The N-acetyl-D-glucosamine repressor NagC of Vibrio fischeri facilitates colonization of Euprymna scolopes

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Summary
To successfully colonize and persist within a host niche, bacteria must properly regulate their gene expression profiles. The marine bacterium Vibrio fischeri establishes a mutualistic symbiosis within the light organ of the Hawaiian squid, Euprymna scolopes. Here, we show that the repressor NagC of V. fischeri directly regulates several chitin- and N-acetyl-D-glucosamine-utilization genes that are co-regulated during productive symbiosis. We also demonstrate that repression by NagC is relieved in the presence of N-acetyl-D-glucosamine-6-phosphate, the intracellular form of N-acetyl-D-glucosamine. We find that gene repression by NagC is critical for efficient colonization of E. scolopes. Further, our study shows that NagC regulates genes that affect the normal dynamics of host colonization.

Introduction
Vibrio fischeri is a Gram-negative bacterium utilized by several species of marine organisms as a source of bioluminescence. Like many of these animals, the Hawaiian squid Euprymna scolopes has evolved an organ that harnesses this bioluminescence while providing shelter and nutrients specifically to this beneficial microbe (McFall-Ngai, 2008). The mutualistic symbiosis is established shortly after squid hatch, and is maintained throughout the lifetime of the animal. A recent transcriptome analysis of the mature symbiosis of adult animals has revealed certain correlated patterns of bacterial gene expression, particularly with metabolic genes predicted to be involved in the utilization of chitin and its monomer, N-acetyl-D-glucosamine (GlcNAc) (Wier et al., 2010).

Chitin is produced in large quantities by many invertebrates to provide structural integrity to their exoskeletons (Keyhani and Roseman, 1999). These chitin polymers are insoluble and as a result, are nutritionally accessible to microbes only by means of chitinases that break the polymer down into its subunits. GlcNAc and chitobiose (GlcNAc)2 are released by exochitinases, which hydrolyse the glycosidic bonds between GlcNAc or chitobiose subunits. As an aminosugar, GlcNAc serves as a source of both carbon and nitrogen, and appears to be a preferred carbon source by many vibrios (Hunt et al., 2008).

Our knowledge of the uptake and metabolism of GlcNAc in Gram-negative bacteria is primarily due to studies of four genes (nagE and nagBAC) within the nag locus of Escherichia coli (Rogers et al., 1988; Plumbridge, 1991; Alvarez-Anorve et al., 2009). Briefly, the nagE gene encodes enzyme IICGlcNAc, IIBGlcNAc and IIAGlcNAc components of a PTS transporter specific for GlcNAc. During its transport into the cytoplasm, GlcNAc is phosphorylated on the 6-carbon within the glucose subunit, resulting in N-acetyl-D-glucosamine-6-phosphate (GlcNAc-6P). GlcNAc-6P is the substrate for the deacetylase NagA, which removes the acetyl group to yield glucosamine-6P. The amine group is then cleaved from glucosamine-6P by the deaminase NagB. NagC is a transcription factor that represses the nag locus by binding to two distinct operator sites upstream of the divergently transcribed nagE and nagBAC operons. Repression of the nag genes by NagC is relieved by allosteric binding of GlcNAc.

Here we show that NagC directly regulates genes involved in the utilization of chitin and GlcNAc in V. fischeri. Mutants unable to repress genes controlled by NagC were outcompeted by wild-type cells during colonization of the squid light organ. The initial selection for strains of V. fischeri that repress genes via NagC could ensure that the squid host retains a symbiont population with the ability to regulate chitin- and GlcNAc-utilization genes as the symbiosis matures.

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Results

NagC regulates chitin- and GlcNAc-utilization genes in V. fischeri

A previous study of the squid–vibrio system revealed that numerous bacterial genes predicted to be associated with the utilization of chitin and GlcNAc are co-regulated during symbiosis (Wier et al., 2010). We hypothesized that this pattern of gene expression arises from the activity of a single bacterial regulator. We selected VF_1598, which is predicted to encode a secreted exochitinase, as a representative gene from the group of co-regulated genes because this gene showed the most pronounced dynamics among the chitin- and GlcNAc-utilization genes in the study mentioned above. Furthermore, VF_1598 is conserved in the V. fischeri fish symbiont MJ11 (VFMJ11_1708), but has diverged in Allivibrio salmonicida LFI1238 to lose the chitin-binding domain (ChtBD3), suggesting that this biological activity of VF_1598 is specific to V. fischeri species. To monitor VF_1598 expression, we constructed a reporter plasmid (pTM314) by cloning the intergenic region upstream of VF_1598, which presumably contains the corresponding promoter, upstream of gfp in pTM267 (Miyashiro et al., 2010). The plasmid pTM267 constitutively expresses mCherry, and has an origin of replication that is maintained in V. fischeri. In cells harbouring pTM314, the GFP/mCherry fluorescence ratio is a quantitative measure of VF_1598 gene expression.

To identify potential regulators of VF_1598, we conjugated pTM314 into a library of approximately 10⁵ transposon mutants of the wild-type V. fischeri strain ES114. We screened 500,000 colonies by fluorescence, and isolated 160 mutants with elevated levels of GFP. Sequencing of the insertion in six of the candidate strains revealed four unique mutants in which the transposon had inserted into either nagA (WPK54) or nagC (WPK84) (Fig. 1A). Although transposon insertions in either gene resulted in elevated VF_1598 expression compared with wild type (Fig. 1B), we assumed that the insertions in nagA are polar on nagC.

To determine whether NagC regulates other chitin- and GlcNAc-utilization genes in V. fischeri, we constructed an in-frame deletion of nagC in ES114 (∆nagC). As with the transposon mutants described above, the level of VF_1598 expression in ∆nagC was higher than wild type (Fig. 1B). Consistent with enhanced VF_1598 expression, we measured approximately sixfold higher levels of exochitinase activity in the supernatants of ∆nagC cultures compared with wild type (Fig. 1C).
Using quantitative reverse-transcriptase PCR (qPCR), we analysed the effect of NagC on other chitin- and GlcNAc-utilization genes that exhibit an expression pattern correlated with the diel rhythm of the symbiosis (Wier et al., 2010). Consistent with our results using pTM314, VF_1598 transcript levels are elevated in a ΔnagC strain compared with wild type (Fig. 2). Furthermore, overexpression of nagC in trans was sufficient to decrease VF_1598 transcription compared with ΔnagC. While VF_0655 and VF_A0143 transcript levels are independent of NagC, VF_2139, VF_A0143 and VF_A0715 are transcriptionally repressed by overexpression of nagC. Examination of the promoter region of these genes revealed potential binding sites upstream of VF_1598, VF_2139 and VF_A0715 but not upstream of VF_A0143 (Fig. S1). Together, these results suggest that NagC can regulate some, but not all, genes predicted to be associated with utilization of chitin and GlcNAc.

NagC controls responses to extracellular GlcNAc

Studies with E. coli have shown that NagC de-represses gene expression in response to the presence of extracellular GlcNAc. Among the genes repressed by NagC are nagE, nagA and nagB, which encode, respectively, the GlcNAc PTS enzyme, deacetylase and deaminase that are required to uptake and convert GlcNAc to fructose-6-phosphate (Plumbridge, 1991). Using qPCR, we have found that nagE, nagA and nagB are repressed by NagC in V. fischeri (Fig. 2). Furthermore, nagB is required for V. fischeri to preferentially utilize GlcNAc, suggesting that the metabolism of GlcNAc is similar to that of E. coli (Fig. S2). We attribute the growth of the nagA mutant WPK54 on GlcNAc to the presence of two additional copies of nagA (VF_0665 and VF_A0999) within the genome of ES114.

To monitor nagA expression in vivo, a two-colour fluorescent reporter plasmid (pTM355) was constructed by cloning the nagA promoter upstream of gfp in pTM267. The exposure of wild-type cells grown under steady-state conditions to medium containing 20 mM GlcNAc was sufficient to induce the expression of nagA 15-fold compared with the medium blank alone (Fig. 3). Compared with wild-type cells, nagA expression in the ΔnagC mutant was 13-fold higher, and induced less than 1.5-fold in the presence of 20 mM GlcNAc. Together, these results show that NagC controls the response of the nag operon to extracellular GlcNAc in V. fischeri.
NagC specifically binds upstream of nagA and VF_1598

In E. coli, NagC represses expression of the nag operon by directly binding two operator sites between the nagB and nagE genes (El Qaidi and Plumbridge, 2008). When bound by NagC, these operator sites, which are separated by 71 bp, lead to the formation of a DNA loop that strengthens the repression of the nagB and nagE promoters (Plumbridge and Kolb, 1998). By examining the corresponding region within the V. fischeri nag locus, we identified two putative binding sites with sequences similar to the consensus sequence of the NagC operator site in E. coli (Fig. S1, VF_0807-VF_0808, boxes 3 and 4). In V. fischeri, as well as in all fully sequenced Vibrio species members, the distance between the two putative operator sites is also 71 bp. This observation not only provides further bioinformatics support of the binding sites in V. fischeri, but also suggests that the cooperativity between the NagC dimers bound at the two sites is a conserved regulatory element of the nag locus. Using 5'-RACE, we have also determined that the transcriptional start site of nagA is located within the proximal NagC operator site (Fig. 4A), which suggests that NagC blocks transcription of the nagAC operon. Interestingly, there are also two potential NagC operator sites located further within the untranslated region of the nagA transcript (Fig. S1, VF_0807-VF_0808, boxes 1 and 2), but their effect on the nagA promoter is unknown at this time.

To study the DNA-binding properties of the V. fischeri NagC, we cloned, expressed, and purified NagC with C-terminal myc and His tags. Previous studies with NagC in E. coli have shown that the C-terminus is not involved in
DNA binding (Pennetier et al., 2008). Using anisotropy to detect protein binding of fluorescently labelled DNA probes, we found that NagC bound the operator sequence proximal to nagA (Fig. 4A). In addition, replacement of two thymine bases in the consensus sequence with two guanine bases abrogated the specific binding of the operator by NagC. Consistent with this result, we found that mutating the same bases in the nagA promoter-reporter plasmid eliminated NagC regulation of nagA (Fig. 5).

Although we identified two putative NagC binding sites upstream of VF_1598 (Fig. S1, VF_1597-VF_1598, boxes 1 and 2), only box 2 is conserved upstream of the VF_1598 homologue in MJ11. Using a DNA probe containing this operator sequence, we found that, as predicted, the presence of NagC increased the anisotropy of the fluorescent probe (Fig. 4A). Together, these results demonstrate that NagC directly binds sites upstream of both nagA and VF_1598.

NagC repression is relieved in response to GlcNAc-6P

In E. coli, DNA binding by NagC is relieved in the presence of GlcNAc-6P (Pennetier et al., 2008). To determine whether the V. fischeri NagC also responds to GlcNAc-6P, we measured binding of NagC to the nagA operator site in the presence or absence of GlcNAc-6P. The presence of 10 mM GlcNAc-6P was sufficient to release NagC (Fig. 4B). In contrast, a variant of NagC containing the mutation E241A, which corresponds to the E244A mutation in the NagC of E. coli that disrupts the binding pocket for GlcNAc-6P (Pennetier et al., 2008), remained bound to the nagA operator site even at 10 mM GlcNAc-6P (Fig. 4B), thereby functioning as a ‘super-repressor’. The E241A mutation did not affect the affinity NagC has for the nagA operator site in the absence of GlcNAc-6P (data not shown).

To test whether the E241A mutation disrupts NagC regulation in vivo, we measured the response of the nagA promoter reporter plasmid pTM355 in cells exposed to GlcNAc. Cells containing nagC-myc-6xHis at the wild-type nagC locus (strain TIM377) displayed a 4.4-fold increase in nagA expression (Fig. 3). In contrast, the nagC(E241A)-myc-6xHis allele (TIM 381) resulted in only a 1.7-fold increase in nagA expression. These results demonstrate that NagC repression is relieved by GlcNAc-6P.

NagC is required for efficient colonization of E. scolopes

The proper regulation of chitin- and GlcNAc-utilization genes is predicted to be important for the long-term interactions between V. fischeri and its natural host E. scolopes (Wier et al., 2010). While the technical aspects of studying long-term interactions within the squid–vibrio symbiosis under laboratory conditions remain challenging, many details concerning the establishment of the symbiosis are known. Therefore, we investigated whether regulation by NagC is important during the initial steps of bacterial colonization of the squid light organ.

A 3 h exposure to seawater containing 30 000 cfu ml⁻¹ of wild-type V. fischeri cells results in the successful colonization of the squid light organ over 90% of the time (Fig. 6). In contrast, only 40% (6/15) of the squid were colonized by the ΔnagC mutant under the same conditions. In two of these six animals the level of colonization (< 10⁴ cfu per squid) was insufficient to result in detectable luminescence (Fig. 6). Increasing the time that the squid are exposed to the inoculum resulted in more successfully colonized
animals (Fig. S3). The colonization defect of the ΔnagC strain was not observed in strains expressing the myc-6xHis-tagged variants of NagC (Fig. S3).

To determine whether the presence of wild-type V. fischeri could abrogate the colonization defect exhibited by the ΔnagC mutant, we performed co-colonization experiments using a GFP-labelled, wild-type strain of V. fischeri as the control strain. Squid were exposed to the inoculum for at least 20 h to allow each strain multiple attempts to colonize the light organ. When co-colonized with the control strain, the ΔnagC mutant was outcompeted by wild-type cells (Fig. 7 and Table 1). This result demonstrates that the presence of wild-type cells fails to complement the colonization defect of the ΔnagC mutant.

Furthermore, the de-repressed genes in the ΔnagC mutant (e.g. the secreted exochitinase VF_1598) do not impede the ability of wild-type cells to colonize and dominate the light organ. In addition, our observation that a ΔnagB mutant, which cannot convert glucosamine-6P to fructose-6P, colonizes as well as wild type (Table 1) suggests the colonization defect of the ΔnagC mutant is independent of GlcNAc metabolism. Increasing the abundance of the ΔnagC mutant 15-fold relative to wild-type cells within the inoculum was necessary to observe a twofold ΔnagC-dominant population within the light organs (Fig. 7 and Table 1). By comparison, the same inoculum bias with wild-type V. fischeri as the test strain led to a 35-fold bias in the V. fischeri population of symbionts.

We predicted that if wild-type cells were exposed to GlcNAc during colonization, then they would phenotypically mimic the ΔnagC mutant, i.e. de-repress any NagC-regulated genes. Consistent with this prediction, in squid exposed to seawater containing 20 mM GlcNAc and a mixed inoculum of the ΔnagC mutant and a GFP-labelled wild-type strain, the competitive advantage of wild type was lost (Table 1). We further hypothesized that the mutant harbouring the super-repressor form of NagC (NagC E241A) would out-compete the wild-type strain in the presence of exogenously added GlcNAc because it would still maintain repression of NagC-regulated genes. When exposed to wild-type and super-repressor strains in the absence of GlcNAc, co-colonized squid contained a slight bias towards the wild-type strain (Table 1). However, as hypothesized, the presence of GlcNAc during colonization was sufficient to reverse the bias of the population within co-colonized squid towards the super-repressor strain (Table 1).

Discussion

In this study, we have identified NagC as an important bacterial regulatory element that functions during the colonization of E. scolopes. Competitive indices of ΔnagC (WP100, open symbols) and wild-type (ES114, closed symbols) strains in animals co-colonized with the GFP-labelled, wild-type strain TIM302. The competitive index is defined as the ratio of test strain to control strain cfu present in the light organ. Error bars indicate 95% confidence intervals with co-colonized animals.

![Fig. 7. Competition experiment comparing pairs of V. fischeri strains during colonization of E. scolopes. Competitive indices of ΔnagC (WP100, open symbols) and wild-type (ES114, closed symbols) strains in animals co-colonized with the GFP-labelled, wild-type strain TIM302. The competitive index is defined as the ratio of test strain to control strain cfu present in the light organ. Error bars indicate 95% confidence intervals with co-colonized animals.](image)

Table 1. Light-organ co-colonization assays.

<table>
<thead>
<tr>
<th>Test strain*</th>
<th>Inoculum ratio</th>
<th>Total inoculum (× 10^4 cfu)</th>
<th>Test onlya (animals)</th>
<th>Control onlya (animals)</th>
<th>Co-colonizedb (animals)</th>
<th>Competitive indexc (test/control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.73</td>
<td>1.1</td>
<td>0</td>
<td>1</td>
<td>29</td>
<td>0.86</td>
</tr>
<tr>
<td>WT</td>
<td>2.8</td>
<td>4.0</td>
<td>11</td>
<td>0</td>
<td>19</td>
<td>2.5</td>
</tr>
<tr>
<td>WT</td>
<td>15</td>
<td>7.5</td>
<td>17</td>
<td>0</td>
<td>13</td>
<td>35</td>
</tr>
<tr>
<td>ΔnagC</td>
<td>1.1</td>
<td>0.74</td>
<td>0</td>
<td>26</td>
<td>3</td>
<td>0.062</td>
</tr>
<tr>
<td>ΔnagC</td>
<td>1.7</td>
<td>2.5</td>
<td>0</td>
<td>6</td>
<td>24</td>
<td>0.26</td>
</tr>
<tr>
<td>ΔnagC</td>
<td>15</td>
<td>6.9</td>
<td>0</td>
<td>3</td>
<td>27</td>
<td>1.9</td>
</tr>
<tr>
<td>ΔnagB</td>
<td>1.5</td>
<td>1.1</td>
<td>0</td>
<td>0</td>
<td>29</td>
<td>0.98</td>
</tr>
<tr>
<td>ΔnagC + GlcNAc</td>
<td>1.0</td>
<td>0.32</td>
<td>4</td>
<td>0</td>
<td>26</td>
<td>1.5</td>
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<td>nagC(E241A) - GlcNAc</td>
<td>1.2</td>
<td>0.63</td>
<td>0</td>
<td>3</td>
<td>27</td>
<td>0.56</td>
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<tr>
<td>nagC(E241A) + GlcNAc</td>
<td>1.2</td>
<td>0.46</td>
<td>6</td>
<td>0</td>
<td>24</td>
<td>2.6</td>
</tr>
</tbody>
</table>

a. For each competition, about 30 animals were exposed for 20 h to mixed inocula containing both the strain indicated in the column (test) and the GFP-labelled, wild-type strain TIM302 (control). At 48 h post inoculation, light organs were homogenized and plated on LBS. Resulting cfu were scored for GFP fluorescence. WT = ES114, ΔnagC = WP100, ΔnagB = JAS101 and nagC(E241A) = TIM381.

b. Light organs were scored as singly colonized by the test (non-fluorescent) or by the control (fluorescent) strain, or as co-colonized by both.

c. The competitive index is defined as the average ratio of test strain to control strain cfu present in co-colonized light organs.
initial colonization of *E. scolopes* by *V. fischeri*. NagC disassociates from DNA in response to intracellular GlcNAc-6P, leading to changes in gene expression. In addition to catabolic genes such as *nagA* and *nagB*, NagC also controls genes that are involved in the conversion of environmental chitin to GlcNAc, such as VF_1598 (exochitinase), VF_2139-VF_2148 (chitobiose-utilization genes), VF_A0143 (N-acetylglucosamine-binding protein) and VF_A0715 (chitodextrinase precursor). Because free monosaccharide sugars are of low abundance (< 100 nM) in seawater (Mopper *et al.*, 1992), we hypothesize that the environmental source of GlcNAc for *V. fischeri* is derived from invertebrate-associated chitin (Keyhani and Roseman, 1999).

In *E. coli*, a mutation in *nagA* results in derepression of NagC due to the accumulation of GlcNAc-6P via peptidoglycan remodelling (White, 1968; Plumbridge, 2009). While it is possible that GlcNAc-6P levels are elevated in doglycan remodelling (*White*, 1968; *Plumbridge*, 2009). *et al.* (Ghosh *et al.*, 2011).

In *Vibrio cholerae*, removal of the two copies of *nagA* is required to demonstrate sensitivity to GlcNAc (Ghosh *et al.*, 2011).

This study contributes to our growing awareness of the nutritional and signalling roles GlcNAc plays in several host–microbe interactions. Recently, transcriptional evidence that the *V. fischeri* population in the mature squid light-organ symbiosis is exposed to chitin as a nutrient has been supported by the discovery of host blood cells carrying particulate chitin in the tissue surrounding the symbionts (Heath-Heckman and McFall-Ngai, 2011). In addition, for both *V. cholerae* and *V. parahaemolyticus*, exposure to GlcNAc leads to the induction of genes associated with GlcNAc utilization (Meibom *et al.*, 2004; Thompson *et al.*, 2011). Recently, the *nagA* and *nagB* genes have been implicated in having an impact on host colonization by *V. cholerae* (Ghosh *et al.*, 2011). In the pathogen *Pseudomonas aeruginosa*, the presence of GlcNAc leads to production of the antibiotic pyocyanin (Korgaonkar and Whiteley, 2011). Interestingly, in this organism, gene expression is activated by the LacI-family transcriptional regulator NagR in response to GlcNAc, highlighting the disparate strategies different bacterial cells expressing NagC-repressed genes while recruiting potential symbionts.

We initiated this study to determine whether specific bacterial regulatory proteins control the patterns of gene expression that were observed in a mature symbiosis. Interestingly, the transcriptomics study of the adult symbiosis did not reveal changes in the expression of *nagA* or *nagB* (*Wier et al.*, 2010), which suggests that symbiont metabolism of GlcNAc is independent of the diel cycle. Nevertheless, we have discovered that the GlcNAc-6P-responsive repressor NagC is important for colonization of the squid light organ by its specific symbiont, *V. fischeri*. We hypothesize that the squid host selects for symbionts containing a functional NagC during the initial steps of colonization, thus ensuring that the bacterial population in the mature symbiosis retains the capacity to regulate the expression of the chitin- and GlcNAc-utilization genes. Future investigation of mature symbioses established with the NagC mutants generated in this study will reveal the role of NagC during the initial development and maintenance of the squid–vibrio symbiosis.

**Experimental procedures**

**Growth and media**

*Vibrio fischeri* strains were grown at 28°C with aeration in LBS broth (Graf *et al.*, 1994) without supplemented glycerol. When necessary, chloramphenicol and erythromycin were used at 2.5 µg ml⁻¹ and 5.0 µg ml⁻¹ respectively.

**Strains and plasmids**

The strains and plasmids used in this study are listed in Table 2, and additional details of their construction are located in Supporting information.

**Transposon mutagenesis and screen**

To generate a transposon-mutant library of *V. fischeri*, the vector pMJM10 (*Studer et al.*, 2008), which contains a Tn5 transposon encoding oriV_pip, and Erm resistance, was conjugated into ES114 via pEVS104 (Stabb and Ruby, 2002), and plated onto LBS with 5.0 µg ml⁻¹ erythromycin. Approximately 100 000 Erm¹ *V. fischeri* colonies that resulted from multiple independent conjugations were pooled and stored in 17% glycerol at ~80°C.

To isolate transposon mutants with enhanced VF_1598 expression, the reporter plasmid pTM314 was conjugated into the transposon-mutant library described above and
plated onto LBS with 2.5 μg ml⁻¹ chloramphenicol. The resulting colonies were screened for GFP levels using a Leica MZFLIII fluorescence dissecting microscope (Leica Microsystems, Wetzlar, Germany), equipped with a GFP2 filter set.

**Fluorescence assay**

Overnight cultures were diluted 1:100 and grown aerobically in LBS broth at 28°C. At OD₆₀₀ ~ 0.7, cultures were cooled quickly using an ice-slurry mix, and 1 ml of samples were centrifuged at 4°C for 5 min at 10 000 g. Cell pellets were resuspended in 350 μl of minimal media without a supplemented carbon source. The OD₆₀₀, GFP fluorescence and mCherry fluorescence of 100 μl of samples were measured in triplicate using a Tecan Genios Pro plate reader (Tecan Group, Mannedorf, Switzerland) as previously described (Miyashiro *et al.*, 2010).

**Exochitinase assay**

Exochitinase activities of 10 μl samples of overnight-culture supernatant were determined using 4-Nitrophenyl N,N′-diacetetyl-β-D-chitobioside (Sigma-Aldrich Corp., St. Louis, MO, USA) according to the manufacturer’s instructions.

**qPCR**

Total RNA extraction and qPCR were performed with cultures grown to OD₆₀₀ ~ 0.7 as previously described (Miyashiro *et al.*, 2010). Significance between samples within each gene was determined by one-way ANOVA and Tukey HSD (SPSS software, version 19). The P-values were adjusted using false discovery rate’s correction (FDR, R software, version 2.12). Primer sequences are listed in Table S1.

**Protein expression and purification**

To purify NagC and NagC(E241A), overnight cultures of *E. coli* strain EC100 harbouring pTM331 and pTM345, respectively, were diluted 1:100 into 500 ml of Luria–Bertani (LB) broth containing 100 μg ml⁻¹ carbenicillin, and grown at 37°C. After 4 h, cells were harvested, lysed by sonication and spun at 4°C for 15 min at 30 000 g. The soluble fraction was incubated 4:1 with 50% Ni-NTA agarose (Qiagen, Valencia, CA, USA) at 4°C. After 1 h, the agarose bed was washed twice and eluted into four 0.5 ml fractions. Protein levels were determined by OD₂₈₀. The fraction containing the highest level of NagC or NagC(E241A) was dialysed into storage buffer [50 mM NaH₂PO₄ (pH 8.0), 600 mM NaCl, 10% glycerol] and stored at 4°C.

### Table 2. Strains and plasmids used in this study.

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<th>Genotype</th>
<th>Reference</th>
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<tbody>
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<td>EC100</td>
<td>F mcrA Δ(mrr-hsdRMS-mcrBC) φø0dialZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galK λ′ rpsL (Str') nupG</td>
<td>Epicentre Biotechnologies</td>
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<td>ES114</td>
<td>Wild-type V. fischeri</td>
<td>Ruby <em>et al.</em> (2005); Mandel <em>et al.</em> (2008)</td>
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<td>CA42</td>
<td>ES114 ΔnagB</td>
<td>This study</td>
</tr>
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<td>JAS101</td>
<td>ES114 ΔnagB Tn7::pEV107</td>
<td>This study</td>
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<td>TIM302</td>
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<td>Miyashiro <em>et al.</em> (2010)</td>
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<td>TIM381</td>
<td>ES114 ΔnagC::nagC(E241A)-myc-6xHis</td>
<td>This study</td>
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<td>pBAD-myc-His</td>
<td>Protein expression vector</td>
<td>Invitrogen Corp.</td>
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<td>pEVS79</td>
<td>pBC SK (+) onT cat</td>
<td>Stabb and Ruby (2002)</td>
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<td>pEVS107</td>
<td>R6Kori onT mini-Tn7 mob erm kan</td>
<td>Shaner <em>et al.</em> (2004)</td>
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<td>pRSETB-mCherry</td>
<td>mCherry expression vector</td>
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<td>pTM214</td>
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<td>pTM314</td>
<td>pTM267 P_r,sar-gfp</td>
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<td>pTM322</td>
<td>pEVS79 ΔnagC</td>
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<td>pTrn59A</td>
<td>lacIq P_r-MCS bia</td>
<td>Armann <em>et al.</em> (1988)</td>
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<td>pVSV105</td>
<td>R6Kori onT(pEVS213) RP4 onT cat</td>
<td>Dunn <em>et al.</em> (2006)</td>
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<td>pXDC34</td>
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NagC-DNA binding measurements

5'-Texas Red-labelled, HPLC-purified DNA oligonucleotides and their unlabelled complements were obtained from Integrated DNA Technologies (IDT, Coralville, Iowa, USA). To generate the double-stranded DNA probes, equimolar levels of complementary single-stranded oligonucleotides were combined in annealing buffer (IDT) and heated to 94°C. After 2 min, the annealing reactions were slowly cooled to room temperature. Each 400 µl of binding reaction contained 10 nM double-stranded DNA probe in binding buffer [20 mM Tris-HCl (pH 7.5), 300 mM KCl, 2.5 mM MgCl₂, 0.5 mg ml⁻¹ BSA, 6 µg ml⁻¹ salmon sperm DNA]. When present, GlcNAc-6P (Sigma-Aldrich Corp.) was used at 10 mM. NagC or NagC(E241A) was added to final concentrations between 10 nM and 3 µM. Reactions were incubated at 28°C for 10 min. Fluorescence polarization (P) of each reaction was measured using a Beacon 2000 fluorescence polarization system (Panvera Corp., Madison, WI, USA). Fluorescence anisotropy (F) was calculated as F = (2 × P)/(3 – P), where P is the polarization of each sample.

5' RACE

Six micrograms of total RNA was extracted from a culture of ΔnagC (WPK100) grown to OD₆₀₀ = 0.6, and subjected to dephosphorylation by Tobacco Acid Pyrophosphatase (TAP; Epicentre Biotechnologies, Madison, WI) for 30 min at 37°C. The RNA oligo RNA-linker was ligated to total RNA using T4 RNA ligase (New England Biolabs, Ipswich, MA) according to the manufacturer’s instructions. cDNA was synthesized using AMV RT (Promega Corp., Madison, WI) according to the manufacturer’s instructions with the nagA-specific primer nagA-L. PCR amplification was performed using the nested primers Adapter and nagA-nested. The single band that was present within the reaction containing TAP, but absent from the TAP-minus control reaction, was subcloned using the TOPO TA Cloning Kit (Invitrogen) and sequenced as recombinated DNA Technologies (IDT, Coralville, Iowa, USA). To mend the TAP-minus control reaction, was subcloned using the TOPO TA Cloning Kit (Invitrogen) and sequenced as recombinated DNA Technologies (IDT, Coralville, Iowa, USA).

Squid colonization experiments

Except for the details described within the text, squid colonization experiments were performed as previously described (Miyashiro et al., 2010).

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**Supporting information**

Additional supporting information may be found in the online version of this article.

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