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Vibrio cholerae NspS, a homologue of ABC-type periplasmic solute binding proteins, facilitates transduction of polyamine signals independent of their transport

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The polyamines norspermidine and spermidine are among the environmental signals that regulate *Vibrio cholerae* biofilm formation. The effects of these polyamines are mediated by NspS, a member of the bacterial periplasmic solute binding protein superfamily. Almost all members of this superfamily characterized to date are components of ATP-binding cassette-type transporters involved in nutrient uptake. Consequently, in the current annotation of the *V. cholerae* genome, NspS has been assigned a function in transport. The objective of this study was to further characterize NspS and investigate its potential role in transport. Our results support a role for NspS in signal transduction in response to norspermidine and spermidine, but not their transport. In addition, we provide evidence that these polyamine signals are processed by c-di-GMP signalling networks in the cell. Furthermore, we present comparative genomics analyses which reveal the presence of NspS-like proteins in a variety of bacteria, suggesting that periplasmic ligand binding proteins may be widely utilized for sensory transduction.

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INTRODUCTION

Periplasmic solute binding proteins (PBPs) comprise a large family of proteins found in the periplasmic space of Gram-negative bacteria and are generally involved in nutrient import as components of ATP-binding cassette (ABC) transporters. These proteins bind a variety of ligands with high affinity, including polyamines, sugars, amino acids, oligopeptides, metals, iron–siderophore complexes and vitamins (Davidson *et al.*, 2008). In most cases, binding of the ligand leads to association of the PBPs with the permease complexes of the ABC transporter located in the cytoplasmic membrane. The ligand is then released and transported into the cytoplasm in a process driven by ATP hydrolysis catalysed by the ATPase component of the transporter (Davidson *et al.*, 2008).

We have previously reported the initial characterization of NspS, a protein in the PBP family that is an activator of biofilm formation in the pathogenic bacterium *Vibrio cholerae* (Karatan *et al.*, 2005). NspS belongs to the bacterial extracellular solute-binding protein family 1, which includes PBPs associated with ABC transporters for polyamines (http://www.ebi.ac.uk/Tools/InterProScan/). Polyamines are short hydrocarbon chains containing two or more amine

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Abbreviations: ABC transporter, ATP-binding cassette transporter; MBP, maltose binding protein; PBP, periplasmic binding protein; TSA, thermal shift assay.

One supplementary table and two supplementary figures are available with the online version of this paper.

groups that are positively charged at physiological pH. They are found in virtually all cells in millimolar quantities and are essential for normal growth of most prokaryotes and eukaryotes (Tabor & Tabor, 1984). In addition to their role in maintaining normal cell growth the polyamines putrescine, spermidine, norspermidine and spermine have been reported to influence biofilm formation in a number of bacteria (Burrell *et al.*, 2010; Goytia *et al.*, 2013; Karatan *et al.*, 2005; Kolodkin-Gal *et al.*, 2012; Lee *et al.*, 2009; McGinnis *et al.*, 2009; Patel *et al.*, 2006).

NspS is a homologue of PotD and PotF, the PBPs of the well-characterized spermidine and putrescine uptake systems, respectively, in *Escherichia coli*. Due to its similarity to PotD and PotF, NspS was initially annotated as a putative spermidine/putrescine ABC transporter binding protein. In recently sequenced genomes, NspS homologues continue to be annotated as having a function in transport in various genome databases. Despite its similarity to the PBPs of transport systems, we hypothesize that NspS is not involved in transport but rather in signal transduction for the following reasons. In many cases, genes encoding components of ABC transporters are found adjacent to each other in an operon. For example, the *V. cholerae* genome contains two *nspS* homologues, *potD1* and *potD2*, found adjacent to the *potA*, *potB* and *potC* genes, encoding the ATPase and permease components of the ABC transporter. PotD1 is responsible for spermidine import while the role of PotD2 has not yet been established (McGinnis *et al.*, 2009). In contrast, the *nspS* gene is adjacent to and has overlapping reading frames with the *mbaA* gene, which encodes a protein that is likely to be involved in signal transduction. MbaA is a putative integral membrane protein containing GGDEF and EAL domains, which are found in enzymes that synthesize or degrade the ubiquitous bacterial secondary messenger cyclic-di-guanylate monophosphate (c-di-GMP). MbaA is also a repressor of *V. cholerae* biofilm formation (Bomchil *et al.*, 2003; Karatan *et al.*, 2005). Therefore, NspS and MbaA have opposite effects on *V. cholerae* biofilms. In addition, the polyamine norspermidine significantly enhances biofilm formation and expression of the *vps* genes, which encode proteins responsible for synthesis of the biofilm polysaccharide in *V. cholerae* (Karatan, *et al.*, 2005). This effect is mediated by the protein NspS, as Δ*nspS* mutants do not respond to norspermidine addition. Δ*mbaA* mutants also do not respond to norspermidine, suggesting that MbaA plays a role in detecting and processing the norspermidine signal in the environment as well. Additionally, spermidine, a polyamine that is one methylene group longer than norspermidine, inhibits *V. cholerae* biofilm formation also in an NspS- and MbaA-dependent manner (McGinnis *et al.*, 2009).

The proximity of the *nspS* and *mbaA* genes, their predicted cellular locations and their effect on biofilm formation has led to the hypothesis that NspS and MbaA make up a signalling complex that regulates *V. cholerae* biofilm formation in response to the presence of norspermidine and spermidine in the environment. Our current working model for this signalling system is depicted in Fig. 1. In the absence of norspermidine or spermidine, NspS interacts with MbaA and downregulates its enzymic activity, allowing for intermediate c-di-GMP levels, *vps* gene expression and propensity to form biofilms (Fig. 1a). Binding of norspermidine to NspS increases the inhibitory effect of NspS on MbaA, leading to an increase in biofilm formation (Fig. 1b). In contrast, binding of spermidine to NspS inhibits its interaction with MbaA, allowing maximal MbaA activity, which decreases c-di-GMP levels and hinders biofilm formation (Fig. 1c).

In this study, our objective was to provide support for our hypothesis that NspS is a signalling protein that communicates polyamine signals to the cell without mediating their transport and to determine whether NspS and MbaA serve as a new paradigm in c-di-GMP signalling.

METHODS

Bacterial strains, plasmids and media. The *V. cholerae* strain used was O139 MO10; more information on the bacterial strains and plasmids used in this study can be found in Table 1. Primers are listed in Table S1 (available in the online Supplementary Material). All experiments were done in Luria–Bertani broth (LB). Primer synthesis and DNA sequencing were performed by Eurofins MWG Operon and the Biotechnology Resource Center at Cornell University, respectively.

Construction of the *nspS* expression vector and purification of NspS. The signal peptide in NspS was predicted to be amino acids 1–33 with the Signal 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) using the default *D* cut-off values. The portion of the *nspS* gene downstream of this sequence was amplified from chromosomal DNA in two separate reactions using primer pairs P228/P231 (reaction 1) and P229/P230 (reaction 2). The amplified products were denatured at 98 °C for 5 min, combined and left to anneal by slow cooling to room temperature to yield sticky ends, as described by Ulijasz *et al.* (1996). These were cloned into *Nco*I/*Xho*I-digested cytoplasmic expression vector pET28b, in-frame with the plasmid encoded C terminus 6× histine tag yielding pNP37. Correct construction and sequence was verified by restriction digests followed by sequencing of several clones. pNP37 was transformed into Shuffle T7 Express cells (New England Biolabs), which are optimized for cytoplasmic production of periplasmic proteins. For protein production, cells were induced at mid-exponential phase with 0.1 mM IPTG and incubated for an additional 18 h at 30 °C. NspS was purified using metal affinity chromatography with a cobalt resin (Thermo Scientific).

Construction of the *mbaA* expression vector and *mbaA*$_{E553A}$ point mutant, and purification of MbaA. To avoid complications resulting from solubility issues with membrane proteins, we chose to assess the enzymic activity of the cytoplasmic portion of MbaA containing the GGDEF and EAL domains (C-terminal 509 residues). The portion of the *mbaA* gene encoding the predicted cytoplasmic residues was amplified from chromosomal DNA using primers PA211 and PA212 which added 5′ *Nde*I and 3′ *Bam*HI sites to the fragment, respectively. The amplified gene fragment was digested with *Nde*I and *Bam*HI and cloned into *Nde*I/*Bam*HI-digested pMAL-c5x (New England Biolabs) downstream of a gene encoding the maltose binding protein (MBP), generating pRC1. Presence of the fusion partner substantially increased the solubility of MbaA as expression of the

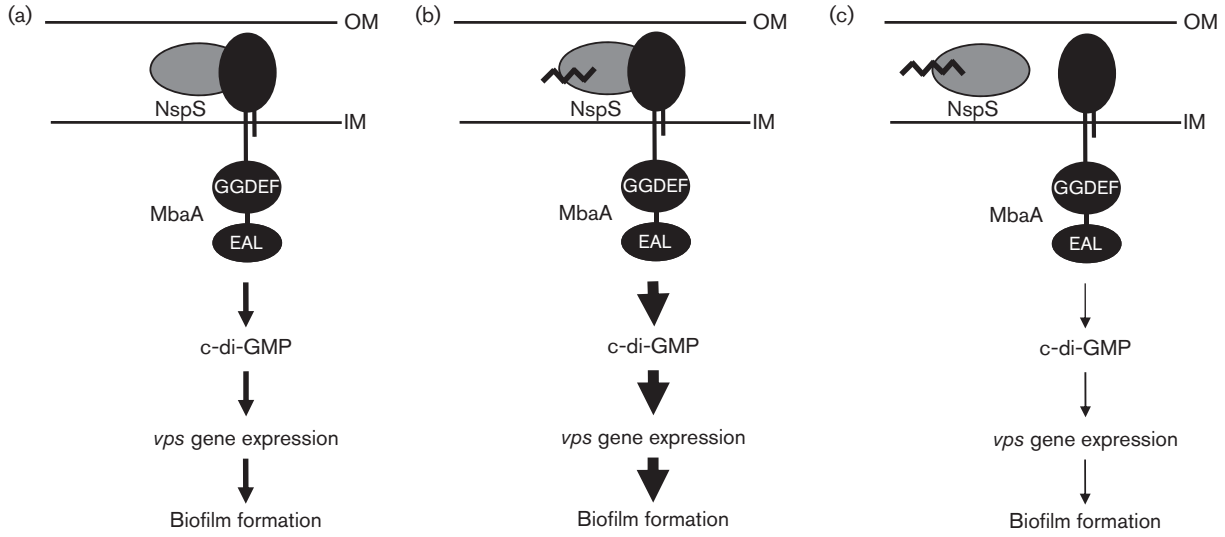


Fig. 1. Working model of the NspS–MbaA signalling complex. Environmental inputs are communicated to the interior of the cell as a change in the enzymic activity of MbaA. This change is reflected in the c-di-GMP levels, which in turn influence *vps* gene expression and biofilm formation. (a) In the absence of a ligand. (b) With norspermidine. (c) With spermidine. Thin, thicker and thickest arrows correlate with low, medium and high c-di-GMP levels, *vps* gene expression, and biofilm formation. Short and long zigzag lines bound to NspS represent norspermidine and spermidine, respectively. IM, inner membrane; OM, outer membrane.

same fragment using pET28b previously had resulted in the majority of the protein accumulating in inclusion bodies. pRC1 was transformed into NEB express cells (New England Biolabs), and correct construction and sequence was verified by colony PCR followed by sequencing of several clones. To produce the MBP–MbaA fusion protein, 1 litre cultures grown in LB with 0.2 % glucose were induced at mid-exponential phase with 0.3 mM IPTG and incubated for an additional 18 h at 30 °C. MBP–MbaA was affinity purified

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype	Reference/source
E. coli		
SM10λpir	*thi thr leu tonA lacY supE recA::RP4-2-Tc::MuλpirR6K;Km*^{r}	Miller & Mekalanos (1988)
SHuffle T7 Express	*fhuA2 lacZ::T7 gene1* [lon] *ompT ahpC gal λatt::*pNEB3-r1-*cDsbC* (Spec^{R}, *lacI*^{q}) Δ*trxB sulA11 R*(*mcr-73::miniTn10*–Tet^{S})2 [dcm] *R*(*zgb-210::Tn10*–Tet^{S}) *endA1* Δ*gor* Δ(*mcrC-mrr*)*114::IS10*	New England Biolabs
NEB express	*fhuA2* [*lon*] *ompT gal sulA11 R*(*mcr-73::miniTn10*–Tet^{S})*2* [*dcm*] *R*(*zgb-210::Tn10*–Tet^{S}) *endA1* Δ(*mcrC-mrr*)*114::IS10*	New England Biolabs
V. cholerae		
PW249	MO10, clinical isolate of *V. cholerae* O139 from India, Sm^{R}	Waldor & Mekalanos (1994)
PW357	MO10 *lacZ::vpsLp→lacZ*, Sm^{R}	Haugo & Watnick (2002)
AK314	MO10 *nspC::kan*, Kan^{R}, Sm^{R}	This study
AK317	MO10 *nspC::kan*, Δ*potD1*, Kan^{R}, Sm^{R}	This study
AK160	MO10 *lacZ::vpsLp→lacZ*, Δ*potD1*, Sm^{R}	This study
Plasmid		
pWM91	*oriR6KmobRP4 lacI pTac tnp miniTn10Km*; Km^{R}, Ap^{R}	Metcalf *et al.* (1996)
pET28b	Kan^{R}	Novagen
pMAL-c5x	Amp^{R}	New England Biolabs
pAR17	pWM91 carrying an internal 981 bp fragment of *nspC* replaced with kanamycin acetyltransferase gene	This study
pRC1	pMAL-c5x::*mbaA*	This study
pRC2	pMAL-c5x::*mbaA*$_{E553A}$	This study
pNP37	pET28b::*nspS*	This study

using an amylose resin (New England Biolabs). To generate a mutant in which the putative catalytic glutamate was changed to alanine, *mbaA* was first amplified in two fragments: the 'up' fragment was amplified using PA211 and PA216 that coded for an A to C nucleotide substitution; the 'down' fragment was amplified using PA215, which was complementary to PA216, and PA212. The up and down fragments were spliced together using overlap extension PCR (splicing by overlap extension) (Ho *et al.*, 1989) and cloned into pMAL-c5x to generate pRC2, which was sequenced to verify the presence of the intended mutation and absence of other mutations.

Thermal shift assay. To determine the binding ability of NspS to various ligands, a thermal shift assay (TSA) was performed essentially as previously described (Giuliani *et al.*, 2008; Niesen *et al.*, 2007). TSA relies on the principle that a protein bound to a ligand has a higher thermal stability than the ligand-free protein. The extent of denaturation is measured using the fluorescent protein-binding dye SYPRO Orange, the fluorescence of which is quenched in aqueous environments. As the protein denatures, more SYPRO Orange binds the protein, resulting in increased fluorescence. This assay has previously been used to identify ligands for ABC-type transporters (Giuliani *et al.*, 2008; Tan *et al.*, 2013). The TSA reaction mixtures contained 20 μM NspS alone or with polyamines in TSA buffer (100 mM HEPES, 150 mM NaCl, pH 7.5). SYPRO Orange (Invitrogen) was used at a concentration of 5×. The reactions were transferred to an Optical 96-well reaction plate, covered with an optical adhesive cover, and analysed in an Applied Biosystems 7300 real-time PCR system using the detection filter for the TAMRA dye. The instrument was set to increase the temperature from 25 to 95 °C in increments of 1 °C min^{-1}. The binding assays were performed in triplicate with multiple biological replicates. Negative controls contained SYPRO Orange, assay buffer and polyamines to ensure no reactions were occurring between the polyamines and SYPRO Orange indicator. Data were analysed in SigmaPlot, where the first derivative of the raw fluorescence values was taken and plotted graphically using Microsoft Excel. The peak is the maximal rate of change of the fluorescence intensity, which is referred to as the melting point (T_m) and is a measure of the thermal stability of the protein. Therefore, an upward shift in T_m shows increased thermal stability and indicates a binding event.

Phosphodiesterase assay. MBP–MbaA or MBP–$MbaA_{E553A}$ (2.5 μM) was mixed with 100 μM c-di-GMP or c-di-AMP (Biolog) and 2 mM $MnCl_2$ in 50 mM Tris, pH 8.5, at a final volume of 100 μl and incubated for 2.5 h at 37 °C. After incubation, reactions were boiled for 5 min and centrifuged through a Nanosep 10 kDa Omega filter for 2 min at 14 000 ***g***. The reaction products were separated using a SUPELCOSIL LC-18 column with a Waters 1525 Binary HPLC pump and were analysed using a Waters 2487 dual λ absorbance detector as described by Ryjenkov *et al.* (2005).

Construction of *nspC*::*kan*, *nspC*::*kan*Δ*potD1* and Δ*nspS*Δ*potD1* mutants. Multiple attempts to make a markerless in-frame deletion in *nspC*, which encodes the last enzyme in the norspermidine biosynthesis pathway, failed. Therefore, we generated an *nspC* mutant by replacing 981 bp of the 1164 bp coding sequence with a kanamycin resistance cassette. The kanamycin aceytltransferase gene was amplified from pKD4 (Datsenko & Wanner, 2000) using primers PA207 and PA208. A 371 bp fragment containing a short 5′ portion of *nspC* and the upstream sequence was amplified using primers P328 and P329. Similarly, a 468 bp fragment containing a short 3′ portion of *nspC* and the downstream sequence was amplified using primers P330 and P331. Primers P329 and P330 contained complementary regions to PA207 and PA208, respectively. The three PCR products were then joined together by splicing by overlap extension. After adding adenines, this product was cloned into pCR2.1-TOPO, and the sequence was verified. This insert was then excised using *Xho*I and *Spe*I, ligated into pWM91 linearized with the same enzymes generating pAR17, and transformed into *E. coli* SM10λpir. *E. coli* SM10λpir containing pAR17 or pMM9 (McGinnis *et al.*, 2009) were mated with wild-type *V. cholerae*, the Δ*potD1* mutant or the Δ*nspS* mutant to generate *nspC*::*kan*, *nspC*::*kan*Δ*potD1* and Δ*nspS*Δ*potD1* mutants, respectively, via double homologous recombination with sucrose selection as described by Metcalf *et al.* (1996).

Extraction, benzoylation and detection of polyamines. Bacteria were grown at 27 °C to mid-exponential phase, pelleted, washed with 1× PBS and resuspended in 10 μl water per milligram wet cell weight. Then, 250 μl of the cell suspension corresponding to 25 mg of cells was lysed using sonication and the cell debris was removed by centrifugation. Cellular proteins were precipitated with 50 % (w/v) trichloroacetic acid and centrifuged, leaving the supernatant containing the polyamines. This supernatant was removed and benzoylated as described previously (McGinnis *et al.*, 2009). Briefly, samples were extracted twice with chloroform, evaporated to dryness and dissolved in 100 μl of 60 % methanol in water. A standard mix containing 0.1 mM of each polyamine was also prepared and benzoylated each time. The resulting set of benzoylated polyamine samples were separated using a Phenomenex Sphereclone ODS column (5 μm, 250×4.6 mm) that was fitted with a 4.0×3.0 mm guard cartridge with the system described above. The runs were performed using a gradient of 45–60 % methanol in water for 30 min with a 10 min isocratic equilibration of 45 % methanol in water.

Biofilm assays. Bacteria were diluted in 0.3 ml LB at an OD_{595} of 0.02 taken using a Bio-Rad MicroPlate Reader model 680 and incubated in borosilicate test tubes for 18 h at 27 °C. After 18 h, planktonic cells were removed. The biofilm was washed once with 0.3 ml of 1× PBS, mechanically disrupted in 0.3 ml of 1× PBS by vortexing with glass beads, and the cell density was measured at OD_{595}. All experiments were performed in triplicate and repeated multiple times to confirm reproducibility.

RNA extraction, cDNA synthesis and RT-PCR. Total RNA was extracted from 5 ml of cells grown to mid-exponential phase using the Ambion RiboPure-Bacteria kit and treated with DNase I for 2 h at 37 °C. One microgram of this RNA was reverse transcribed with random primers using the Protoscript First Strand cDNA Synthesis kit (New England Biolabs). Negative controls were also performed without reverse transcriptase to ensure a lack of genomic DNA contamination. The cDNA was then used in a PCR with gene-specific primers designed to amplify approximately 300 bp regions.

Bioinformatics. To determine if *nspS* and *mbaA* genes were conserved in other members of the genus *Vibrio*, we utilized the Pathosystems Resource Integration Center (PATRIC) website (http://patricbrc.org/portal/portal/patric/Home; last accessed 4 October 2013) (Gillespie *et al.*, 2011). To determine if *nspS*-like/*mbaA*-like gene pairs were present in genomes of other bacteria in a different genomic context, we performed a preliminary genome region comparison analysis using the 672 bacterial genomes available in the Comprehensive Microbial Resource (J. Craig Venter Institute; http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi; last accessed 24 September 2013).

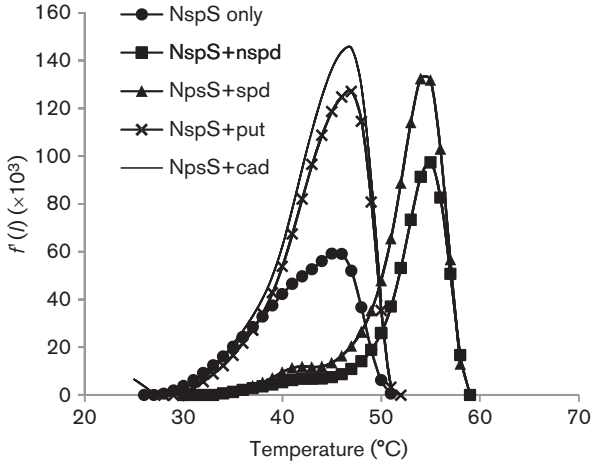
RESULTS

NspS interacts with norspermidine and spermidine

To determine if norspermidine and spermidine mediate their effect on *V. cholerae* biofilms by directly interacting

with NspS, we evaluated the ability of NspS to bind these polyamines using a TSA. This assay operates on the principle that a ligand binding protein will be more thermally stable when bound to its ligand and thus will have a higher T_m. In the ligand-free state, NspS had a T_m of approximately 47 °C (Fig. 2). Presence of norspermidine and spermidine increased the melting point of NspS by approximately 10 °C, indicating a binding event (Fig. 2). The NspS homologue PotD has been shown to bind the diamine putrescine in addition to the triamine spermidine, albeit with less affinity (Kashiwagi *et al.*, 1990, 1996). To determine if NspS is also capable of interacting with putrescine, we performed the binding assay with this polyamine as well as another diamine, cadaverine. Putrescine and cadaverine did not increase the thermal stability of NspS when used at the same concentration, indicating the increase in T_m was polyamine specific (Fig. 2). These results indicate that the triamines norspermidine and spermidine can interact with NspS whereas the diamines putrescine and cadaverine cannot.

The presence of norspermidine and spermidine in the culture medium leads to enhanced and hindered accumulation in biofilms, respectively, as previously reported (Fig. S1) (Karatan *et al.*, 2005; McGinnis *et al.*, 2009). The results of the binding assays suggested that putrescine and cadaverine should not affect *V. cholerae* biofilm formation. To test this prediction, biofilm assays were conducted in the presence of putrescine and cadaverine. Consistent with the results of the TSA, the presence of these two polyamines did not affect biofilm formation (Fig. S1). These results support the hypothesis that the effect of norspermidine and spermidine on biofilms is the result of direct binding to NspS.



Norspermidine: $H_2N(CH_2)_3NH(CH_2)_3NH_2$
Spermidine: $H_2N(CH_2)_4NH(CH_2)_3NH_2$
Putrescine: $H_2N(CH_2)_4NH_2$
Cadaverine: $H_2N(CH_2)_5NH_2$

Fig. 2. Binding of polyamines to NspS. TSAs were conducted in the presence or absence of polyamines. Denaturation profile of NspS without polyamines (NspS only) and with norspermidine (nspd), spermidine (spd), putrescine (put) and cadaverine (cad) are shown. The *y*-axis $f'(I)$ is the rate of change of total fluorescence intensity (I). Peaks of the curve represent T_m, melting temperature, which was used to compare the stability of NspS under the assay conditions. Assays were performed in triplicate and means were plotted. Assays were repeated at least twice with protein purified from different cultures to ensure reproducibility; a representative graph is shown. Chemical formulas of the polyamines used are presented below the graph.

The *nspS* and *mbaA* genes are in an operon

In bacteria, genes functioning in the same pathway are often found in operons. Therefore, we wanted to determine whether *nspS* and *mbaA* are co-transcribed to add weight to our hypothesis that NspS is a sensor and MbaA is a signal transducer, which interact to regulate biofilm formation. We reverse-transcribed the RNA extracted from wild-type *V. cholerae* cells and amplified the junctions between *nspS*/*mbaA* and *mbaA*/VC0702 using primers that annealed to adjacent genes (Fig. 3). We have previously shown that the deletion of VC0702, the third gene in the predicted operon, does not affect biofilm formation under the conditions where deletion of *nspS* or *mbaA* has pronounced effects on biofilms (Karatan *et al.*, 2005). The protein encoded by VC0702 was later shown to be an NTPase capable of cleaving dITP and dUTP (Ni *et al.*, 2006). However, the role of VC0702 in *V. cholerae* physiology remains unknown. We detected products for all of the junctions, indicating that *nspS*, *mbaA* and VC0702 reside in an operon. Because proteins encoded by genes in an operon interact and/or work together in the same pathway, this result lends support to the hypothesis that NspS and MbaA work together to regulate biofilm formation.

MbaA is a c-di-GMP phoshpodiesterase

Our working model predicts that NspS and MbaA work together to regulate c-di-GMP levels in *V. cholerae*. MbaA is predicted to be a phosphodiesterase because the canonical GGDEF motif in this protein is altered to SGDEF, suggesting the GGDEF domain is not likely to have diguanylate cyclase activity. In addition, the increased propensity of *mbaA* mutants to accumulate in a biofilm is consistent with phenotypes of phosphodiesterase mutants, which have local or global increases in c-di-GMP levels (Römling *et al.*, 2013). To confirm that MbaA could indeed contribute to c-di-GMP signalling by hydrolysing this molecule, we performed c-di-GMP phosphodiesterase assays with purified MbaA. We separated the reaction products by HPLC and compared the HPLC traces with c-di-GMP and pGpG standards to identify peaks (Fig. 4a). MbaA was able to break down c-di-GMP to pGpG, confirming that it is a phosphodiesterase (Fig. 4b). To ensure the phosphodiesterase activity was associated with the EAL domain, we constructed a mutant, $MbaA_{E553A}$, in

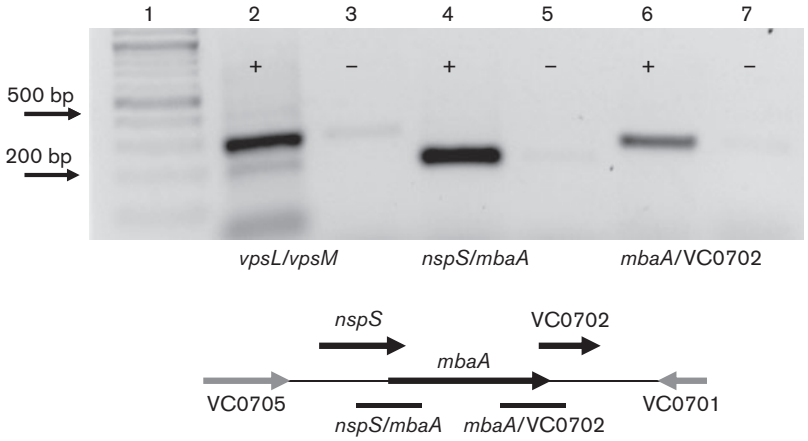


Fig. 3. *nspS*/*mbaA*/VC0702 are co-transcribed: gene junctions between *nspS*/*mbaA*, *mbaA*/VC0702 and *vpsL*/*vpsM* (control) were amplified from cDNA reverse-transcribed from *V. cholerae* total RNA. The *vpsL* and *vpsM* genes are the first two genes of the *vpsII* (or *vpsL*) operon encoding proteins responsible for synthesis and transport of the biofilm exopolysaccharide. Lane 1, ladder; lanes 2 and 3, intergenic region between *vpsL*/*vpsM* amplified using PA108 and PA109; lanes 4 and 5, intergenic region between *nspS*/*mbaA* amplified using PA 112 and PA113; lanes 6 and 7, intergenic region between *mbaA*/VC0702 amplified with PA110 and PA111. +/−, Presence or absence, respectively, of reverse transcriptase in the cDNA synthesis reactions. Amplified regions are shown as black bars under the cartoon of the chromosomal region. The *vpsL*/*vpsM* region is not shown. The image was taken using Alpha Imager and reversed with the program software for better resolution.

which the catalytic glutamate residue was changed to an alanine. As expected, this altered protein was incapable of degrading c-di-GMP to pGpG (Fig. 4c). We also tested the activity of MbaA against c-di-AMP, another cyclic dinucleotide utilized by bacteria. MbaA was not able to break down c-di-AMP, indicating it is a c-di-GMP-specific phosphodiesterase (Fig. 4d).

NspS is not involved in spermidine transport

If NspS is a sensor that communicates extracellular levels of polyamines to MbaA, then it may not be involved in transport. Alternatively, it may be a bi-functional protein involved in both signalling and transport. To distinguish between these two possibilities, we investigated the role of NspS in polyamine transport, starting with spermidine uptake. We have previously shown that PotD1, a protein that has 66 % amino acid identity to *E. coli* PotD, is responsible for spermidine import in *V. cholerae* (McGinnis *et al.*, 2009). In the absence of PotD1, NspS cannot support spermidine uptake at low concentrations of spermidine (10–40 μM in different batches of LB broth used), indicating that it does not play a role in high-affinity spermidine transport. However, these results do not preclude the possibility that NspS is part of a low-affinity transport system for spermidine. To test this hypothesis, we analysed the polyamine content of the Δ*potD1* strain grown in high concentrations of spermidine (1 mM). The presence of 1 mM spermidine in the culture medium resulted in accumulation of a small amount of spermidine in the cell, suggesting that NspS could indeed be a low-affinity binding protein utilized for spermidine import (data not shown). To test this hypothesis, we constructed a Δ*nspS*Δ*potD1* double mutant and assessed its ability to uptake spermidine. In the absence of additional spermidine in the growth medium, this mutant contained putrescine, diaminopropane, cadaverine and norspermidine (Fig. 5a). All of these polyamines can be produced by *V. cholerae*, but spermidine has to be imported from the culture medium under the conditions of this experiment. (Lee *et al.*, 2009; McGinnis *et al.*, 2009). Growing this mutant in the presence of 1 mM spermidine still led to accumulation of spermidine in the cell (Fig. 5b). These results indicate that NspS is not responsible for spermidine transport. It is possible, however, that *V. cholerae* has a second protein in addition to PotD1 that is capable of mediating spermidine transport at high concentrations of this molecule.

PotD1, but not NspS, is responsible for norspermidine import

Next, to rule out the involvement of NspS in norspermidine transport, we first eliminated norspermidine synthesis in the cell. We disrupted the *nspC* gene, which codes for the enzyme that catalyses the last step of norspermidine synthesis. As expected, this mutant was not able to produce norspermidine (Fig. 5c). Addition of norspermidine to the growth medium of this mutant restored the presence of norspermidine in the cell, indicating that the norspermidine uptake system was intact (Fig. 5d). Exogenous norspermidine also eliminated spermidine uptake, corroborating the results previously reported by us and others (Lee *et al.*, 2009; McGinnis *et al.*, 2009). The competition between spermidine and norspermidine transport suggested that PotD1

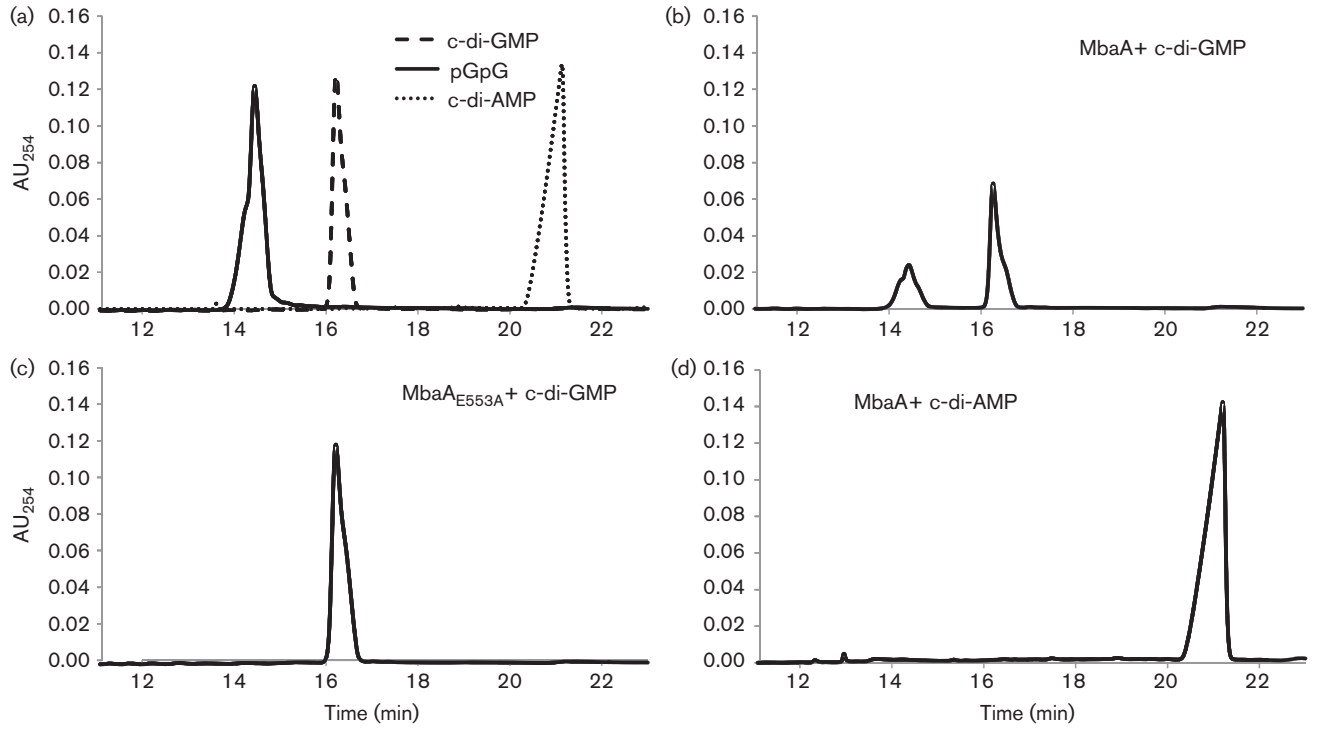


Fig. 4. MbaA is a c-di-GMP phosphodiesterase. Phosphodiestrase assays were conducted as described in Methods and the reaction products were separated by HPLC. (a) c-di-GMP, pGpG and c-di-AMP standards. (b) MbaA with c-di-GMP. (c) $MbaA_{E553A}$ with c-di-GMP. (d) MbaA with c-di-AMP. Graphs shown are representative of data from different experiments using protein purified from at least three separate cultures. AU_{254}, absorbance units at 254 nm.

may be responsible for norspermidine uptake as well. To investigate this possibility, we constructed a double mutant, *nspC*::*kan*Δ*potD1*, which synthesized putrescine, diaminopropane and cadaverine and was unable to import spermidine (Fig. 5e). The addition of norspermidine to the growth medium did not restore norspermidine levels in the cell, indicating that this mutant cannot import norspermidine (Fig. 5f). This result shows that PotD1 is the sole protein responsible for norspermidine uptake under the conditions of our experiment. In addition, because the *nspS* gene remains intact in this mutant our results also confirm that NspS does not play a role in norspermidine import.

The *nspS* and *mbaA* genes are conserved in a subset of vibrios

The presence of norspermidine signal detection systems have not been reported in other microbes. To determine if norspermidine-responsive signal transduction systems might be utilized by other members of the genus *Vibrio*, we searched for *nspS* and *mbaA* in the genomes of other vibrios. We found that *nspS* and *mbaA* were conserved in almost all *V. cholerae* isolates as well as many other members of this genus (Fig. S2). In these genomes, the gene identity and gene order upstream and downstream of the *nspS* and *mbaA* gene pair were also completely conserved. The gene upstream of *nspS* is annotated as chorismate mutase/prephenate dehydratase; the genes downstream of *mbaA* are annotated as inosine/xanthosine triphosphatase (VC0702), putative *trp* operon repressor (VC0701) and soluble lytic murein transglycosylase (VC0700), respectively. Several species contained the *mbaA* gene but not the *nspS* gene while retaining the synteny downstream and upstream of *mbaA*. Others did not harbour either *nspS* or *mbaA*; however, again the gene identity and gene order upstream and downstream of this region were completely conserved. Our results indicate that *nspS* and *mbaA* are not conserved in all members of the genus *Vibrio*; however, approximately 50 % of species of the genus *Vibrio* whose genomes have been sequenced should have the ability to respond to norspermidine using the NspS/MbaA system. Conversely, absence of *nspS* and *mbaA* from a significant number of vibrios suggests that these organisms may not have a need for detecting these polyamines in their environment.

nspS-like/*mbaA*-like gene pairs are present in the genomes of diverse *Proteobacteria*

Conservation of the *nspS*/*mbaA* gene pair in a number of *Vibrio* genomes prompted us to investigate whether

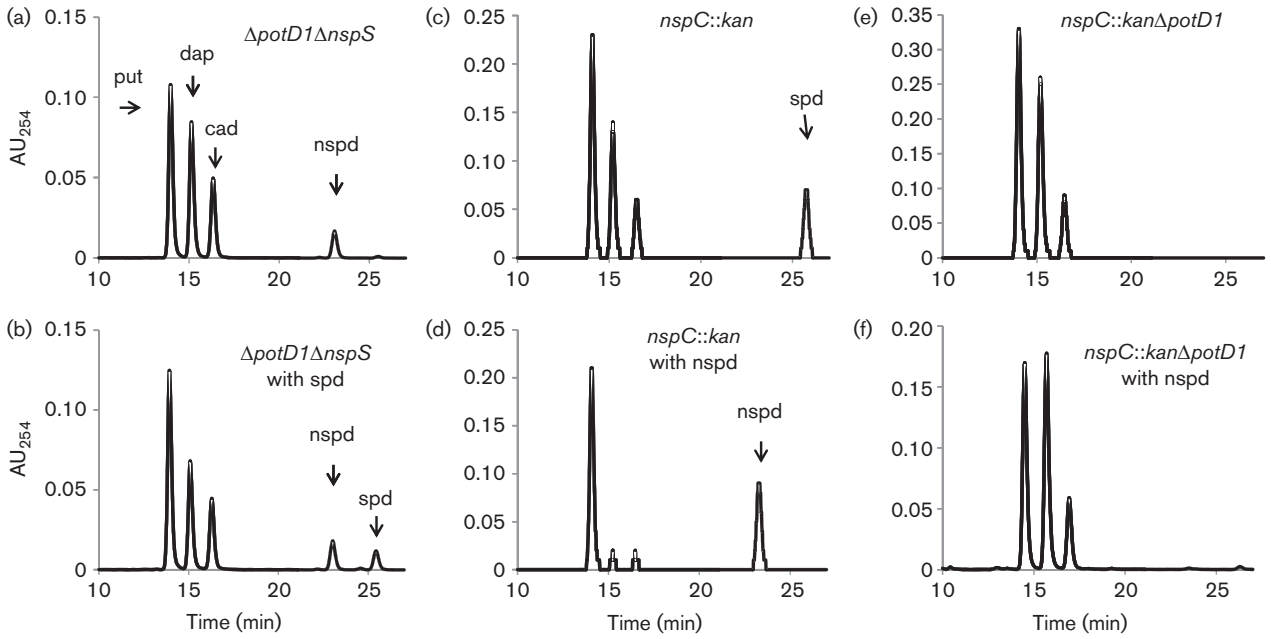


Fig. 5. The role of NspS in spermidine and norspermidine transport. Polyamines were extracted, derivatized by benzoylation and analysed by HPLC as described in Methods. Only data obtained between 10 and 27 min of a 40 min run are plotted for clarity. (a) Δ*nspS*Δ*potD1* mutant. (b) Δ*nspS*Δ*potD1* mutant with 1 mM spermidine. (c) *nspC*::*kan* mutant. (d) *nspC*::*kan* mutant with 1 mM norspermidine. (e) *nspC*::*kan*Δ*potD1* mutant. (f) *nspC*::*kan*Δ*potD1* mutant with 1 mM norspermidine. Peaks labelled in the wild-type chromatogram correspond to putrescine (put), diaminopropane (dap), cadaverine (cad), norspermidine (nspd) and spermidine (spd). AU_{254}, absorbance units at 254 nm. At least three biological replicates were performed; data from one representative experiment are shown.

potential signalling systems composed of periplasmic solute binding proteins and integral membrane proteins containing GGDEF/EAL domains could be a paradigm in bacterial signalling. Genome region comparisons were performed using the sequenced bacterial genomes available in the Comprehensive Microbial Resource database (http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi). This preliminary analysis produced 13 different species belonging to *Proteobacteria* that contain genes encoding PBPs adjacent to predicted integral membrane proteins containing GGDEF/EAL domains (Table 2). The predicted ligands for the PBPs as determined by the annotations available on the genome pages included putrescine, spermidine, phosphate, phosphonate, nitrate, sulphonate and bicarbonate for more detailed annotations as well as anions, amino acids, peptides, and amines for less detailed annotations. In all cases, the gene encoding the PBP was followed by another gene encoding a GGDEF/EAL domain protein that either had overlapping reading frames with the first gene or was only a few base pairs (3–13) downstream of this gene, an arrangement consistent with an operon structure.

DISCUSSION

The second messenger c-di-GMP is extensively utilized in bacteria to regulate cellular physiology in response to external or internal signals. The *V. cholerae* genome encodes more than 60 proteins that potentially contribute to the c-di-GMP pools in the cell. Yet, the signals detected directly or indirectly by these proteins remain largely unknown. Our previous work had shown that norspermidine and spermidine are two exogenous signals that are likely to influence cellular c-di-GMP levels in *V. cholerae* as a result of their interaction with the putative NspS/MbaA signalling system (Karatan *et al.*, 2005; McGinnis *et al.*, 2009). In this study, we demonstrate that both norspermidine and spermidine bind NspS. Other spermidine-binding PBPs have been reported; however, to our knowledge NspS is the first norspermidine-binding PBP that has been identified. Norspermidine greatly stimulates biofilm formation whereas spermidine has the opposite effect; therefore, these polyamines are antagonistic signals both of which are detected by NspS. We have also shown that the *nspS* and *mbaA* genes constitute an operon, providing evidence for the hypothesis that NspS and MbaA work together to process these signals. How NspS mediates the response to norspermidine and spermidine and whether this involves a direct interaction with MbaA is currently under investigation in our laboratory.

The ability of *V. cholerae* to both synthesize and detect norspermidine leads to the intriguing possibility that this molecule could be used in quorum sensing. This would

Table 2. *nspS*/*mbaA*-like gene pairs in other bacteria

Organism	PBP	Predicted ligand	GGDEF/EAL	Proximity
Vibrio cholerae	*nspS*	Norspermidine/spermidine	*mbaA*	Overlap
Psychromonas ingrahamii	Ping_1238	Spermidine/putrescine	Ping_1239	8
Hahella chejuensis	HCH_06688	Spermidine/putrescine	HCH_06689	4
Shewanella sediminis	Ssed_2394	Spermidine/putrescine	Ssed_2393	Overlap
Pseudomonas stutzeri	PST_0371	Spermidine/putrescine	PST_0370	13
Sinorhizobium meliloti	SMc_00991	Putrescine	SMc_00991	3
Magnetospirillum magneticum	amb_1105	Phosphate/phosphonate	amb_1104	3
Nitratiruptor sp. SB155-21	NIS_1757	Nitrate/sulphonate/bicarbonate	NIS_1758	Overlap
Thiomicrospira crunogena	Tcr_1221	Phosphonate	Tcr_1222	Overlap
Vibrio parahaemolyticus	VPA_1753	Alkylphosphonate	VPA_1754	Overlap
Vibrio parahaemolyticus	VPA_1512 (ScrB)	S-signal	VPA_1511 (ScrC)	Overlap
Shewanella paeleanna	Spea_3650	ESBF-3	Spea_3649	13
Xanthomonas oryzae pv. *oryzae*	X000RF_2004	Phosphate	X000RF_2004	Overlap
Xanthomonas campestris pv. *campestris*	xcc-b100_1903	Anions	xcc-b100_1904	3

require the cells to export norspermidine into the environment. We have previously investigated this possibility; however, we did not detect norspermidine in the spent culture media of cells grown in a variety of conditions (Parker *et al.*, 2012). Therefore, norspermidine does not appear to be exported by *V. cholerae* in its unmodified form. In many cases, polyamines are modified by acetylation prior to being exported (Igarashi & Kashiwagi, 2010). Polyamine acetylation has not been studied in *V. cholerae*; however, the presence of acetyl-norspermidine has been reported in *Vibrio parahaemolyticus* (Yamamoto *et al.*, 1989). It is possible that acetyl-norspermidine is indeed synthesized and exported by *V. cholerae* and detected by NspS to serve as a measure of cell density.

In this study, we have also shown that PotD1 is responsible for norspermidine import in *V. cholerae*. To our knowledge, this is the first identification of a PBP responsible for norspermidine import in bacteria. The ability to synthesize norspermidine *de novo* as well as to import it from the environment implies that this polyamine plays an important role in *V. cholerae* physiology. Norspermidine forms the backbone of the *V. cholerae* siderophore vibriobactin, implicating its importance especially in iron-limited environments (Keating *et al.*, 2000). In addition, deletion of the *nspC* gene reduces the growth rate in this organism, indicating norspermidine synthesis is required for normal growth (Lee *et al.*, 2009). Whether this molecule plays other roles in *V. cholerae* physiology in addition to its effect on growth, biofilm formation and iron acquisition remains to be studied.

The *nspS* and *mbaA* genes are conserved in a number of species in the genus *Vibrio*, indicating they should have the ability to respond to norspermidine and spermidine using the NspS/MbaA system. Being able to detect these polyamines is likely to be important for these organisms and may give insight into their interactions with other organisms in their environment. Norspermidine and spermidine are both produced by prokaryotes and eukaryotes. Norspermidine is made by many *Vibrio* species as well as other bacteria and archaea (Christensen *et al.*, 2012; Hamana, 1997; Hamana & Itoh, 2001; Hamana *et al.*, 2001). It is also the major polyamine found in aquatic invertebrates such as sea urchins, sea cucumbers and bivalves, as well as some aquatic plants and algae (Hamana *et al.*, 1991, 1998, 2004). Spermidine is a common polyamine synthesized by many different bacteria and almost all eukaryotes (Tabor & Tabor, 1984). Therefore, norspermidine and spermidine may mediate interactions of some members of the genus *Vibrio* with other organisms containing these polyamines.

Because of its similarity to PotD and PotF, the PBPs of the *E. coli* ABC transporters for spermidine and putrescine, NspS has been annotated as having a function in polyamine transport. We have conclusively demonstrated that NspS does not play a role in the import of norspermidine or spermidine. Hence, NspS appears to be primarily used in signal transduction. Our results also underline the importance of consideration of genomic context in addition to sequence similarity to provide more accurate annotations for genes whose functions have not yet been studied.

Although rare, examples of PBPs involved in signal processing have been reported. A number of PBPs that are part of ABC transporters also play a role in sensory transduction by directly associating with membrane-bound components of signalling complexes. In *E. coli*, maltose, ribose and glucose binding proteins, which are involved in the transport of these sugars, also bind distinct chemotaxis receptors when complexed with their respective sugars (Eym *et al.*, 1996; Gardina *et al.*, 1992; Shilton *et al.*, 1996). In *Agrobacterium tumefaciens*, ChvE, the sugar binding protein of the MmsAB transporter, also regulates virulence by interacting with the sensor kinase VirA (Hu *et al.*, 2013; Zhao & Binns, 2011). Thus, these bi-functional PBPs

coordinate nutrient uptake with cellular responses such as chemotaxis and virulence. In *Vibrio harveyi*, the PBP LuxP directly associates with the membrane-bound two-component sensor kinase LuxQ to communicate information about cell density (Neiditch *et al.*, 2005). Binding of autoinducer-2 to LuxP affects its interaction with LuxQ, which in turn changes from a kinase to a phosphatase. In this case, LuxP is involved in sensing of autoinducer-2, but not its transport.

Our genomic analyses identified additional periplasmic binding proteins that are likely to be involved in signal transduction. The genes encoding these proteins were always adjacent to *mbaA*-like genes, suggesting that they are likely to be co-regulated. One of these gene pairs encode ScrB and ScrC of the *V. parahaemolyticus* ScrABC system that has been recently characterized (Trimble & McCarter, 2011). In this system, ScrA, a putative periplasmic enzyme, is thought to process an autoinducer signal termed the S-signal, which can interact with the periplasmic solute binding protein of the system, ScrB. ScrB is then thought to interact with ScrC, a membrane-bound GGDEF/EAL protein, to regulate c-di-GMP levels and swarming in response to cell density. NspS, ScrB and the other periplasmic solute binding proteins may constitute a subfamily of PBPs that are either utilized in signal transduction only or are bi-functional proteins utilized in signalling as well as transport. Distinguishing between these possibilities will have to await more in-depth characterization of these proteins.

Our results suggest that a variety of environmental signals can be processed by NspS/MbaA-like sensor/transducer pairs. The resulting information can feed into c-di-GMP regulatory networks to influence c-di-GMP-regulated phenotypes in diverse bacteria (Fig. 6). We propose these NspS-like sensors and MbaA-like transducers constitute a new paradigm in bacterial signalling.

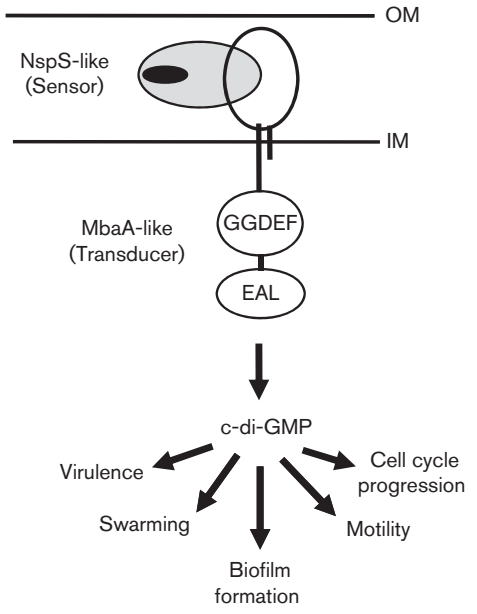


Fig. 6. Model of c-di-GMP signalling complexes made up of NspS-like sensors and MbaA-like transducers. Extracellular ligands (black oval) bind NspS-like sensors that are involved in signalling, which in turn changes the interactions between these and their cognate MbaA-like transducers, leading to changes in their enzymic activities. This results in alteration of local or global c-di-GMP levels and affects a variety of responses. IM, inner membrane; OM, outer membrane.

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