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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 IIC^{GlcNAc}, IIB^{GlcNAc} and IIA^{GlcNAc} 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-6P.

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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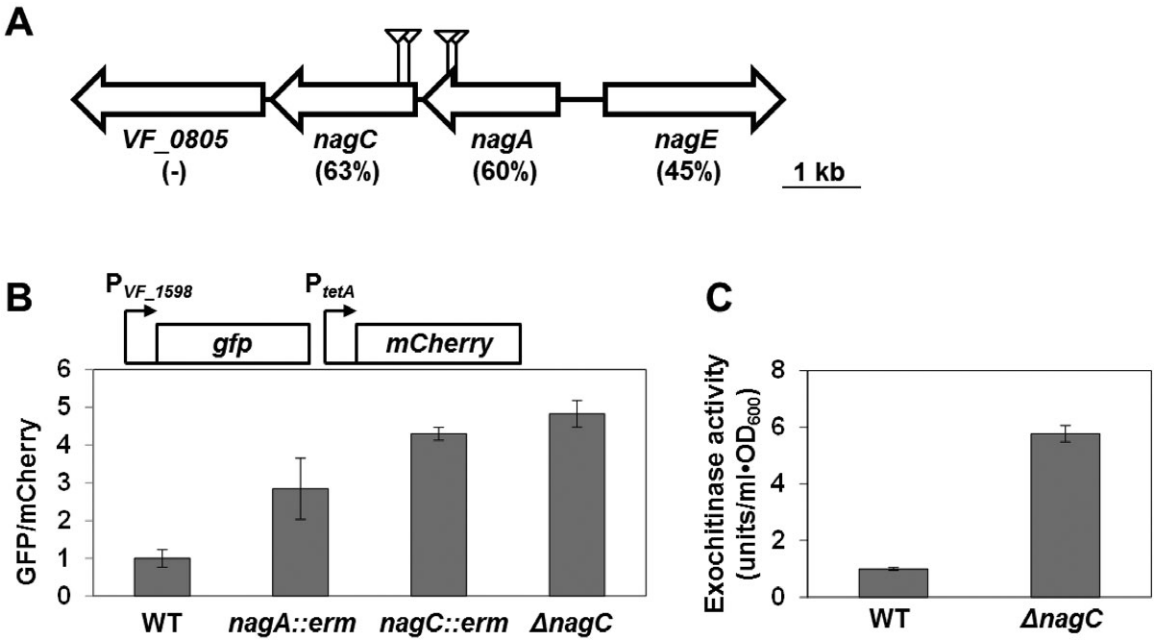


Fig. 1. Regulation of exochitinase gene *VF_1598* by NagC.
A. The *nag* locus of *V. fischeri* strain ES114. Triangles indicate the positions of unique transposon insertions in mutants with elevated *VF_1598* gene expression. Percentages below gene IDs are amino acid identities in comparison with *E. coli* homologues. Unlike in *E. coli*, in *Vibrio* sp., *nagB* is located at a distance from the *nag* locus.
B. GFP/mCherry fluorescence ratios of various *V. fischeri* strains harbouring the *VF_1598* promoter reporter plasmid pTM314. WT = ES114; *nagA::erm* = WPK54; *nagC::erm* = WPK84; and Δ*nagC* = WPK100. Error bars indicate ± one SD.
C. Exochitinase activities in supernatants of *V. fischeri* cultures. WT = ES114; and Δ*nagC* = WPK100. Error bars indicate ± one SD.

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 10^5 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 *VF_0807* (WPK54) or *VF_0806* (WPK84) (Fig. 1A). *VF_0807* and *VF_0806* are co-transcribed and encode *N*-acetylglucosamine-6-phosphate deacetylase (NagA) and GlcNAc transcriptional repressor (NagC) respectively. 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 (Δ*nagC*). As with the transposon mutants described above, the level of *VF_1598* expression in Δ*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 Δ*nagC* cultures compared with wild type (Fig. 1C).

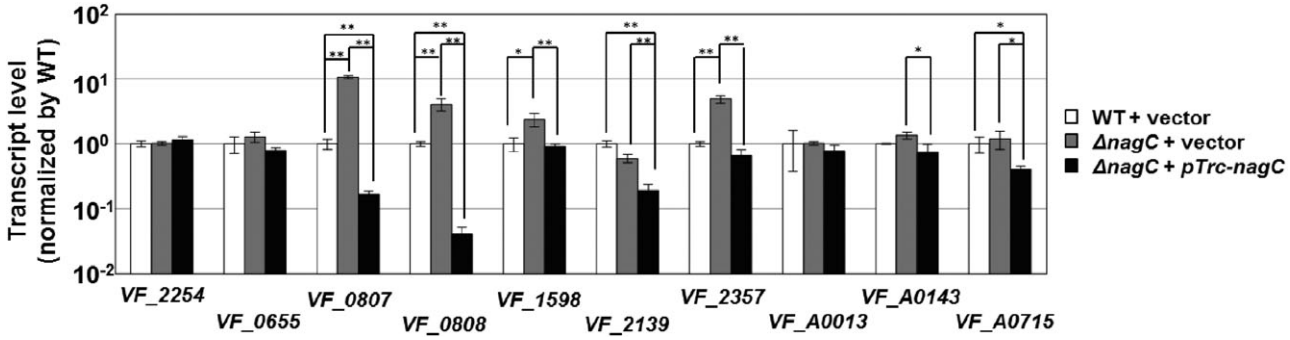


Fig. 2. Regulation of chitin- and GlcNAc-utilization genes by NagC. Expression levels of several *V. fischeri* genes measured by qPCR in wild type (ES114) and Δ*nagC* (WPK100) harbouring pTM214 (vector control) and Δ*nagC* (WPK100) harbouring pTM360 (*pTrc-nagC*).
VF_2254 = RpoD; VF_0655 = endochitinase; VF_0807 = NagA; VF_0808 = NagE; VF_1598 = exochitinase;
VF_2139 = chitoologosaccharide-binding protein; VF_2357 = NagB; VF_A0013 = chitin-binding protein;
VF_A0143 = *N*-acetylglucosamine-binding protein; and VF_A0715 = chitodextrinase precursor. Values are of triplicate biological replicates and normalized by wild-type levels. Error bars indicate ± one SD. One-way ANOVA and Tukey HSD comparisons with significance are shown with **P*-value < 0.05; and ***P*-value < 0.005, where *P*-values are adjusted using FDR's correction.

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 Δ*nagC* strain compared with wild type (Fig. 2). Furthermore, overexpression of *nagC in trans* was sufficient to decrease *VF_1598* transcription compared with Δ*nagC*. While *VF_0655* and *VF_A0013* 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-6P (Plumbridge, 1991). Using qPCR, we have found that *nagE*, *nagA* and *nagB* are regulated 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 Δ*nagC* 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*.

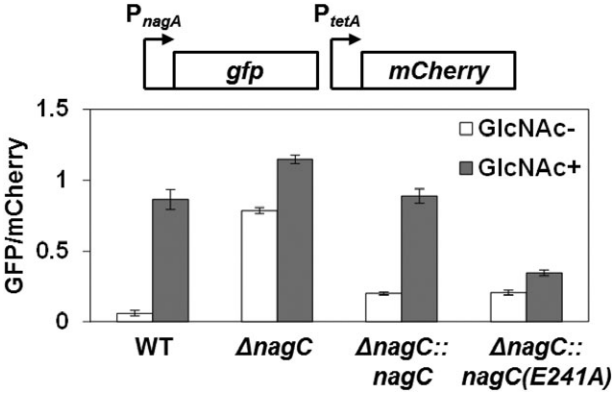
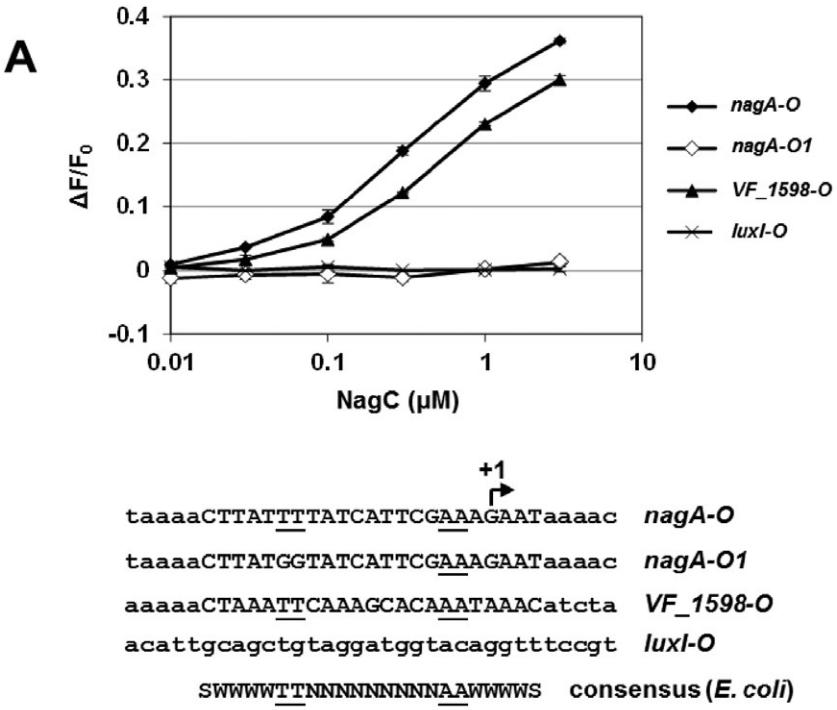


Fig. 3. Effect of GlcNAc on expression of *nagA* in various *V. fischeri* strains. GFP/mCherry fluorescence ratios of various *V. fischeri* strains harbouring the *nagA* promoter reporter plasmid pTM355 grown in the presence or absence of 20 mM GlcNAc.
WT = ES114; Δ*nagC* = WPK100; Δ*nagC::nagC* = TIM377; and Δ*nagC::nagC(E241A)* = TIM381. The *nagC* alleles in TIM377 and TIM381 have myc and 6x-His affinity tags at the C-terminal ends. Error bars indicate ± one SD.



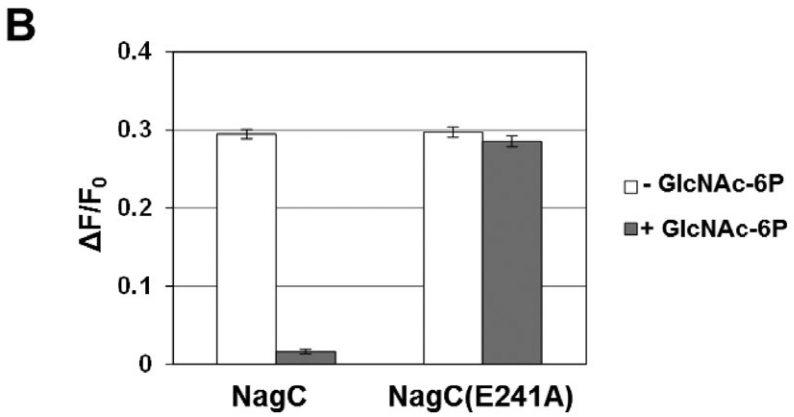


Fig. 4. *In vitro* DNA binding by NagC.
A. *In vitro* NagC binding assay. The fractional change in anisotropy of fluorescently labelled, double-stranded probes is plotted against the concentration (in μM) of NagC added. Points and bars represent mean and range of two independent binding reactions. Sequences of the probes are shown below the plot. The arrow indicates the transcriptional start site of *nagA* determined by 5′ RACE.
B. *In vitro* binding of 1 μM wild-type (NagC) or mutant [NagC(E241A)] NagC protein, as determined by fluorescence anisotropy, in response to the absence (white bars) or presence (grey bars) of 10 mM GlcNAc-6P. Error bars indicate ± one SD.

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 *Vibrionacaeae* 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

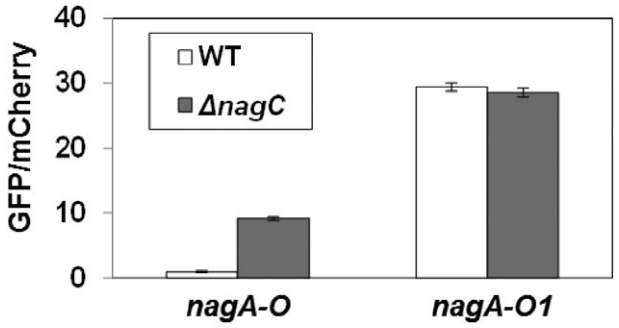


Fig. 5. Impact of NagC operator site on *nagA* expression. GFP/mCherry fluorescence ratios of *V. fischeri* strains harbouring *nagA*-promoter reporter plasmids containing wild-type (*nagA-O*, pTM355) or mutated (*nagA-O1*, pTM361) NagC operator sites. WT = ES114; and Δ*nagC* = WPK100. Error bars indicate ± one SD.

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 Δ*nagC* mutant under the same conditions. In two of these six animals the level of colonization (< 10^4 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

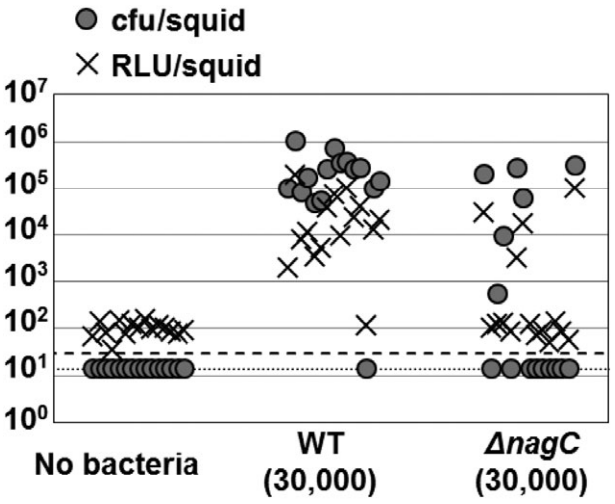


Fig. 6. Single-strain, 3 h, light-organ colonization assay. Symbiont population levels (cfu), and relative luminescence levels (RLU), of individual animals at 48 h post inoculation with either wild type (ES114) or the Δ*nagC* mutant (WPK100) are shown as circles and crosses respectively. The detection limit for cfu is indicated by the dotted line, and for luminescence is indicated by a dashed line.

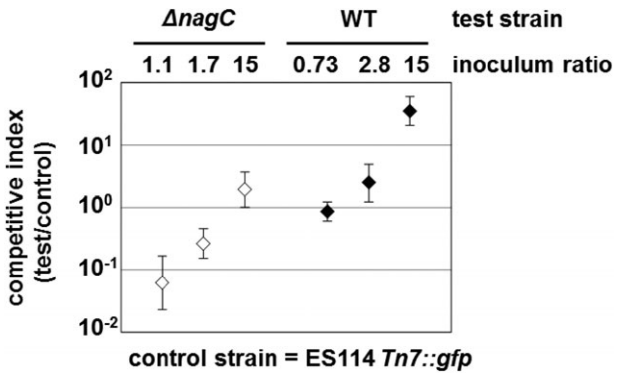


Fig. 7. Competition experiment comparing pairs of *V. fischeri* strains during colonization of *E. scolopes.* Competitive indices of Δ*nagC* (WPK100, open symbols) and wild-type (ES114, closed symbols) strains in animals co-colonized with the GFP-labelled, wild-type strain TIM302. The competitive index 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.

animals (Fig. S3). The colonization defect of the Δ*nagC* 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 Δ*nagC* 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 Δ*nagC* 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 Δ*nagC* mutant. Furthermore, the de-repressed genes in the Δ*nagC* 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 Δ*nagB* mutant, which cannot convert glucosamine-6P to fructose-6P, colonizes as well as wild type (Table 1) suggests the colonization defect of the Δ*nagC* mutant is independent of GlcNAc metabolism. Increasing the abundance of the Δ*nagC* mutant 15-fold relative to wild-type cells within the inoculum was necessary to observe a twofold Δ*nagC*-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 Δ*nagC* 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 Δ*nagC* 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

Table 1. Light-organ co-colonization assays.

Test strain[a]	Inoculum ratio (test/control)	Total inoculum (× 10^4 cfu)	Test only[b] (animals)	Control only[b] (animals)	Co-colonized[b] (animals)	Competitive index[c] (test/control)
WT	0.73	1.1	0	1	29	0.86
WT	2.8	4.0	11	0	19	2.5
WT	15	7.5	17	0	13	35
Δ*nagC*	1.1	0.74	0	26	3	0.062
Δ*nagC*	1.7	2.5	0	6	24	0.26
Δ*nagC*	15	6.9	0	3	27	1.9
Δ*nagB*	1.5	1.1	0	0	29	0.98
Δ*nagC* + GlcNAc	1.0	0.32	4	0	26	1.5
nagC(E241A)-GlcNAc	1.2	0.63	0	3	27	0.56
nagC(E241A) + GlcNAc	1.2	0.46	6	0	24	2.6

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, Δ*nagC* = WPK100, Δ*nagB* = 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 dissociates 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 the *nagA* transposon mutant WPK54, the ability of this mutant to grow on GlcNAc (Fig. S2) suggests that the other two *nagA* genes are functional and can catabolize GlcNAc-6P. 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 microbes have employed to sense and respond to environmental sources of GlcNAc. Finally, the chitin-related Nod factors produced by rhizobia are key signalling molecules throughout the development of the *Rhizobium*–legume symbiosis (Hamel and Beaudoin, 2010).

At this time, we are uncertain which NagC-controlled factor(s) is responsible for the significant colonization defect associated with the Δ*nagC* (i.e. unrepressed) mutant. However, our results from co-colonization experiments with wild-type and Δ*nagC* strains show: (i) the presence of wild-type cells does not abrogate the colonization defect of the Δ*nagC* mutant, and (ii) the Δ*nagC* mutant does not impede the ability of wild-type cells to colonize the light organ. These findings suggest that the squid host is able to actively and specifically discourage 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 Tn*5* transposon encoding *ori*$V_{R6K\gamma}$ 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 Erm^R *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

Table 2. Strains and plasmids used in this study.

Strain	Genotype	Reference
EC100	*F⁻ mcrA* Δ*(mrr-hsdRMS-mcrBC) Φ80dlacZ*Δ*M15* Δ*lacX74 recA1 endA1 araD139* Δ*(ara, leu)7697 galU galK λ⁻ rpsL (Str^R) nupG*	Epicentre Biotechnologies
ES114	Wild-type *V. fischeri*	Ruby *et al.* (2005); Mandel *et al.* (2008)
CA42	ES114 Δ*nagB*	This study
JAS101	ES114 Δ*nagB Tn7*::pEVS107	This study
TIM302	ES114 *Tn7::gfp erm*	Miyashiro *et al.* (2010)
TIM377	ES114 Δ*nagC::nagC-myc-6xHis*	This study
TIM381	ES114 Δ*nagC::nagC(E241A)-myc-6xHis*	This study
WPK54	ES114 *nagA::erm*	This study
WPK84	ES114 *nagC::erm*	This study
WPK100	ES114 Δ*nagC*	This study
Plasmid	**Genotype**	**Reference**
pBAD-myc-His	Protein expression vector	Invitrogen Corp.
pEVS79	pBC SK (+) *oriT cat*	Stabb and Ruby (2002)
pEVS104	R6Kori RP4 *oriT trb tra kan*	Stabb and Ruby (2002)
pEVS107	R6Kori *oriT mini*-Tn*7 mob erm kan*	McCann *et al.* (2003)
pRSETB-mCherry	*mCherry* expression vector	Shaner *et al.* (2004)
pTM214	pVSV105 *lacI^q* P_{trc}-*mCherry*	This work
pTM267	Promoterless-*gfp* reporter plasmid	Miyashiro *et al.* (2010)
pTM314	pTM267 P_{VF_1598}-*gfp*	This work
pTM322	pEVS79 Δ*nagC*	This work
pTM331	pBAD *nagC-myc-6xHis*	This work
pTM345	pBAD *nagC(E241A)-myc-6xHis*	This work
pTM346	pEVS79 Δ*nagC::nagC-myc-6xHis*	This work
pTM350	pEVS79 Δ*nagC::nagC(E241A)-myc-6xHis*	This work
pTM355	pTM267 P_{VF_0807}-*gfp*	This work
pTM360	pVSV105 *lacI^q* P_{trc}-*nagC*	This work
pTM361	pTM267 $P_{VF\_0807\text{-}O1}$-*gfp*	This work
pTrc99A	*lacI^q* P_{trc}-MCS *bla*	Amann *et al.* (1988)
pUX-BF13	R6Kori *tns bla*	Bao *et al.* (1991)
pVSV105	R6Kori *ori*(pES213) RP4 *oriT cat*	Dunn *et al.* (2006)
pXDC34	pEVS79 Δ*nagB*	This work

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 OD_{600} ~ 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 OD_{600}, 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'*-diacetyl-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 OD_{600} ~ 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} carbinicillin, 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 OD_{280}. The fraction containing the highest level of NagC or NagC(E241A) was dialysed into storage buffer [50 mM NaH_2PO_4 (pH 8.0), 600 mM NaCl, 10% glycerol] and stored at 4°C.

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 $MgCl_2$, 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 $F = (2 \times P)/(3 - P)$, where *P* is the polarization of each sample.

5′ RACE

Six micrograms of total RNA was extracted from a culture of Δ*nagC* (WPK100) grown to $OD_{600} = 0.6$, 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 recommended by the manufacturer.

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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