

Corrected and republished from: “*Vibrio fischeri* Possesses Xds and Dns Nucleases That Differentially Influence Phosphate Scavenging, Aggregation, Competence, and Symbiotic Colonization of Squid”

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ABSTRACT Cells of *Vibrio fischeri* colonize the light organ of *Euprymna scolopes*, providing the squid bioluminescence in exchange for nutrients and protection. The bacteria encounter DNA-rich mucus throughout their transition to a symbiotic lifestyle, leading us to hypothesize a role for nuclease activity in the colonization process. In support of this, we detected abundant extracellular nuclease activity in growing cells of *V. fischeri*. To discover the gene(s) responsible for this activity, we screened a *V. fischeri* transposon mutant library for nuclease-deficient strains. Interestingly, only one strain, whose transposon insertion mapped to nuclease gene *VF_1451*, showed a complete loss of nuclease activity in our screens. A database search revealed that *VF_1451* is homologous to the nuclease-encoding gene *xds* in *Vibrio cholerae*. However, *V. fischeri* strains lacking *xds* eventually revealed slight nuclease activity on plates upon prolonged incubation. This led us to hypothesize that a second secreted nuclease, identified through a database search as *VF_0437*, a homolog of *V. cholerae dns*, might be responsible for the residual nuclease activity. Here, we show that Xds and/or Dns are involved in essential aspects of *V. fischeri* biology, including natural transformation, aggregation, and phosphate scavenging. Furthermore, strains lacking either nuclease were outcompeted by the wild type for squid colonization. Understanding the specific role of nuclease activity in the squid colonization process represents an intriguing area of future research.

IMPORTANCE From soil and water to host-associated secretions such as mucus, environments that bacteria inhabit are awash in DNA. Extracellular DNA (eDNA) is a nutritious resource that microbes dedicate significant energy to exploit. Calcium binds eDNA to promote cell-cell aggregation and horizontal gene transfer. eDNA hydrolysis impacts the construction of and dispersal from biofilms. Strategies in which pathogens use nucleases to avoid phagocytosis or disseminate by degrading host secretions are well-documented; significantly less is known about nucleases in mutualistic associations. This study describes the role of nucleases in the mutualism between *Vibrio fischeri* and its squid host *Euprymna scolopes*. We find that nuclease activity is an important determinant of colonization in *V. fischeri*, broadening our understanding of how microbes establish and maintain beneficial associations.

KEYWORDS *Vibrio*, squid, symbiosis, nuclease

Most host-bacterial symbiotic associations are comprised of multiple bacterial species co-mingling within a single host, which often makes it more difficult to resolve complex interactions. Far less common are symbiotic relationships that contain

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one host and only one bacterial species, an example being the relationship between the luminous bacterium *Vibrio fischeri* and its squid host *Euprymna scolopes* (1). Cells of *V. fischeri* colonize the microvilli-lined crypts of the squid light organ. In order to reach this tissue, cells aggregate near the opening of mucus-lined pores. These cells must then detach from the aggregates and swim through mucus-filled ducts into crypts of the light organ. Marine animal-secreted mucus is a source of extracellular DNA (eDNA) (2, 3). Extracellular DNA can strengthen the adhesivity of the mucus due to entanglement within the mucus mesh network (4). The mucus secreted by *E. scolopes* contains sugars and nucleotides that attract *V. fischeri* cells (5, 6). DNA found in the mucus may help with the structure of the mucus and provide nutrients to migrating *V. fischeri* cells. In support of this hypothesis, interactions between *V. fischeri* and the epithelium-lined crypts enhance mucus production from the tissue (7). The importance of eDNA-containing squid mucin secretions in the colonization process led us to speculate on the role of nuclease activity in colonization.

Given the similarities between light organ and human intestinal epithelium, it is likely that *V. fischeri* requires many of the same colonization determinants as *Vibrio cholerae* (8). For example, the global regulator of luminescence and symbiotic competence, *litR*, in *V. fischeri* is homologous to *hapR* in *V. cholerae* (9). In *V. cholerae*, the *hapR* gene encodes a global regulator of many genes required for host colonization, including one encoding the secreted nuclease Dns (10, 11). That cells of *V. fischeri* possess a HapR homolog led us to hypothesize a potential role for a Dns homolog in *V. fischeri*.

Marine bacteria, including members of the Vibrionaceae, produce nucleases as a survival mechanism (12). Some bacteria secrete nucleases as a mechanism to heighten colonization fitness through means of DNA degradation and repair, disrupting host defense mechanisms, biofilm formation, nutrient acquisition, and natural transformation (10, 11, 13–15). For example, infection by *V. cholerae* causes an inflammatory response in the human intestines by inducing the proliferation of neutrophils that produce extracellular traps (i.e., NETs) to combat the bacteria. In turn, Xds and Dns are secreted by the cells to degrade the DNA component of the NETs, allowing evasion of phagocytosis (14). Similarly, the innate immune system of *E. scolopes* produces neutrophil-like hemocytes that adhere to and phagocytize bacteria, which *V. fischeri* must avoid to colonize squid (16). Based on these observations, we hypothesized that secreted nuclease activity may contribute to a variety of symbiosis-related activities (e.g., nutrient acquisition, hemocyte avoidance, etc.) thus enhancing the ability of *V. fischeri* to colonize the squid.

In this study, we identified homologs of both Xds and Dns in *V. fischeri* and provided data on the important roles they play in bacterial physiology, evolution, and host colonization. Specifically, Dns appears to be more important in cell-cell aggregation, natural competence, and squid colonization, while Xds is more important for eDNA degradation and phosphate scavenging.

RESULTS

The *V. fischeri* nucleases are Xds and Dns homologs

The *V. fischeri* genome encodes two secreted nucleases, VF_1451 and VF_0437, that are homologous to Xds and Dns, respectively, nucleases of *V. cholerae* (Fig. 1A and B). The Xds homologs shared 46% identity across the entire protein (62% similarity), while the Dns homologs shared 66% identity (77% similarity). Analysis of the amino acid sequences of Xds and Dns in *V. fischeri* using SignalP 5.0 (17) revealed a signal peptide cleavage site in each protein, suggesting that they are most likely secreted (Sec/SPI scores: 0.97 and 0.99, respectively). Genes flanking the *xds* homologs in *V. fischeri* and *V. cholerae* are different (Fig. 1C). Conversely, the *dns* homologs share genomic synteny (Fig. 1D).

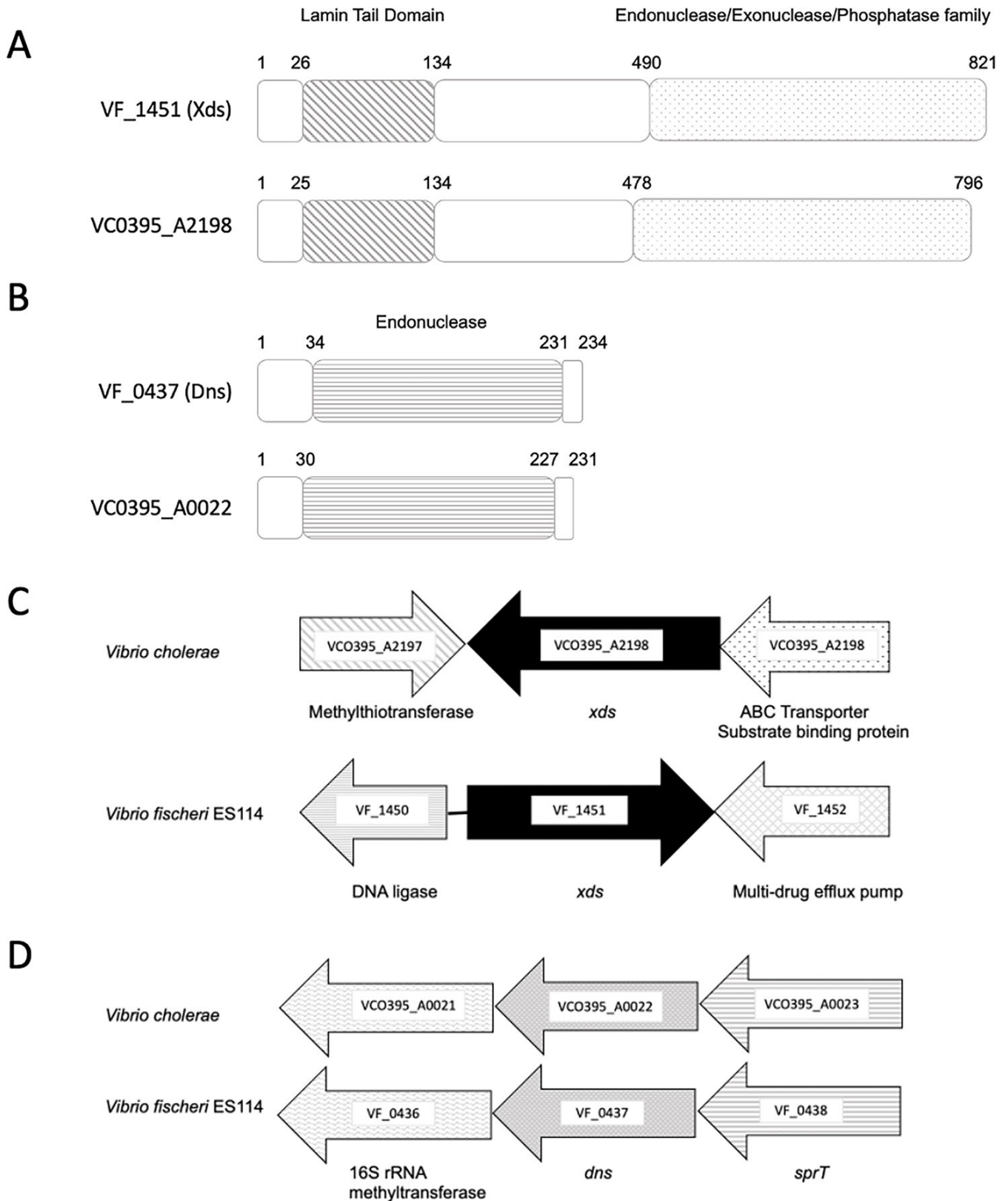


FIG 1 *V. fischeri* possesses two putative secreted nucleases that are homologs of Xds and Dns nucleases in *V. cholerae*. (A) The Xds proteins in *V. fischeri* ES114 and *V. cholerae* VC0395 share two functional domains, the Lamin Tail Domain (LTD; diagonal stripes) and the Endonuclease/Exonuclease/Phosphatase family (stippled). (B) The Dns proteins share an Endonuclease I functional domain (horizontal stripes). The numbers listed above in the protein cartoon represent amino acid numbers. (C) Genes flanking the *xds* homologs are different, while the genes flanking *dns* in both species are homologs (D).

Xds is the predominant secreted nuclease in *V. fischeri*

Inactivation of *xds* abolished the nuclease activity observed for wild-type strain ES114 after 24 hours of incubation on DNase agar. This defect could be restored by expression of *xds* in *trans* (Fig. 2A). After prolonged incubation, the *xds* mutant eventually displayed secreted nuclease activity not as apparent in the *xds dns* double mutant (Fig. 2B). These findings are supported by quantitative data obtained from liquid cultures. When cells were grown to OD₆₀₀ values of 0.5 and 1.0, compared to wild type, inactivation of *xds* reduced average nuclease activity about 15- to 30-fold, respectively, while average nuclease activity in the *dns* mutant was not statistically different (Fig. 2C). Addition of ethylenediaminetetraacetic acid (EDTA) completely abolished nuclease activity in cell-free supernatants of strain ES114, suggesting that Xds, and possibly Dns, requires divalent cations for activity (Fig. 3A). Furthermore, average nuclease activity was reduced by about 36% with the addition of excess phosphate to the growth medium (Fig. 3B).

Xds degrades linear and plasmid DNA

To confirm and extend these findings, we also assessed extracellular nuclease activity by mixing cell-free supernatants from *V. fischeri* strains with either PCR (linear) or plasmid (supercoiled; pUC19) DNA and visualizing degradation using gel electrophoresis (Fig. 4). Within 5 minutes, all Xds⁺ strains (i.e., *xds*⁺, *dns*, ES114, and *dns*⁺) either partially or completely degraded both linear (PCR) and plasmid DNA (Fig. 4A and B). After 30–60 minutes, the Xds⁺ strains completely degraded either type of DNA. Strains that lacked Xds (i.e., *xds* and *xds-dns*) exhibited markedly reduced DNA degradation during the incubation periods we tested. Together, these data support the hypothesis that Xds is a key protein involved in degrading both linear and supercoiled DNA in *V. fischeri*.

Cells of *V. fischeri* can use eDNA as their sole phosphate source

Inactivation of *xds*, and to a lesser extent *dns*, resulted in an overall reduced growth rate and cell yield for *V. fischeri* strains grown with eDNA as the sole phosphate source (Fig. 5A). Specifically, log phase Xds⁻ cells (i.e., *xds*, *xds-dns*) required roughly 4 hours to double, while Xds⁺ cells (i.e., ES114, *dns*) doubled in just over 1 hour. Furthermore, after 16 hours, the Xds⁻ strains reached an OD₆₀₀ of only about half that of the Xds⁺ strains. However, OD₆₀₀ for the *dns* mutant at 16 hours was about 15% lower (~1.0 vs ~0.85) than that of the wild-type strain ES114 despite starting at roughly the same OD₆₀₀. All strains grew similarly in Tris-minimal medium (TMM) with K₂HPO₄ (Fig. 5B), while none of the strains grew in the absence of a phosphate source (Fig. 5C). Taken together, *V. fischeri* can use eDNA as a phosphate source that is dependent on Xds, and to a lesser extent, Dns.

Xds might play a role in mucin degradation

Cells of *V. fischeri* encounter abundant DNA-containing mucin upon colonizing squid (5). To determine whether nuclease might play a role in degrading the DNA component of mucin, strains were grown on a medium containing mucin as the sole nutrient source. Consistent with our other findings, and unlike the wild-type strain, the Xds⁻ strains (i.e., *xds* and *xds-dns*) were unable to degrade the DNA component of mucin after 24 hours (Fig. 6). Expression of *xds* in *trans* (i.e., *xds*⁺) restored full wild-type activity.

Dns controls cell-cell aggregation in *V. fischeri*

Enhanced aggregation in strains lacking *dns* but not *xds* was apparent after extended incubation (i.e., 5–7 days) in static liquid culture (Fig. 7). Thus, we conclude that (1) eDNA may contribute to biofilm formation by *V. fischeri* that occurs following extended static growth, and (2) Dns, but not Xds, appears to function to inhibit eDNA-dependent biofilm formation.

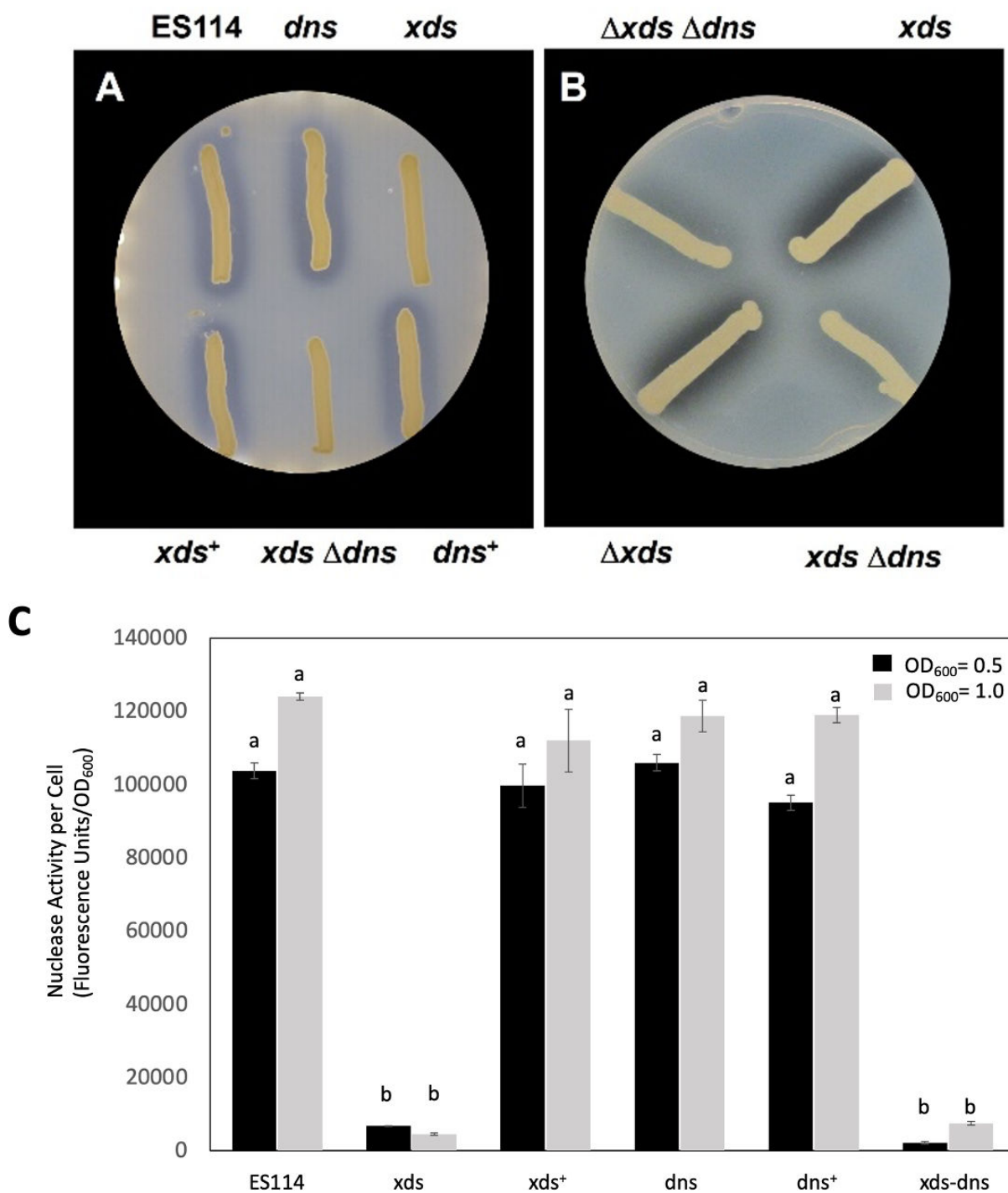


FIG 2 Nuclease activity in strains of *V. fischeri*. (A) Strains lacking *xds* (i.e., *xds* and *xds*-*dns*) were unable to degrade DNA in DNase agar after 24 hours at 28°C, but expression of *xds* in *trans* in the *xds* single mutant (*xds*⁺) restored nuclease activity. Strains used are as follows: ES114, *dns* (Δ *dns*::FRT), *xds* (*xds*::miniTn5), *xds*⁺ (*xds* + pVSV105 *xds* cm^R), *xds* *dns* (*xds*::miniTn5 Δ *dns*::FRT), *dns*⁺ (Δ *dns*::FRT P*nrdR* *dns*). (B) After 6 days, a halo could be visualized around the *xds* mutant. Strains are as follows: Δ *xds* Δ *dns* (KV8865), *xds* (*xds*::miniTn5), *xds* Δ *dns* (*xds*::miniTn5 Δ *dns*::FRT), and Δ *xds* (KV8811). (C) Nuclease activity in cells grown with shaking in lysogeny broth salt (LBS) to an OD₆₀₀ of either 0.5 or 1.0. Cell-free culture supernatants were mixed with DNaseAlert reagents to measure nuclease activity. Each bar represents the average of five replicates (with standard error); these results are representative of three separate experiments. Different letters indicate statistically significant differences based on analysis of variance (ANOVA; *P*-value < 0.05).

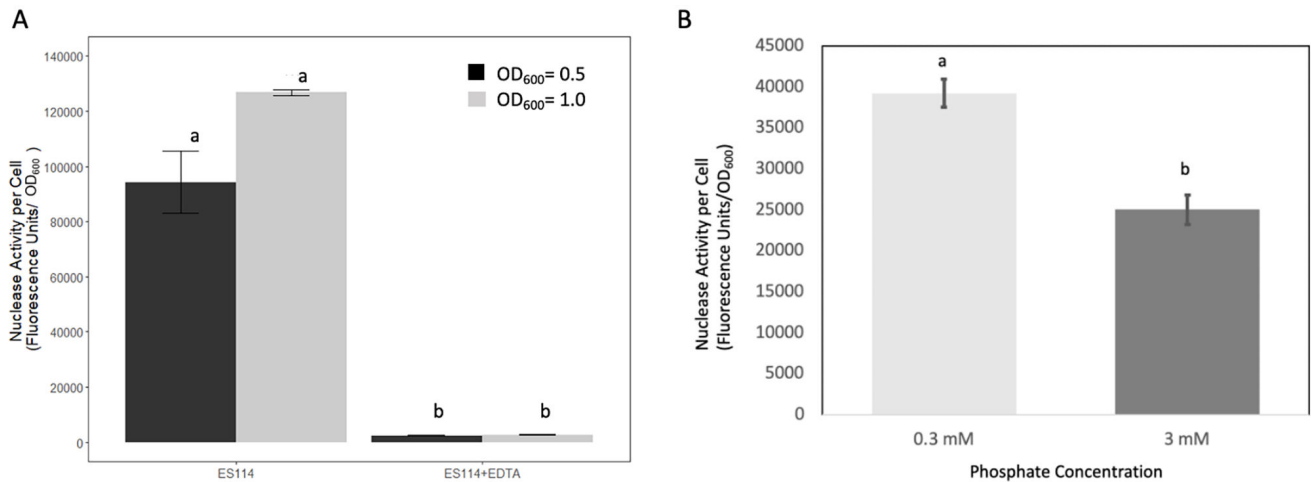


FIG 3 Effect of EDTA and phosphate on *V. fischeri* DNase activity. (A) Wild-type (ES114) cells were grown with shaking in LBS to an OD₆₀₀ of 0.5 and 1.0. Cell-free supernatants with and without added EDTA (10 mmol/L) were mixed with DNaseAlert reagents to measure nuclease activity. Each bar represents the average of five replicates (with standard error); these results are representative of three separate experiments. (B) Cells were grown in a minimal medium containing either 0.3 or 3 mM phosphate and then assayed for nuclease activity as described previously. Each bar represents the average of five replicates from three experiments (with standard error). Different letters indicate significant differences based on ANOVA. (P -value < 0.05).

Dns acts as a barrier to natural transformation

Our previous work demonstrated a role for *dns* in *tfoX*-induced competence (18) but did not evaluate the impact of the loss of Xds or both nucleases. As previously reported, the inactivation of *dns* increased transformation efficiency by ~2 orders of magnitude (Fig. 8). Surprisingly, despite its greater effect on extracellular nuclease activity, loss of Xds did not alter transformation efficiency: it was not statistically different from that of the wild-type strain, although it trended slightly higher in some of our experiments. Similarly, the *xds-dns* strain exhibited a comparable efficiency to that of the *dns* mutant. Complementation of the *dns* mutant by the introduction of *dns* at a non-native site substantially reduced the transformation efficiency, such that no transformants were obtained (Fig. 8A). To confirm that the complemented strain was still competent to take up DNA, we introduced into the strain (and its controls) a different *tfoX* overexpression plasmid, one that we previously showed to induce a higher transformation frequency (18). We then repeated the competence experiments with the wild type, *dns* (i.e., KV8807), and

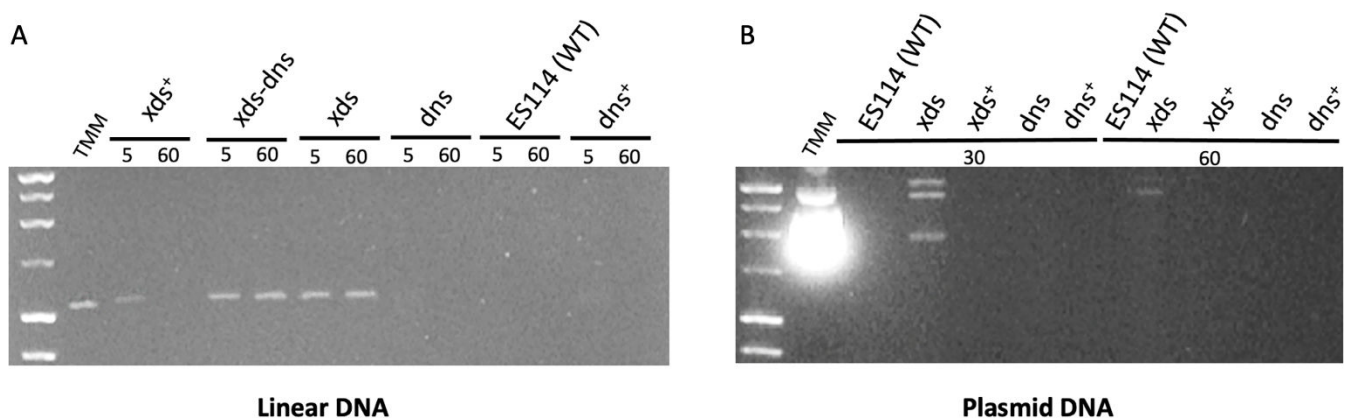


FIG 4 Degradation of eDNA by *V. fischeri* strains. Strains were cultured in Tris-minimal medium (TMM) to an OD₆₀₀ of approximately 2.0. Either sterile TMM or cell-free supernatant from each strain was mixed with (A) ~0.2 μg of PCR DNA (linear) or (B) 1 μg of plasmid (pUC19) incubated at 28°C for the times (in minutes) indicated, and then visualized on a 1% agarose gel.

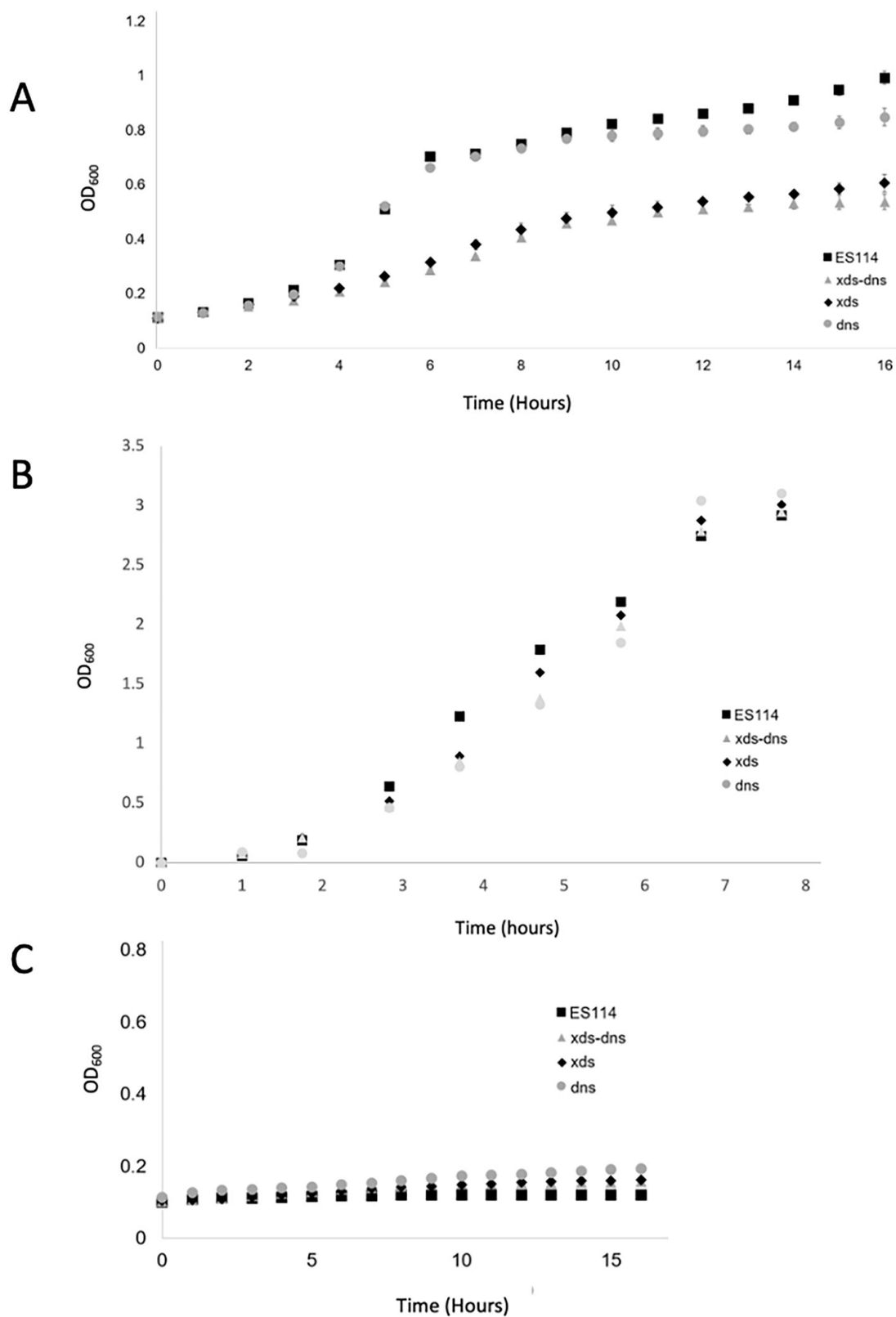


FIG 5 Secreted nucleases are essential for optimal growth when eDNA is the only source of phosphate. Cells were grown at 28°C with shaking in TMM containing (A) sheared salmon sperm DNA (1.0 mg mL^{-1}) or (B) K_2HPO_4 , as the sole phosphate source, or (C) no phosphate. Error bars represent averages (\pm standard error) for three separate trials.

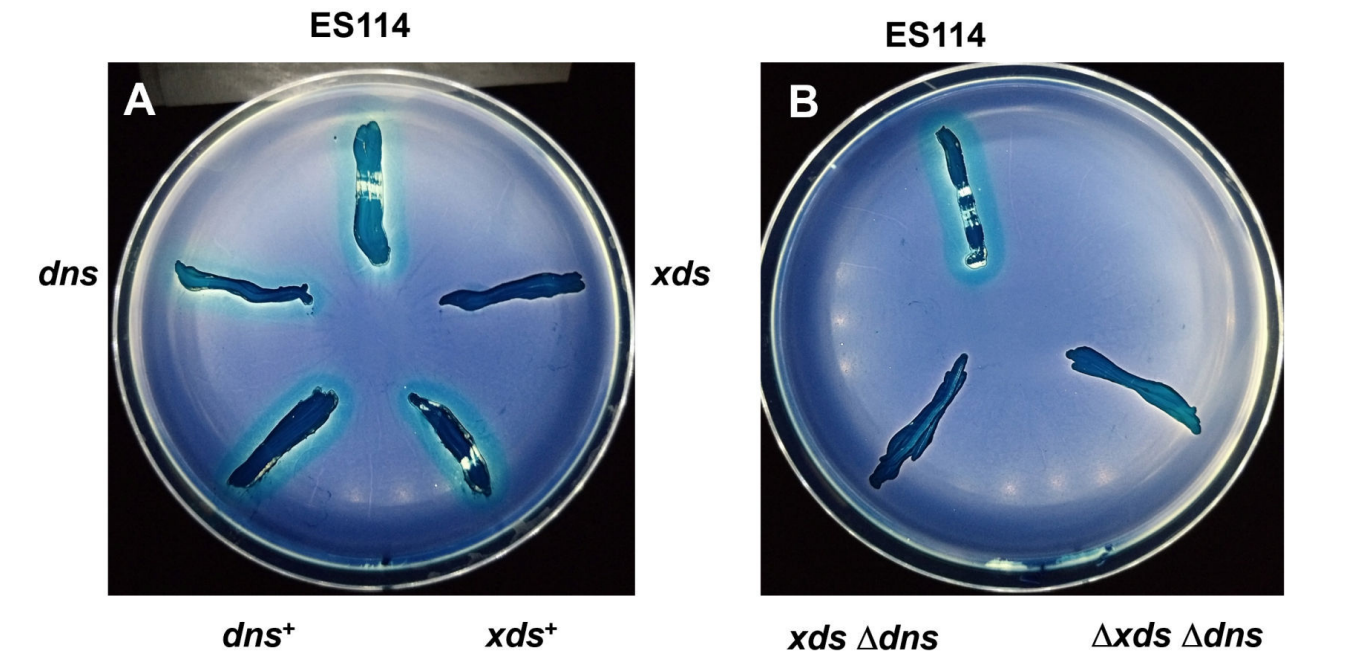


FIG 6 Xds is required to digest DNA in mucin. Digestion of mucin at 24 hours. Strains are as follows: (A) ES114, *dns* ($\Delta dns::FRT$), *xds* (*xds::miniTn5*), *dns+* ($\Delta dns::FRT$ *PnrdR dns*), *xds+* (*xds* + pVSV105 *xds* cm¹). (B) ES114, *xdsΔdns* (*xds::miniTn5Δdns::FRT*), $\Delta xds \Delta dns$ (KV8865).

complemented *dns* strains (i.e., *dns*⁺ KV10273). Under these genetic conditions, low numbers of transformants were obtained for the complemented *dns* mutant, indicating the strain was capable of DNA uptake (Fig. 8B). Together, these data reveal that Dns is the key nuclease controlling competence.

Xds and Dns are required for efficient colonization of *E. scolopes*

Single strain colonization assays performed in triplicate revealed that cells of *V. fischeri* lacking *xds* or *dns* were like the wild type in that they colonized squid with 100% efficiency by 24 hours post-infection (Fig. 9A). Relative to the wild type, the *xds* strain produced roughly equivalent amounts of light per squid, while the *dns* strain emitted on average about 20-fold less light per squid by 48 hours post initial exposure. Because we did not detect a luminescence defect in either the *xds* or *dns* strains (Fig. 9B), these findings suggest that inactivation of *dns* results in reduced colonization efficiency. In competition experiments, combining the wild type with either nuclease mutant in equal proportions, differences in colonization efficiency were magnified. The wild-type strain

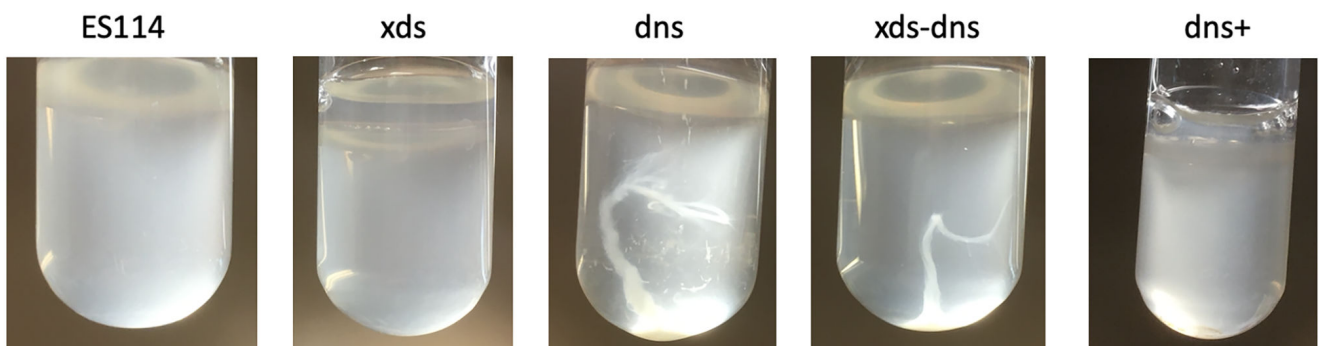


FIG 7 *Dns* mutant cells display a hyper-aggregation phenotype. Cells were grown in a minimal medium without shaking for up to 1 week, then gently mixed to visualize aggregation. Only strains lacking *dns* (i.e., *dns* and *xds-dns*) showed an aggregation phenotype.

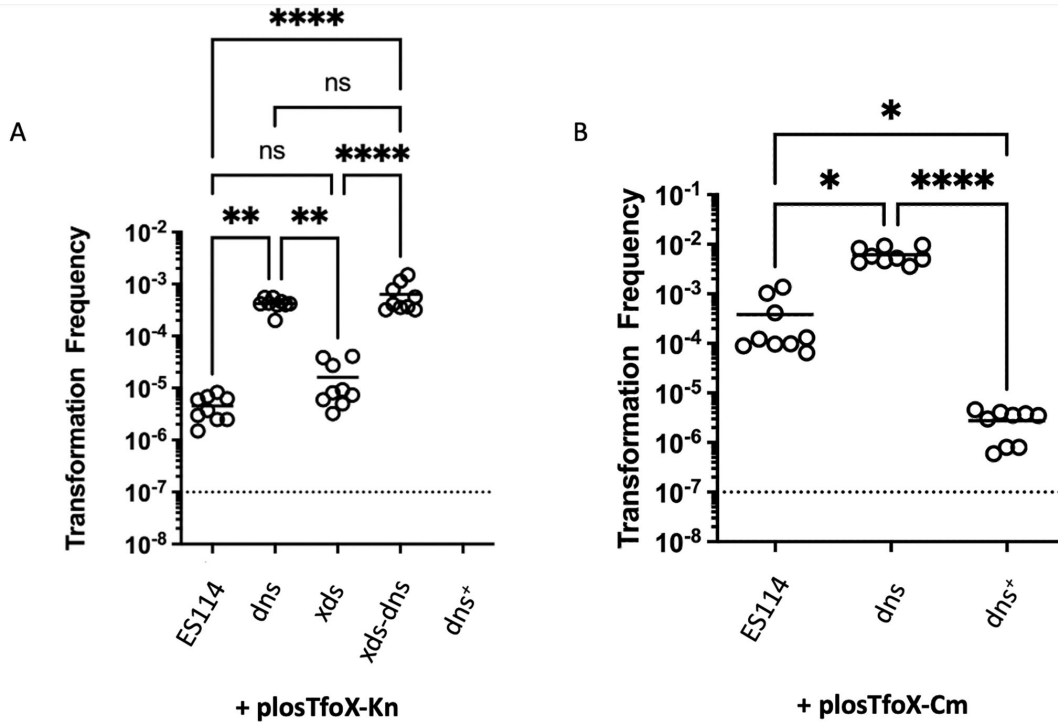


FIG 8 The *dns* mutant is hyper-transformable. Strains ES114, *dns* (KV8807), *xds* (KV8811), *xds-dns* (KV8865), and *dns*⁺ (KV10273) carrying a *tfoX* expression plasmid with either kanamycin (A) or chloramphenicol resistance (B) were grown at 28°C with shaking in TMM to an OD₆₀₀ of approximately 0.5. Aliquots of each culture were exposed to purified DNA containing an antibiotic resistance marker. Cells were plated on selective media, transformants were counted to estimate transformation frequency, and an ANOVA test was performed assuming a normal distribution. The dotted lines indicate the limit of detection. *, *P* < 0.05; **, *P* < 0.005; ****, *P* < 0.00005. n.s. = not significant

outcompeted the *xds* and *dns* strains in about 75% and 92% of squid, respectively; indeed, for three squids, the *dns* mutant was excluded (Fig. 10). These data indicate that expression of *dns*, and to a lesser extent *xds*, provides a colonization advantage to *V. fischeri*.

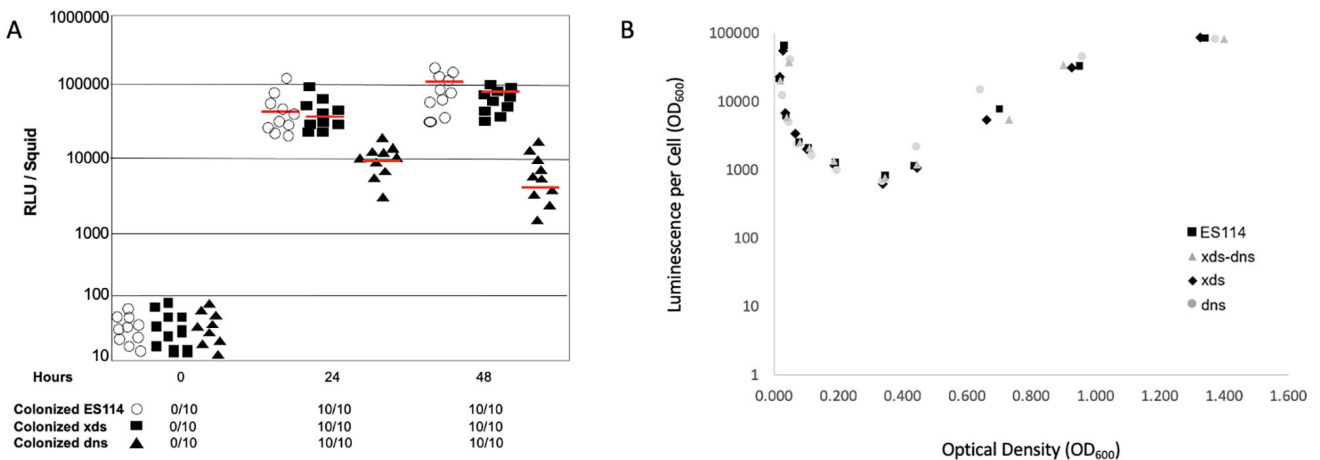


FIG 9 Single-strain squid colonization. (A) Closed squares, closed triangles, and open circles represent bioluminescence emission per squid infected with either *xds*, *dns*, or the wild-type strain ES114, respectively. Red lines represent average relative light units (RLU) per squid over the 48 hours. The number of colonized squid was determined by comparing RLU emissions between aposymbiotic squid and the test strains. (B) Nuclease is not required for normal growth or luminescence in culture. All results shown are typical for three independent trials.

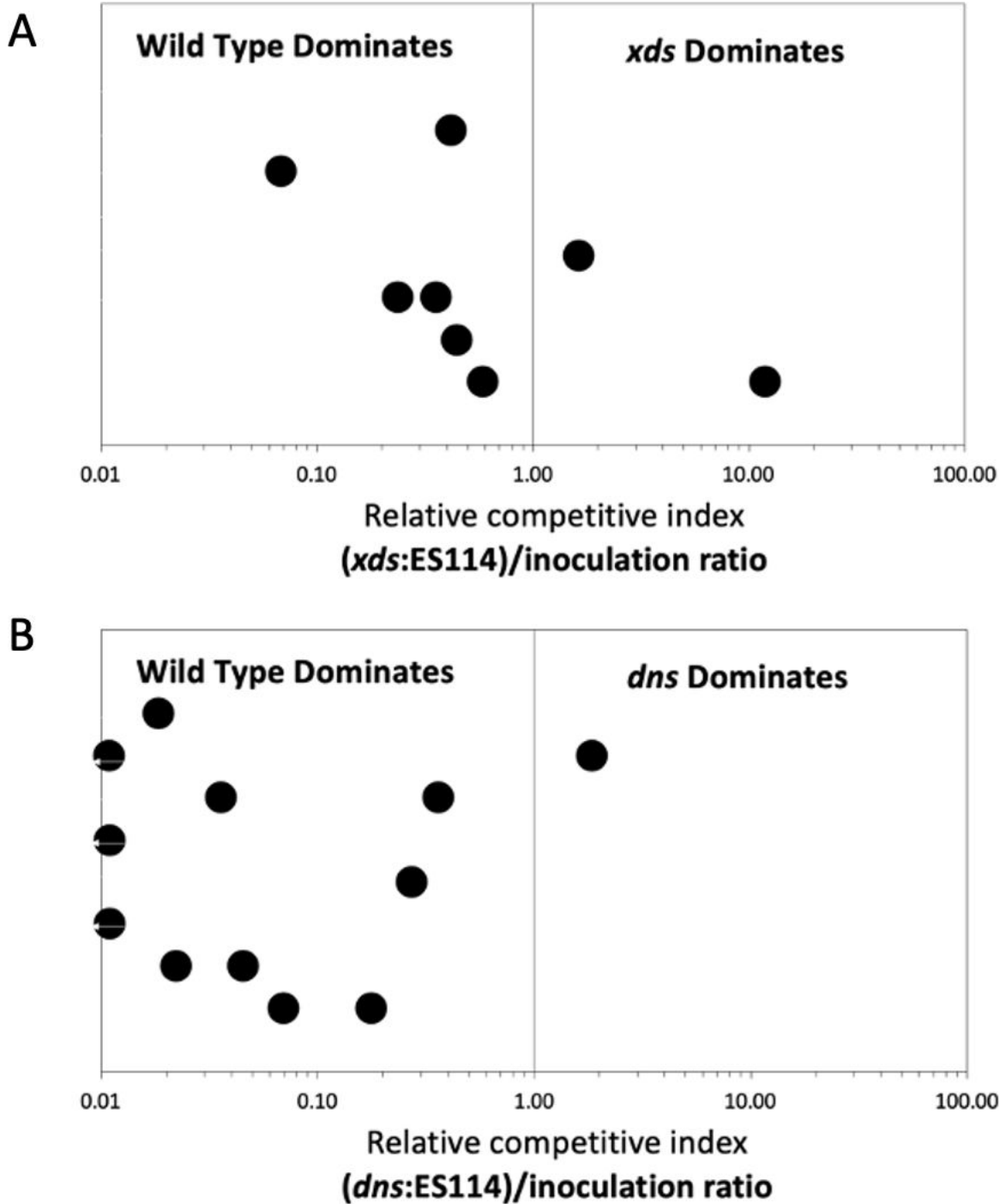


FIG 10 Nucleases are required for optimal squid colonization. Juvenile squid were exposed to an inoculum containing a 1:1 ratio of wild-type *V. fischeri* and either the *xds* (A) or *dns* (B) mutant. At 48 hours post-infection, the ratio of mutant to wild-type bacteria in each light organ was assessed by plating squid light organs. The results are expressed as the relative competitive index (RCI). An RCI above 1.0 indicates that the mutant was dominant. These results are representative of three separate trials.

DISCUSSION

V. fischeri produces two nucleases that are homologs of the exonucleases Xds and Dns first characterized in *V. cholerae* (10, 11). Xds activity was the predominant nuclease detected in culture, with Dns activity being most apparent in an *xds* mutant background after extended incubation. Xds of *V. cholerae* require divalent cations (i.e., Ca^{2+} and Mg^{2+})

(19), and this seems to be the case for *V. fischeri* as well, as secreted nuclease activity was completely abolished by the addition of EDTA. Both nucleases possess signal peptide cleavage sites, suggesting that they are secreted by the general secretion (Sec) protein export pathway. Closer homology between the *dns* homologs in *V. fischeri* and *V. cholerae* is consistent with their genomic synteny and shared roles in biofilm formation and natural transformation (10, 11). Similarly, the relatively greater evolutionary distance in Xds proteins is consistent with their lack of synteny. Still, the role of Xds in *Pseudomonas aeruginosa*, *V. cholerae*, and *V. fischeri* (i.e., degradation of eDNA to nucleotides for nutritional purposes) is conserved (15, 19).

In *V. cholerae*, *dns* is negatively regulated at high cell density by HapR (10), which is homologous to LitR in *V. fischeri* (9). However, evidence from microarrays revealed that LitR does not affect either *xds* or *dns* expression at OD₆₀₀ values of up to 2.0 (20). Consistent with those data, the loss of Dns did not substantially alter nuclease activity when assessed at either OD of 0.5 or 2. However, Dns activity might become important at later stages of growth, as evidenced by the late impact of the loss of Dns on the growth of eDNA (Fig. 5A), plasmid DNA digestion (Fig. 4B), eDNA digestion on DNase agar (Fig. 2B), and biofilm aggregate formation (Fig. 7). Whether this late requirement for Dns is due to transcriptional control via LitR that was not apparent from the microarray or is due to another factor remains to be determined. In *V. cholerae*, *xds* is regulated by the two-component system PhoR/PhoB and phosphate limitation (21). Our findings suggested that nuclease activity is phosphate-dependent (Fig. 3B), but additional work will be required to unravel the regulatory mechanisms for the two *V. fischeri* nucleases.

Both *V. fischeri* nuclease mutants exhibited colonization defects, with the *dns* mutant being more severely impaired. These mutants displayed multiple phenotypes to differing extents, including biofilm formation and the ability to degrade and/or grow on eDNA. Any or all of these traits could be critical for normal symbiotic colonization. For example, biofilm formation is critical for cells of *V. fischeri* to initiate the squid colonization process (22). Initially, cells are collected in aggregates outside of the light organ pores and must eventually detach and swim into the light organ interior (5). Deletion of *rscS*, encoding a sensor kinase that promotes biofilm formation, severely impaired squid colonization, while overexpression enhanced it (22, 23). In this study, the *dns* mutant displayed an increase in cell-cell aggregation, potentially due to the presence of excess eDNA on the bacterial surface (24). However, unlike with *rscS* overexpression leading to excess biofilm and superior colonization, the *dns* mutant was associated with a severe colonization defect, suggesting that hyper-aggregation may be detrimental. While biofilm formation (aggregation) is essential in the early phases of colonization, *V. fischeri* cells also need to detach from the aggregate and/or squid mucus to reach the light organ interior where colonization proceeds (5). Thus, it is possible that the hyper-aggregation of the *dns* mutant impaired cellular detachment, resulting in fewer cells reaching the light organ interior.

Alternatively, the importance of Dns in colonization could stem from a role in generating/altering a specific structure of the biofilm. For example, biofilms produced by many bacteria are laden with extracellular DNA that can be degraded and remodeled by secreted nucleases to increase colonization fitness when bacteria enter a host (11). In addition, host secretions may contribute to biofilm formation, adherence, and/or inhibit dissemination (25). Pus and mucus secretions contain eDNA, which can strengthen adhesivity (25, 26). Pathogenic microbes such as *V. cholerae* cells are proposed to utilize extracellular nucleases to degrade mucus secreted in the host small intestine (27). *V. fischeri* may likewise use nucleases to degrade or alter bacterial eDNA and/or squid-secreted mucus. While our findings suggested that Xds is primarily responsible for degrading the DNA component of mucus, it is possible that symbiosis-specific conditions enhance the production or activity of Dns. Alternatively, the less severe defect of the *xds* mutant during competitive colonization experiments may stem from exogenous complementation by the wild-type strain present in the same location.

Other known eDNA-related host mechanisms include the production of neutrophil (PMN) extracellular traps (NETs) (28). In *V. cholerae*, both Xds and Dns contribute to the degradation of NETs, promoting colonization of the human gastrointestinal tract (14). In the squid symbiosis, PMN-like hemocytes are recruited to the light organ interior by newly colonizing cells of *V. fischeri* (29). One role of hemocytes is to remove undesirable microbes from the light organ interior, although there is currently no evidence of an interaction between *V. fischeri* and eDNA-containing NETs.

The Dns and Xds nucleases may also be important in symbiotic colonization due to their role in nutrient acquisition (Fig. 5A). Microbes such as *V. cholerae* and *P. aeruginosa* use Xds homologs to scavenge phosphate from eDNA in nutrient-poor environments (15, 19). The heterogeneous distribution of phosphate in the squid light organ suggests that there is selective pressure to optimize phosphate scavenging, potentially via eDNA nuclease activity (30).

Lastly, we also investigated the relationship between nuclease secretion and natural transformation. Secreted nucleases affect natural competency in a wide range of bacteria, such as *V. cholerae* and *Staphylococcus aureus* (10, 31). For example, the inactivation of *dns* in *V. cholerae* resulted in a hypertransformable phenotype (10). Similarly, the inactivation of *dns*, but not *xds*, resulted in a hypertransformable phenotype by *V. fischeri* (Fig. 8). Xds catalyzes readily demonstrable, fairly rapid degradation of linear and plasmid eDNA (Fig. 4). Thus, on the surface it is surprising that the major secreted nuclease Xds did not impact transformation. However, these data corroborate work in *V. cholerae* (10) and demonstrate that the roles of the two nucleases are distinct, potentially in part due to a difference in where these proteins localize. Our findings support a model in which Xds is secreted, while Dns is periplasmic but leaks into the environment over time. Future studies will determine whether Dns in *V. fischeri* is localized to the periplasmic as in *V. cholerae* (32). Taken together, these findings contribute to our understanding of competence in *V. fischeri*, which has far-reaching implications for understanding how this microbe evolves and how it can be manipulated in the laboratory.

This study thus reveals a range of activities associated with secreted nucleases that are vital to the beneficial squid symbiont *V. fischeri*. These activities include phosphate acquisition, cell-cell aggregation, natural competence, and host colonization. Investigations into specific aspects of the squid-*Vibrio* association, such as aggregation and avoidance of hemocyte NETs, should allow us to bore down on the mechanisms by which nucleases might influence these critical colonization processes.

MATERIALS AND METHODS

Strains and media

Strains and plasmids used in this study are listed in Table 1. The parent *V. fischeri* strain used in this study (e.g., ES114) was isolated from *E. scolopes* (33) and was grown aerobically using various media at 24°C–28°C. For molecular biology and enzyme assay applications, *V. fischeri* cells were cultured in lysogeny broth salt (LBS) containing 10 g of tryptone, 5 g of yeast extract, 20 g of NaCl, and 20 mM Tris-hydrochloride (Tris HCl, pH 7.5), per liter of sterile water. All squid colonization and bioluminescence assays used seawater tryptone (SWT) which contained 5 g of tryptone, 3 g of yeast extract, 3 mL of glycerol, and 700 mL of artificial seawater (ASW) per liter of sterile water. *Escherichia coli* was cultured in LB-Miller which contained 10 g tryptone, 5 g yeast extract, and 10 g NaCl. Agar was added at 15 mg mL⁻¹ for media solidification for plating. Where necessary, antibiotics chloramphenicol, erythromycin, and kanamycin were added to growth media at 2.5, 5, and 100 µg mL⁻¹, respectively, for *V. fischeri* and 30, 100, and 150 µg mL⁻¹, respectively, for *E. coli*. DNase activity was assessed using DNase Agar (Thermo Fisher), as well as porcine mucin agar containing 1% mucin (Sigma-Aldrich), 500 mL of ASW per liter of sterile water, 5% Tris-HCl (1 M, pH 7.4), 0.3% glycerol, and 0.006% K₂HPO₄, and 1.5% agar. To visualize nuclease activity, DNase and Mucin agar plates were

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Descriptor	Source or reference
Strains		
ES114	Wild type	33
xds (KV7913)	<i>xds::miniTn5 erm^R</i>	This study
xds (KV8811)	<i>Δxds::FRT-erm</i>	This study
dns (KV9606)	<i>Δdns::FRT</i>	This study
dns (KV8807)	<i>Δdns::FRT-erm</i>	18
xds ⁺ (KV10274)	<i>xds</i> + pVSV105/ <i>xds cm^R</i>	This study
dns ⁺	<i>dns</i> + pVSV105/ <i>dns cm^R</i>	This study
dns ⁺ (KV10273)	<i>Δdns::FRT IG (yeiR-FRT-Erm/glmS)::PnrdR dns</i>	This study
xds-dns (KV10492)	<i>xds::miniTn5 erm^R/Δdns::FRT</i>	This study
xds-dns (KV8865)	<i>Δxds::FRTΔdns::FRT</i>	This study
Plasmids		
pESV104	Helper plasmid (<i>tra⁺, mob⁺, kn^R</i>)	38
pVSV105	Shuttle vector (<i>cm^R</i>)	40
pEVS170	miniTn5 delivery plasmid (<i>erm^R</i>)	38
plosTfoX-Cm	TfoX overexpression (<i>cm^R</i>)	36
plosTfoX-Kn	TfoX overexpression (<i>kn^R</i>)	37

treated with either 1N HCl or 0.1% Amido black in 3.5 M acetic acid, respectively. Mucin plates were de-stained with 1.2 M acetic acid. The liquid medium for aggregation assays was a modified HEPES-minimal medium that contained ASW, 0.1% (wt/vol) ammonium chloride, 0.0058% K₂HPO₄, 10 μM ferrous ammonium sulfate, 100 mM HEPES, pH 7.5, and 0.03% casamino acids. For natural transformation and *in vitro* luminescence experiments, *V. fischeri* strains were grown in TMM (100 mM Tris, pH 7.5, 300 mM NaCl, 0.1% ammonium chloride, 10 mM *N*-acetylglucosamine, 50 mM MgSO₄, 10 mM KCl, 10 mM CaCl₂, 0.0058% K₂HPO₄, 10 μM ferrous ammonium sulfate).

Constructing nuclease mutants and complementation

Mutants were constructed either by random transposon mutagenesis (*xds*; 34, 35) or by TfoX-mediated transformation and in-frame allelic replacement [*dns*, *dns* (KV8807), *xds* (KV8811), *xds-dns*, and *xds-dns* (KV8865)], and complemented *dns*⁺ (KV10273; 36, 37) strains were constructed by TfoX-mediated transformation. Transposon delivery to create the *xds* strain was carried out using an *E. coli* donor strain (carrying pEVS170) and *E. coli* helper strain CC118 *λpir* carrying pESV104 (38). Two thousand mutants were streak purified on LBS and then patched onto DNase agar. After 24-hour growth, plates were flooded with 1N HCl to detect a patch lacking nuclease activity. For TfoX-mediated transformation and allelic exchange, we introduced a *tfoX* overexpression plasmid from *E. coli* via triparental conjugations using an *E. coli* strain containing the *tfoX* overexpression plasmid, a pEVS104 (38)-containing *E. coli*, and the recipient *V. fischeri* strain. Strains containing the *tfoX* plasmid were then transformed with either PCR DNA derived from PCR Splicing by Overlap Extension (SOE) reactions or from genomic DNA obtained from a strain carrying an antibiotic-marked deletion or insertion. DNA fragments for PCR SOE were constructed as previously described (39) using high-fidelity PCR enzyme EMD Millipore KOD DNA polymerase and primers listed in Table 2. The reactions allowed for the construction of a fragment that contains an antibiotic resistance cassette flanked by sequences homologous to ~500 bp sequences up and downstream of the gene/region of interest. The PCR or genomic DNA was transformed into *tfoX* overexpression strains, and the resulting cultures were plated onto a selective medium. Colonies that arose were passaged onto the same selective medium once more and then onto a non-selective medium to facilitate the loss of the unstable overexpression plasmid. Strains were confirmed to have the insertion or deletion of interest via a PCR reaction using outside primers. The *dns* complementation cassette was sequenced (ACGT, Inc., Wheeling, IL)

TABLE 2 Primers used in this study

Name	Sequence	Purpose
Xds-Ndel-F	CCCCATATGGAGCTCTGCCAGATTTGTAG	Complement VF_1451 (<i>xds</i>)
Xds-Ndel-R	CCCCATATGTGCAAAGTGGTGCAGAAACC	Complement VF_1451 (<i>xds</i>)
Dns-Ndel-F	CCCCATATGCGGTACGTGCCATAATCGA	Complement VF_0437 (<i>dns</i>)
Dns-Ndel-R	CCCCATATGTTTCGATTACACGACCAACG	Complement VF_0437 (<i>dns</i>)
VIPCR-F	CCTAGAGCGGCCGAGA	Tn localization in VF_1451 (<i>xds</i>)
VIPCR-RBio	Biotin-ACTGGCCGTCGTTTTACAG	Tn localization in VF_1451 (<i>xds</i>)
MoSeq-F	AGATGTGTATAAGAGAC	Tn localization in VF_1451 (<i>xds</i>)
2508	CTTCCAGCCATAACTCTCC	Delete VF_1451 (<i>xds</i>); strain KV8811
2509	taggcggccgcactaagatggACTTTCCATAGTTGAGTTATATGAA	Delete VF_1451 (<i>xds</i>); strain KV8811
2089	CCATACTTAGTGGCGCCCTA	Delete VF_1451 (<i>xds</i>); strain KV8811
2090	CCATGGCCTTCTAGGCCTATCC	Delete VF_1451 (<i>xds</i>); strain KV8811
2510	ggataggcctagaagccatggACGTAAATAACCGTTATAAAGAGC	Delete VF_1451 (<i>xds</i>); strain KV8811
2511	GCAAGATTACATCGTTAAAGCC	Delete VF_1451 (<i>xds</i>); strain KV8811
2090	CCATGGCCTTCTAGGCCTATCC	Complement VF_0437 (<i>dns</i>); strain KV10273
2290	AAGAAACCGATACCGTTTACG	Complement VF_0437 (<i>dns</i>); strain KV10273
3352	ggataggcctagaagccatggAGGAGGACTACTTACTGAATTAATACAATCATCAGTAG	Complement VF_0437 (<i>dns</i>); strain KV10273
3353	taggcggccgcactaagatggaGGAGGTTAAAGTATGCTTTTTCAACC	Complement VF_0437 (<i>dns</i>); strain KV10273
2196	tccatacttagtgccgccccta	Complement VF_0437 (<i>dns</i>); strain KV10273
1487	GGTCGTGGGGAGTTTTATCC	Complement VF_0437 (<i>dns</i>); strain KV10273

to ensure no mutations arose. To remove antibiotic cassettes (Erm^R or Trim^R) from the insertion mutants and complementation strains, we performed a triparental mating with the *V. fischeri* strain of interest, an *E. coli* strain bearing pEV5104, and an *E. coli* strain bearing pKV496 containing the flippase that recognizes FRT sites as previously described (38), followed by selection for pKV496 (Kan^R). Conjugants were then assessed for sensitivity to Erm or Trim. Sensitive colonies were confirmed by PCR to have lost the antibiotic cassette and were passaged non-selectively to facilitate the loss of pKV496.

The *dns* and *xds-dns* mutants were constructed using PCR SOE to remove *dns* through a series of PCRs (39). For in-frame allelic replacement, the resolution of the FRT-flanked antibiotic resistance cassettes was achieved using the flippase plasmid pKV496 (39). For complementation of either *xds* or *dns*, the gene (including the ribosome binding site through the stop codon) was amplified, inserted into pVSV105 plasmid (40), and then mobilized into the recipient cells by tri-parental mating. Primers for Xds (Xds-Ndel-F/R) and Dns (Dns-Ndel-F/R) complementation are listed in Table 2.

Determining the site of Tn5 insertion in *V. fischeri* strains

Transposon insertion was localized in nuclease mutants as described previously (35). Genomic DNA was extracted from nuclease mutants, cut with *Sau3AI*, and self-ligated to form closed circular DNA fragments. Next, fragments were subjected to iPCR using primers VIPCR-F and VIPCR-RBio to amplify DNA flanking the transposon insertion, then amplicons were sequenced using primer MoSeq-F and a pyrosequencer to identify the flanking DNA (Table 2).

eDNA degradation assay

All strains of *V. fischeri* were incubated in TMM overnight shaking at 225 rpm at 28°C to an optical density at 600 nm (OD₆₀₀) of about 2.0. One milliliter of culture was pelleted by centrifugation. The cell-free supernatant was withdrawn and combined with purified PCR or plasmid DNA. The mixtures were incubated for 5 to 60 minutes at 28°C. The degree of DNA degradation was assessed on a 1% agarose gel.

Quantitative nuclease assay

Nuclease activity was quantified using the DNaseAlert QC System (ThermoFisher), according to the manufacturer's instructions. *V. fischeri* strains were grown with shaking in LBS at 28°C to OD₆₀₀ readings of 0.5 and 1.0. One-milliliter aliquots of culture were pelleted by centrifugation. Eighty microliters of each supernatant were combined with 10 µL of 10× NucleaseAlert buffer and 10 µL DNaseAlert Substrate in wells of a microtiter plate and incubated at 37°C for 60 minutes. Fluorescence was measured using a Cytation 5 Imaging Reader (BioTek Instruments, Inc.). DNase I (2 U µL⁻¹) was utilized as a positive control. To measure the effects of a chelating agent on nuclease activity, some supernatants were also combined with 10 mmol L⁻¹ EDTA.

Role of nuclease activity in phosphate scavenging

Strains were grown shaking at 225 rpm in TMM overnight. TMM was prepared as a complete medium (i.e., containing all nutrients to support *V. fischeri* cells), or without a phosphate (i.e., K₂HPO₄) source. Media without a phosphate source was supplemented with exogenous salmon sperm DNA to a final concentration of 1.0 mg mL⁻¹. Cells of nuclease mutants and wild-type *V. fischeri* were inoculated into separate wells of a microtiter plate. Initial OD₆₀₀ was ~0.015 for all cultures. The cells were grown at 28°C in a Cytation 5 imaging Reader (BioTek Instruments, Inc.) shaking at 237 rpm for 20 hours. Optical density readings at 600 nm were taken every 10 minutes.

Transformation assay

Transformation assays were performed as described in Cohen et al. (18), using ES114 (wild-type strain), and the following genetically modified strains: *dns* (KV8807), *xds* (KV8811), *xds-dns* (KV8865), and *dns*⁺ (KV10273). In brief, *V. fischeri* strains carrying *tfoX* overexpression plasmids with kanamycin (*plstfoX-Kn*) (37) or chloramphenicol (*plstfoX-Cm*) (36) resistance were grown overnight in TMM containing the appropriate antibiotic. In ES114-derived strains, *plstfoX-Kn* promotes a transformation efficiency over 10× lower than that induced by *plstfoX-Cm* and thus permits experimental observations of increased transformation efficiency such as seen with a *dns* mutant (18). Cells were subcultured into fresh TMM and grown to an OD₆₀₀ of about 0.5. Cells were inoculated with chromosomal DNA (~0.5 µg) from strain KV8300 (Δ *fliQ*::Frt-Trim) (39) or left untreated and incubated at room temperature for 30 minutes without shaking. LBS (0.5 mL) was added to the cells in TMM and the cultures were grown with shaking at 28°C for at least 90 minutes. The cultures were then serially diluted or left undiluted and 100 µL of each serial dilution was plated onto antibiotic plates (LBS containing trimethoprim) for selection of transformants. The plates were incubated overnight at 28°C. Total colony counts were performed to assess transformation efficiency at each dilution. Untreated control samples were diluted and plated onto LBS plates alone to obtain total colony-forming units (CFU). As described in Cohen et al. (18), we calculated transformation frequency by multiplying the number of antibiotic-resistant transformants by the dilution factor and dividing by the total calculated CFU.

Squid colonization

Juvenile *E. scolopes* were collected within minutes of hatching. Hatchlings were removed with plastic eye droppers and held in ASW at 22°C for at least 30 minutes before

inoculating the water with 3,000 CFUs of either wild-type *V. fischeri* the *xds* mutant, or the *dns* mutant. The individual squid were then washed in sterile seawater and placed in their scintillation vial filled with 5 mL of ASW. Luminescence emission for each squid was measured for up to 48 hours using a GloMax 20/20 luminometer. Luminescence emission was used to assess colonization effectiveness. For co-colonization assays, squid was exposed to roughly a 1:1 mixture of the wild-type strain and either mutant for 12 hours; squid was then washed and transferred to symbiont-free seawater for the remainder of the experiment. After 48 hours, squid light organs were plated on selective and non-selective media to assess the relative proportions of each strain.

***In vitro* luminescence and growth**

Strains were grown with shaking at 28°C in SWT. At regular intervals, aliquots of each culture were assayed for cell density in a spectrophotometer and luminescence in a GloMax 20/20 luminometer.

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Pat M. Fidopiastis, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review and editing.

DATA AVAILABILITY

Supporting data will be made available upon reasonable request.

ETHICS APPROVAL

All animal experiments were conducted in compliance with protocol number A18-029 approved by the Institutional Animal Care and Use Committee, University of Connecticut.

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