revealed novel biofilm regulation and beneficial colonization factors at the cell envelope. To our knowledge, our study is the first global functional analysis in *V. fischeri* and expands opportunities for systems biology approaches at the host-microbe interface in a valuable reductionist model of microbiota colonization.

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Results

Mutagenesis of *V. fischeri* and INSeq. To identify colonization determinants, it was first necessary to develop tools to globally analyze mutant pool composition in *V. fischeri*. The *Bacteroides thetaiotaomicron* mutagenesis vector pSAM was modified so that its resistance cassette and transposase promoter functioned in *V. fischeri* (10, 12). The resulting vector, *V. fischeri* mariner transposon delivery vector (pMarVF1), can be conjugated from *Escherichia coli* into *V. fischeri*, generating stable erythromycin-resistant insertions (Fig. S1). With this approach, we generated a library of over 41,000 independent erythromycin-resistant insertions (T_n) in MJM1100. This is the “input” library for our analysis (Fig. 1A). Using the INSeq high-throughput method, the transposon–chromosome junctions were subsequently captured, subjected to limited amplification, and sequenced (10, 12) (SI Materials and Methods and Table S2), facilitating the identification of over 41,000 independent genomic insertion sites with high concordance across 10 technical replicates (Fig. 1B and Fig. S2).

Essential and Nonessential Genes in Culture. The mutagenesis approach affords the opportunity to identify potentially essential genes as those with reduced transposon insertion in the input mutant population. We quantified the transposon abundance (i.e., Illumina read counts) from the 5′-most 90% of each gene, and normalized those counts to the respective sample in a counts-per-million (cpm) measure. Genes that exhibited a low cpm (normalized to the size of the gene) represented likely essential genes and operons (including nonessential genes that exhibit polar effects on the downstream essential genes). To identify these loci, the transposon counts were plotted against the size of each respective gene. Mariner transposons insert at TA dinucleotides, so for gene size we used the count of such dinucleotides in the gene. We observed three clusters of genes (Fig. 1C): two clusters of likely essential genes included those with no transposon hits and those with a low number of normalized hits (0.03–4.5, depending on gene size). The third cluster exhibited significantly higher levels of transposon counts, and the genes in this cluster were therefore labeled as putative nonessential genes. Genes with fewer than nine TA dinucleotides were too small to reliably determine essentiality.

Onto the plot in Fig. 1C we mapped the *V. fischeri* genes that were orthologs of essential genes in *E. coli* MG1655, the organism for which the best curated list of essential genes exists (13). Most *E. coli* essential genes mapped to the clusters with no or relatively few transposon counts in *V. fischeri* (175 of the 230 orthologs encoded in *V. fischeri*), supporting their assignment as putative essential genes in *V. fischeri*. For the *V. fischeri* genes that are orthologs of essential *E. coli* genes, 55 fell in the higher (putative nonessential) gene cluster. For 36 of these genes, 85–100% of hits fell in the 3′ half of the gene, suggesting that the mutant allele still encodes a (partially) functional truncated protein. Therefore, we classified these genes as “putative essential” in *V. fischeri*. The remaining 19 genes had a more even distribution of hits across each gene, arguing that the gene is not essential in *V. fischeri*. The 19 essential *E. coli* genes that we predict to have nonessential *V. fischeri* orthologs include multiple genes whose products function as part of the same complex or biochemical pathway, supporting this assignment. Independent analysis using published software further confirmed their assignment as nonessential, as described in SI Materials and Methods. The genes that are predicted to be nonessential in *V. fischeri* include genes that have been demonstrated to be nonessential in the related species *Vibrio cholerae*: genes for riboflavin biosynthesis (*ribABDE*), phospholipid biosynthesis (*plsB*), other metabolic functions (*asd*, *can*, *cca*), cell division (*mukBEF*), and secretion (*secDF*) (Table S3). Mutants in multiple *V. fischeri* *muk* genes were identified in a previous study, and in the VFS (*Vibrio fischeri* sublibrary) collection 9 of the 19 predicted nonessential genes

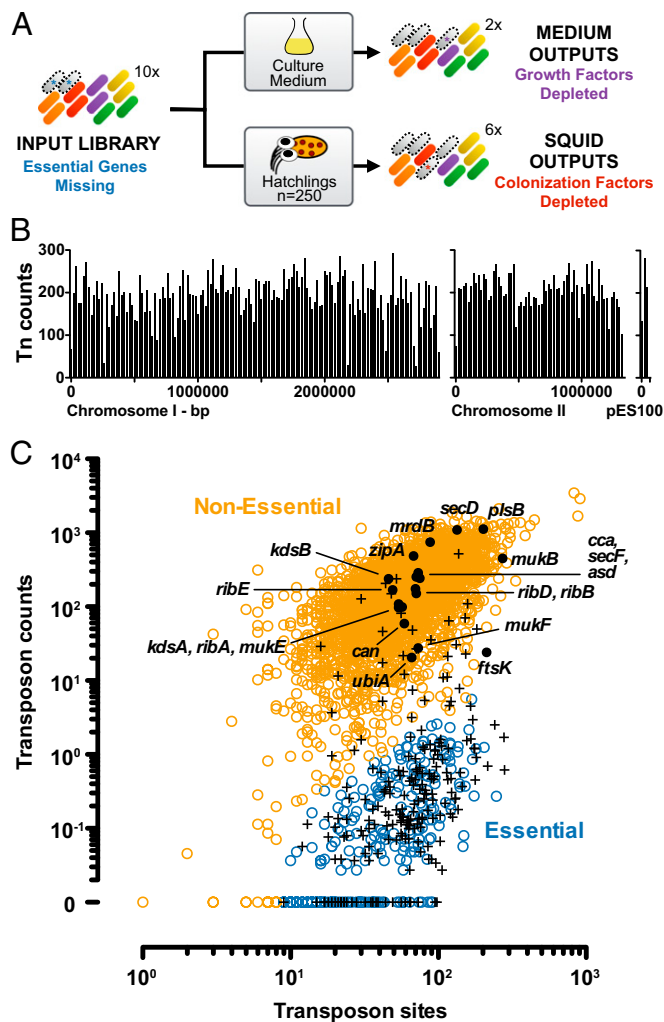


Fig. 1. Use of insertion sequencing to identify putative essential genes in *Vibrio fischeri*. (A) Overall approach. Dense transposon mutagenesis was performed in *V. fischeri*. This “input library” was then passaged, either through colonization in the hatchling squid during a 3-h inoculation and 48-h total colonization time or for an equivalent number of generations ($n = 15$) in LBS medium in vitro. Identification of genes that were missing in the input library and comparison of the output libraries to the input library enabled categorization of *V. fischeri* genes as putative colonization factors, as putative growth factors (i.e., required for normal medium growth), or as essential genes. (B) The mariner transposon developed for this purpose inserted randomly across the *V. fischeri* chromosomes and plasmid. Normalized transposon counts per 10,000-bp bin are plotted from the input library. (C) Data points represent individual *V. fischeri* ES114 genes, with the normalized transposon counts per million (cpm) and transposon sites (TA dinucleotides) in the 5′-most 90% of each gene. Orange circles are predicted nonessential genes and genes for which there is not sufficient information. Blue circles are predicted essential genes that lack an *E. coli* ortholog ($n = 244$). Plus signs are genes for which the *E. coli* ortholog is essential and the *V. fischeri* ortholog is predicted to be essential ($n = 211$). Solid black circles represent genes for which the *E. coli* ortholog is essential and the *V. fischeri* ortholog is predicted to be nonessential ($n = 19$).

harbor Tn5 transposon insertions (Table S3) (8). Included in the list of essential *E. coli* genes that are not essential in *V. fischeri*—and that are essential in *V. cholerae* or for which there exist conflicting data in different strains or studies—are multiple genes involved in cell division (*ftsK*, *mrdB/rodA*, *zipA*), LPS biosynthesis (*kdsA*, *kdsB*), and ubiquinone biosynthesis (*ubiA*) (Table S3). Comparative analysis of the essential genes therefore suggests conserved and unique features of *Vibrio* and *V. fischeri* biology for

these factors. Importantly for this study, these results highlight that the INSeq analysis identifies relevant signal within a mutant library, and we therefore proceeded to examine genes that were depleted following squid colonization.

Identification of Squid Colonization Factors. For experimental inoculation of *E. scolopes* bacterial concentrations of 10^3 – 10^6 cfu/mL are typically used (14, 15). It was demonstrated that higher inoculum concentrations led to a greater diversity of strains within the light organ, so we used an inoculum of 2×10^5 cfu/mL (16, 17). To ensure that sufficient strain diversity could be achieved in the light organ, we colonized squid with a defined 96-mutant library and then analyzed the individual animal output samples by INSeq. Each animal contained over 80 of the initial 96 mutants (Fig. S24), revealing that even with some initial bottleneck we can obtain a large number of independent mutant strains in an individual animal. We estimated that with 250 animals, we could conservatively sample 20,000 mutants (250 animals \times 80 mutants minimum per animal). We therefore sought to analyze the distribution of the input mutant library following colonization in six replicates of 250 animals each, which would give average coverage of at least 35-fold per nonessential gene. In many systems, the natural route of inoculation is bypassed to investigate colonization *in vivo*, in contrast to inoculation of *E. scolopes* where bacteria are introduced to the seawater and then acquired by the host's natural mechanisms. We observed some noise in the squid output libraries (Figs. S2B and S3), which likely reflects some variation in the extent to which the host bottleneck

influenced symbiont diversity across the large number of animals in our analysis.

To avoid the study of mutants that were simply defective for growth, the input library was passaged in LB salt (LBS)-rich medium and the 115 genes that exhibited >10-fold mutant depletion (Fig. 2A). We proceeded to use the dynamics of known colonization mutants to train our analysis to identify true, novel colonization factors. First, we observed that in most cases, mutants in known colonization factors were observed in every squid output library, and they were diminished in each library with respect to their relative abundance in the input library (Fig. 2C and Fig. S3). In contrast, 967 genes were represented by mutants that were not sampled in at least one squid output library, and because known squid colonization factors were not highly represented in this dataset, we suspect that some of these factors are artifacts (i.e., seawater growth defective; or low representation in the input library and therefore dramatic sampling bias in the squid). For subsequent analysis, we therefore took a conservative approach and focused on the 2,291 genes for which mutants were sampled in all squid libraries, and for which representation in each output library diminished by at least 1.8-fold (Fig. 2B). This set of 380 squid-depleted mutants captured over 75% of known mutants during the first two of the three defined stages of squid colonization (i.e., initiation and accommodation; Fig. 2C and Fig. S3), validating the analytical criteria applied. The INSeq squid-depleted mutants identified only 20% of known persistence-stage colonization factors (Fig. 2C), and finer-scale analysis of these factors revealed significant variation across the

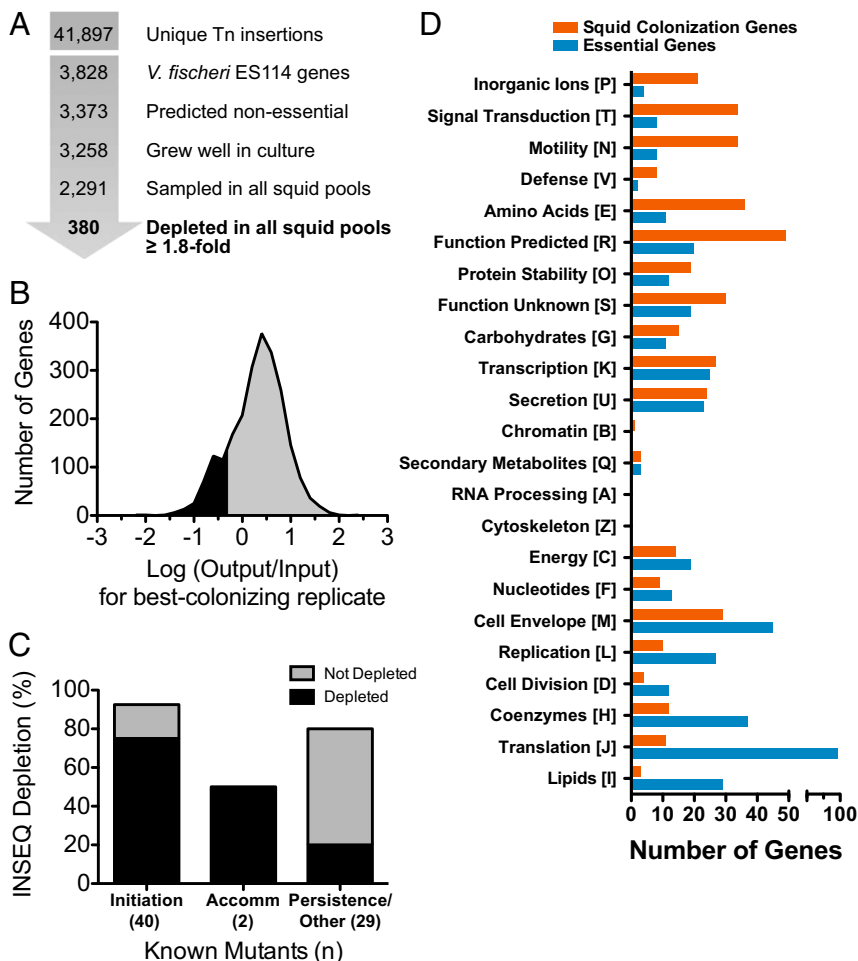


Fig. 2. INSeq analysis identifies squid colonization factor candidates. To identify putative colonization factors, the dynamics of the 41,897 mariner transposon library was followed during squid colonization. (A) Flowchart of the manner by which *V. fischeri* genes were categorized during the analysis. The 155 rRNA and tRNA genes were removed before the analysis shown. Genes that were classified as essential or that grew poorly in LBS medium (depleted by >1 log over 15 generations) were excluded from analysis in the squid host. The remaining 3,258 genes included 2,291 genes with mutants that were sampled in all squid output libraries. This conservative filter ensured that the genes assayed were abundant enough to colonize squid in the experimental conditions provided. (B) These genes were plotted by their best colonizing replicate (i.e., highest count for the mutant), and 380 genes had transposon counts that were depleted in all squid output libraries by at least 1.8-fold. This set of 380 genes represents the colonization factor candidates presented in this report. (C) For each category of colonization mutant, the percentage of the genes that were identified in the screen is shown by the black bars. The gray bars represent the percentage of genes that did not display depletion, and the remaining percentage indicates genes that were not assayed fully in squid (e.g., *in vitro* growth defect). Additional detail is provided in Fig. S3. (D) COG categorization of depleted genes. COG categories that are present in *V. fischeri* are shown, and plotted for each, the number of essential genes (blue) and the number of significantly squid-depleted genes (red).

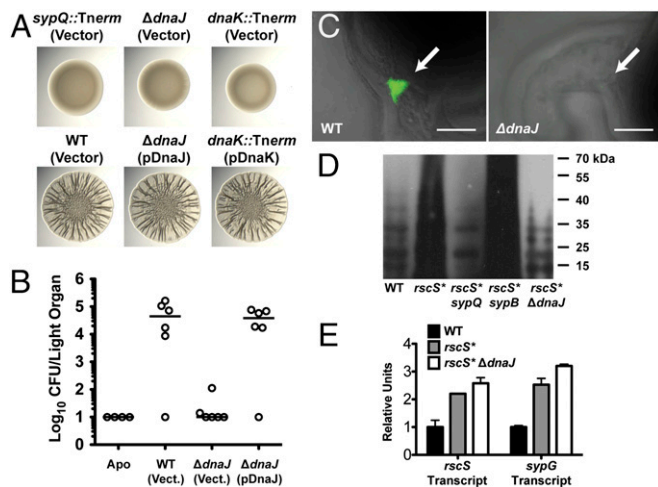


Fig. 4. The DnaJ/DnaK chaperone system regulates Syp biofilm formation. (A) Biofilm formation, as assayed by wrinkled colony formation, is not observed in *dnaJ* or *dnaK* mutant strains but is complemented when the interrupted gene is provided in *trans*. (B) Single-strain colonization of *E. scolopes* squid by each of the indicated strains; Apo, aposymbiotic. Hatchling squid were inoculated with 2×10^3 cfu/mL bacteria, washed at 3 h and 24 h, and assayed at 48 h. (C) Aggregate formation in the squid ciliated epithelial field by strains of the indicated genotype. Hatchling squid were inoculated with 2×10^6 cfu/mL bacteria that constitutively express GFP from the pVSV102 plasmid and assayed at 3 h by epifluorescence microscopy. Large aggregates are readily apparent in the wild-type colonized animals (arrow) but only isolated cells are observed in the isogenic Δ *dnaJ* mutant (arrow). (Scale bars, 50 μ m.) (D) Syp polysaccharide immunoblot with biofilm-specific antibodies. (E) QRT-PCR demonstrating the relative abundance for *rscS* and *sypG* transcripts in the indicated strains.

involved in the biofilm phenotype. We observed that a *dnaK*::*Tnrm* mutant exhibited a significant defect in wrinkled colony formation, and that the phenotype was complemented by *dnaK*⁺ *in trans* (Fig. 4A). The *dnaK*::*Tnrm* strain exhibited a growth defect, as did *dnaK* mutants in the INSeq dataset (Fig. S5 and Dataset S1). However, DnaJ and DnaK were similarly required for heat tolerance, arguing that it is reasonable to model the DnaJ/DnaK chaperone machine in *V. fischeri* on mechanisms proposed from other organisms (19). We therefore proceeded to probe the role of the DnaJ/DnaK system using the Δ *dnaJ* allele.

Based on the severe biofilm defect observed in culture, we predicted that the Δ *dnaJ* mutant would exhibit a colonization defect even in the absence of competition. We inoculated squid hatchlings with the mutant strain for 3 h and observed at 24 h that the Δ *dnaJ* mutant failed to colonize the light organ (Fig. 4B). The complemented strain was indistinguishable from wild type. This result is similar to that observed for other biofilm mutants, supporting a role for DnaJ during biofilm formation *in vivo*. We proceeded to test directly whether DnaJ influences biofilm development in the host mucociliary field. We colonized squid hatchlings with GFP-labeled bacteria for 3 h and then imaged the dissected light organ by epifluorescence microscopy. In contrast to the wild-type strain, which exhibited large aggregates of ~20 μ m diameter, the Δ *dnaJ* mutant was present only as single cells (Fig. 4C). These data support a role for DnaJ in regulating biofilm formation *in vivo* during the initiation stage of squid colonization.

We next asked whether DnaJ regulates polysaccharide production or polysaccharide secretion. The Syp exopolysaccharide (EPS) is readily detected with an anti-EPS antibody, and upon Western blot analysis a strong signal was observed for EPS produced, regardless of whether it is secreted or retained in the cell (20). The Syp polysaccharide that is synthesized in an RscS-

dependent manner is not observed in a strain harboring the Δ *dnaJ* allele (Fig. 4D). The phenotype resembled that of a strain with an interruption in the SypQ glycosyltransferase, which is required for polysaccharide production (20). The canonical pathway that activates the Syp biofilm relies on a transcriptional regulatory cascade to activate four σ^{54} -dependent promoters at the *syp* locus (18). In a biofilm-induced background, we observed high levels of transcription at *sypG*, even in the absence of DnaJ (Fig. 4E). Together, these results suggest that DnaJ acts at a step downstream of *syp* transcription to allow polysaccharide production.

Discussion

The mechanisms by which beneficial bacteria become reproducibly established in their animal hosts are poorly understood. Here we describe a functional genomic screen to elucidate novel genetic factors required for establishment of *V. fischeri* in its squid host. Overall our study is unique in several respects. First, this work represents to our knowledge the first global *V. fischeri* genetic screen during squid colonization, a natural binary system in which the host innate immune system is intact and colonization occurs through the native pathways. Second, we present functional characterization of three factors that influence biofilm regulation, including a connection between the widely conserved stress-responsive DnaJ/DnaK chaperone system and biofilm development. Third, we identify many colonization factors that provide the basis for mechanistic investigation of the acquisition of beneficial microbes by their cognate animal hosts.

We describe 380 candidate colonization factors, and as proof of principle we rediscovered over 75% of the known colonization initiation and accommodation factors (Fig. 2C). Mutants that affect the later persistence stage were not well represented in this analysis. We therefore predict that most of the previously unidentified factors will map to the initiation or accommodation stages, a claim that is supported by the discovery of three biofilm (i.e., initiation) regulators in the initial set of validated factors.

There is an increasing appreciation that metal concentrations are critical during host colonization. It is established that copper is an effective antimicrobial agent *ex vivo*, and there is evidence that it may play a similar role *in vivo*. Pathogens that lack copper efflux systems colonize poorly in the mammalian lung, possibly because of an inability to detoxify increased levels of copper in the host tissue (21). Detoxification of intracellular copper is achieved by efflux via P-type ATPase and RND (resistance-nodulation-cell division) family efflux systems (22). We observed that *V. fischeri* lacking the P-type ATPase CopA or components of the RND efflux system CusCFBA exhibited significant colonization defects in the INSeq analysis and in 1:1 colonizations. Oxygen in squid is carried by hemocyanin, a metalloprotein that uses a pair of copper ions to reversibly bind the oxygen. Copper coordination is predicted to be conserved in *E. scolopes*, and there is intimate communication between host and microbe, including known involvement of hemocyanin and oxygen (23). Copper may therefore be critical to the dialogue that leads to establishment of a secure and robust partnership.

Our screen identified a role for the two-partner secretion system TamAB (YtfMN) during host colonization. This system was identified as a colonization determinant in signature-tagged mutagenesis screens in *Proteus mirabilis* and *Klebsiella pneumoniae* (24, 25). From work in *Enterobacteriaceae* the TamAB system has been suggested to function in the translocation of autotransporters; however, the *V. fischeri* ES114 genome does not have any annotated autotransporters (26). This suggests the presence of an unannotated autotransporter in *V. fischeri*—which our data would argue play an important role during host colonization—or a novel role for the TamAB secretion system. In other systems it is not clear how TamAB impacts host colonization, and the *V. fischeri* model provides an opportunity to examine this further.

The discovery of genes with unknown or predicted functions as required for robust host colonization provides an opportunity to interrogate how uncharacterized gene products influence colonization behavior. Some of these genes (*yhcB* and *yafD*) exhibit no defects in culture and influence host colonization with such dramatic phenotypes that they exert them within 48 h during both massive competition and during 1:1 competitions. The *Vibrio*–squid system is therefore a sensitive one in which to interrogate the function of uncharacterized gene products during host association.

Previous reports in bacteria and yeast suggest a possible role for various chaperone systems in biofilm development. Increased expression of chaperone genes is observed in environmental biofilms (27) and in *Pseudomonas aeruginosa* tobramycin-treated biofilms (28). However, the functional role of these chaperones has been difficult to examine. DnaK influences biofilm development in *Listeria monocytogenes* (29), and treatment of *Histophilus somni* biofilms with anti-GroES blocking antibodies leads to reduced biofilm formation (30). Furthermore, depletion experiments in *Candida albicans* have identified roles for Hsp90 (HtpG) and Hsp104 (ClpB) in regulating biofilm formation, arguing for a broad connection between protein folding and biofilm development. Here we demonstrate that the DnaJ/DnaK chaperone system regulates symbiotic biofilm formation in *V. fischeri*, and that aggregation in host tissue is abolished in the absence of the system. Prior reports have connected nitric oxide signaling to the size of the bacterial biofilm-like aggregates at this stage (31, 32). Further investigation of the basis of the DnaJ/DnaK aggregation

defect should examine how microbial proteostasis regulates biofilm formation upon encountering host immune challenges.

Materials and Methods

Detailed methods are provided in *SI Materials and Methods*.

Mariner Transposition and INSeq Analysis in *V. fischeri*. The *V. fischeri* mariner transposon delivery vector pMarVF1 was constructed to facilitate INSeq analysis in *V. fischeri* ES114. The suicide delivery vector carries an INSeq-compatible transposon, which encodes an erythromycin-resistance cassette and a backbone that includes an R6K γ origin of replication, RP4 transfer origin, beta-lactamase (i.e., ampicillin resistance), and expresses the Himar1C9 transposase from the ES114 *nrdR* promoter.

Squid Colonizations. Colonization of hatchling squid is detailed fully in *SI Materials and Methods*. For the assays performed, the inoculum concentrations, inoculation time, and total colonization time are as follows: INSeq screen: 2×10^5 cfu/mL, 3 h and 48 h; in vivo 1:1 competitions: 2×10^3 cfu/mL, 3 h and 48 h; single-strain colonization assays (DnaJ): 2×10^3 cfu/mL, 3 h and 24 h; and visualization of aggregates: 2×10^6 cfu/mL, 3 h and 3 h.

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