 doi:10.20944/preprints202207.0204.v1

Article

The *Sinorhizobium Meliloti* Nsps-Mbaa System affects Biofilm Formation, Exopolysaccharide Production and Motility in Response To Specific Polyamines

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Abstract: Biofilm formation is important in the free-living and symbiotic lifestyles of *Sinorhizobium meliloti*. Here we show that the *S. meliloti* NspS-MbaA system is homologous to the c-di-GMP modulating NspS-MbaA system described in *Vibrio cholerae* and modulates biofilm formation, exopolysaccharide (EPS) production and motility in *S. meliloti* in response to exogenous polyamines. Biofilm and EPS production in quorum sensing-deficient wild type strain 1021 decreased in cultures containing putrescine, 1,3-diaminopropane or spermine, but were unaffected in a 1021 *nspS* mutant (1021 nspS). Decreases in biofilm formation caused by these polyamines paralleled decreases in EPS production and increases in *nspS* transcription. Cell aggregation did not differ markedly between strain 1021 and 1021 nspS. The *S. meliloti* quorum sensing-proficient Rm8530 wild type strain and *nspS* mutant (Rm8530 nspS) produced similar levels of biofilm under control conditions and 3.2- and 2.2-fold more biofilm in cultures with norspermidine, but these changes did not correlate with EPS production. Rm8530 nspS aggregated up to several fold more than the wild type under most conditions. Changes caused by polyamines in swimming and swarming motility were not generally dependent on NspS-MbaA in either 1021 or Rm8530. We speculate that the *S. meliloti* NspS-MbaA system could modulate biofilm formation in response to environmental polyamines.

Keywords: cyclic di-GMP; polyamine sensing; NspS-MbaA. *S. meliloti*; biofilm; exopolysaccharides; motility

1. Introduction

The α-proteobacterium *Sinorhizobium meliloti* forms in an economically and agriculturally important nitrogen-fixing symbiosis with plants of the genus *Medicago*. To establish the symbiosis, *S. meliloti* must move through the bulk soil and rhizosphere to the root, synthesize nodulation factors and exopolysaccharides (EPS) and form aggregates or biofilms on the root [1-4]. Later stages of the symbiosis involve penetration into the root tissue by individual *S. meliloti* cells, culminating in the formation of root nodules that house the intracellular, nitrogen-fixing microsymbiont [3]. The symbiotic process as a whole thus involves transitions between motile and biofilm lifestyles [5,6].

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Bacterial biofilms consist of a self-generated extracellular matrix composed of EPS, proteins, and extracellular DNA. Biofilm formation and EPS production by *S. meliloti* and other nodule rhizobia are important not only for symbiosis with legume hosts but also for withstanding abiotic stress conditions encountered in the soil [7-9]. Bacterial aggregates are clumps of cells that often form in liquid cultures and can exist independently or initiate the formation of attached biofilms [10].

The production of succinoglycan (EPS I), galactoglucan (EPS II), mixed-linkage ß-glucan and arabinose-containing polysaccharides are important for biofilm formation in *S. meliloti*, and their production is regulated in response to environmental conditions like phosphate, iron and nitrogen availability, and osmolarity [5,7,11,12]. Here we generically refer to these polysaccharides as EPS. Increases in EPS production generally result in more biofilm formation [12].

The second messenger bis-(3′-5′) cyclic diguanosine monophosphate (c-di-GMP) is a key intracellular regulator that controls the transition to and from the biofilm state in *S. meliloti* and many other bacteria [13,14]. C-di-GMP is produced by diguanylate cyclases (DGCs) and degraded by phosphodiesterases (PDEs). DGCs contain conserved GGDEF motifs and PDEs have EAL or HD-GYP motifs. Many c-di-GMP metabolizing enzymes contain both DGC or PDE domains while others contain only one. Their c-di-GMP synthesizing and/or degrading activity is modulated by sensory domains or proteins that respond to environmental cues. High intracellular concentrations of c-di-GMP favor the production of EPS, fimbriae, pili, and adhesins that contribute to biofilm formation and aggregation [11,12,15]. *S. meliloti* contains 20 proteins with GGDEF and/or EAL motifs but most of the environmental signals that modulate their activity remain unknown [14,16].

EPS production in *S. meliloti* is also regulated by quorum sensing (QS) and other regulatory pathways. The ExpR/SinI QS system controls EPS II and ß-glucan biosynthesis and affects biofilm formation. EPS I production, biofilm formation and motility are also controlled by the ExoR-ExoS/ChvI signalling pathway.Although the transcriptional regulator MucR also affects EPS I and EPS II synthesis it does not greatly alter biofilm formation nor does *mucR* transcription respond to environmental cues [5,12,17,18]. ExoR, MucR and ExpR also participate in regulating motility[5,7,14).

In the cholera pathogen *Vibrio cholerae*, low levels of c-di-GMP enhance motility and decrease biofilm formation and high levels have the opposite effect. The *V. cholerae* QS system modulates c-di-GMP levels and biofilm formation in response to cell density [19]. Biofilm formation is also controlled by the NspS-MbaA signaling system, which affects c-di-GMP levels in response to specific polyamines [20,21]. The *V. cholerae* NspS is a periplasmic solute-binding homolog of the polyamine ABC-transporter solute binding

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protein PotD. MbaA is composed of a large N-terminal periplasmic domain flanked by two transmembrane regions and tandem GGDEF and EAL cytoplasmic domains at the C terminus. The current model of the system is that a conformational change in NspS caused by binding norspermidine (NSpd) increases its association with MbaA and elevates the latters DCG activity, thus increasing c-di-GMP levels and biofilm formation. Unliganded NspS or NspS bound to spermidine (Spd) or spermine (Spm) does not interact with MbaA, allowing higher PDE activity, decreased c-di-GMP levels and less biofilm formation. NSpd produced by *V. cholerae* and other vibrios as well as by some aquatic eukaryotes would trigger the formation of biofilm communities of *Vibrio* species in this environment. In the mammalian gut, Spd or Spm generated by members of the microbiota would inhibit biofilm formation and confer mobility on *V. cholerae* to colonize the host and express virulence factors [21,22].

We previously hypothesized that the NspS and MbaA paralogs in *S. meliloti* functioned in polyamine sensing and biofilm modulation [23,24]. In this work, we validate this hypothesis by showing that biofilm formation, EPS production and motility in *S.meliloti* are differently affected by polyamines in the wild type and mutant strains in which the NspS-MbaA system is inactivated, and that these responses are also influenced by QS. These results demonstrate the importance of both the NspS-MbaA and QS systems in modulating c-di-GMP dependent phenotypes in response to polyamine signals. These results suggest that *S. meliloti* could respond to environmental polyamines in order to modulate biofilm formation and motility in a manner that enhances its fitness in that environment.

2. Materials and Methods

2.1. Bacterial strains, plasmids, media and reagents

The bacterial strains and plasmids used in this study are listed in Table 1. Wild-type *S. meliloti* strain Rm8530 is identical to strain 1021 except that it has a functional copy of the transcriptional regulator gene *expR*, which is required for QS and promotes the production of EPS II in addition to EPS I [26]. PY and LB complex media, and MMSN minimal medium with succinate and NH_4Cl as carbon and nitrogen sources, respectively, were described previously [31]. Putrescine ·2HCl (Put; $H_2N(CH_2)_4NH_2$), cadaverine (Cad; $H_2N(CH_2)_5NH_2$), spermine (Spm; $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$), spermidine (Spd; $H_2N(CH_2)_3NH(CH_2)_4NH_2$), 1,3-diaminopropane (DAP; $H_2N(CH_2)_3NH_2$) and norspermidine (NSpd; $H_2N(CH_2)_3NH(CH_2)_3NH_2$) were purchased from Sigma (St. Louis, MO, USA) and homospermidine·3HCl (HSpd; $H_2N(CH_2)_4NH(CH_2)_4NH_2$) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Aqueous 200 mM polyamine stock solutions were adjusted to pH 6.8, filter sterilized and added to cultures

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to a final concentration of 0.1 mM. When required, antibiotics were used at the following concentrations (μg ml^{-1}): gentamicin (Gm), 15; kanamycin (Km), 50; spectinomycin (Sp), 100; and streptomycin (Sm), 200.

Table 1. Strains and plasmids used in this study.

Bacterial strain	**Relevant characteristics**	**Source of reference**
E. coli DH5α	Strain for cloning	Laboratory collection
S. meliloti 1021	Wild type. Sm^r derivative of wild type strain SU47, Sm^r Nal^r	[25]
S. meliloti Rm8530	Wild type. *expR*$^+$ derivative of *S. meliloti* 1021, Sm^r	[26]
S. meliloti 1021 nspS	1021 *smc00991*::ΩSp, Sm^r Sp^r	This study
S. meliloti Rm8530 nspS	Rm8530 *smc00991*::ΩSp, Sm^r Sp^r	This study
Plasmids		
pBBR1MCS-53	ΔplacZ pBBR1MCS-5 derivative with promoterless *gusA*, Gm^r	[27]
pBB53nspS::gusA	Transcriptional *smc00991*::*gusA* fusion in pBBMCS-53	This study
pJQ200SK+	Suicide vector for gene replacement, Gm^r	[28]
pHP45ΩSp	Source of ΩSp element	[29]
pJQ-nspS::ΩSp	*nspS* gene interruped with ΩSp, cloned in pJQ200SK$^+$	This study
pRK2013	Helper plasmid, Km^r	[30]
pTopo	pCR2.1Topo vector for cloning PCR products, Km^r	Invitrogen
pTopo-nspS	Contains PCR-amplified nspS coding sequence and flanking nt	This study
pTopo-PRnspS	pTopo containing 152 nt of the *nspS* coding sequence and 306 upstream nt	This study

 doi:10.20944/preprints202207.0204.v1

2.2. DNA manipulations

Standard protocols were used to grow *E. coli* and for DNA isolation, restriction digests, cloning, and transformation [32]. Bacterial conjugations were done as described previously [31]. DNA sequences were obtained from GenBank (www.ncbi.nlm.nih.gov/gene/). PCR amplifications were done using Dream Taq PCR master mix (Thermo Fisher, Waltham, MA, USA).

2.3. Mutant construction

To inactivate the *S. meliloti* 1021 *nspS* gene, its ORF including 598 and 338 upstream and downstream nt, respectively, was amplified by PCR using primers NspS-F and NspS-R (Table S1, supplemental material). The PCR amplification consisted of an initial denaturing step at 95°C for 3 min followed by 3 cycles of 95°C for 30 seconds, 56 °C for 30 seconds, and 72°C for 2 min, with a final extension at 72°C for 10 min. and the 1.98 kb product cloned into pTopo to generate plasmid pTopo-nspS (Table 1). The insert from pTopo-nspS was excised with *Sal*I and *Xba*I and ligated into suicide vector pJQ200SK+ cut likewise to give plasmid pJQ-nspS. The ΩSp element from pHP45ΩSp was inserted into the unique *Bam*HI site in the *nspS* gene in pJQ-nspS to give pJQ-nspS::ΩSp. This plasmid was conjugated separately into *S. meliloti* wild type strains 1021 and Rm8530 by triparental mating and double recombinants were selected by sucrose selection [31]. The resulting 1021 *nspS*::ΩSp mutants in the 1021 (*expR*$^-$) and Rm8530 (*expR*$^+$) genetic backgrounds were confirmed by Southern blotting [33] and designated 1021 nspS and Rm8530 nspS, respectively.

2.4. Construction of a nspS *transcriptional fusion with the β-glucuronidase (*gusA*) gene*

The 5′ portion of the *S meliloti nspS* gene and its putative promoter sequence were amplified with primers pNspS-F and pNspS-R (Table S1, supplemental material). The PCR cycling program included an initial denaturing step at 95°C for 5 min followed by 35 cycles of 95°C for 30 seconds, 54 °C for 30 seconds, and 72°C for 1 min., and a final extension at 72°C for 10 min. The PCR product contained 152 nt of the *nspS* coding sequence and 306 nt upstream of the *nspS* start codon. The product was cloned into pTopo to generate plasmid pTopo-PRnspS. The promoter region-5′-*nspS* fragment was excised from pTopo-PRnspS with *Kpn*I and *Xho*I and ligated into vector pBBR1MCS-53 to obtain plasmid pBB53nspS::gusA. The correct transcriptional orientation of the fusion was confirmed by restriction enzyme digestion with *Kpn*I and *Xho*I. The fusion plasmid was transferred to *S. meliloti* 1021 by triparental mating.

2.5. β-glucuronidase (Gus) assays

 doi:10.20944/preprints202207.0204.v1

Cultures of *S. meliloti* 1021/pBB53nspS::gusA were grown in MMSN minimal medium without or with 0.1 mM of an exogenous polyamine for 16 h at 30°C with shaking at 200 rpm. Gus activity was determined by measuring the production of *p*-nitrophenol from the *p*-nitrophenyl β-D-glucuronidase substrate with quantification based on total protein [31]. One unit (U) of activity is defined as the production of 1 nmol of product min^{-1} mg $protein^{-1}$. Strain 1021 containing pBBR1MCS-53 without an insert lacked Gus activity.

2.6. Motility assays

Swarming assays were done in Bromfield medium [34] containing 0.5% Difco Noble Agar (Beckman, Dickinson and Co., Sparks, MD, USA). *S. meliloti* cultures grown in PY were diluted and plated on PY and incubated for 3 days at 30°C. Individual colonies of the *S. meliloti* strains were stabbed into the swarming media with a toothpick and incubated at 30°C for 72 h. Swarm zones were quantitated by taking the average of two sides of a rectangle that framed the zone [35]. Swimming assays were performed in Bromfield medium containing 0.3 % Noble agar as described previously [36].

2.7. Biofilm assays

Biofilm formation by cultures grown in borosilicate glass tubes was determined by crystal violet (CV) staining essentially as described by O´Toole and Kolter [37]. Overnight cultures of *S. meliloti* strains were grown in 50 ml PY medium with appropriate antibiotics and cells were washed twice in MMSN and diluted to an OD_{595} of 0.2. Three milliliters of suspension with or without 0.1 mM of an exogenous polyamine were added per glass tube and incubated for 72 h at 80 rpm, 30°C. Bacterial growth was quantified by OD_{595} measurement before the removal of the planktonic cells by gentle aspiration. Biofilms were stained with 3 ml 0.1% CV for 15 min. Tubes or wells were rinsed three times with water and air-dried. The CV was solubilized with 3 ml of 95% ethanol and the absorbance at 595 nm determined. Biofilm formation was calculated as the A_{595} of the crystal violet solutions divided by the OD_{595} of the cultures.

2.8. EPS quantification

Samples of supernatants of the tube cultures used for biofilm assays were taken to quantify total hexose content by the anthrone method with glucose as a standard [38]. Preliminary experiments showed that quantitation of EPS obtained by precipitation with isopropanol, drying and weighing [39] gave qualitatively similar results to those obtained with the total carbohydrate measurements (results not shown).

2.9. Autoaggregation assays

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Autoaggregation assays were done as described by Sorroche et al. [15]. Briefly, overnight cultures of *S. meliloti* strains were grown in 50 ml PY medium with appropriate antibiotics and cells were washed twice in MMSN, diluted to an OD_{595} of 0.2 in 50 ml MMSN and grown for 48 h. Five ml of bacterial suspension was transferred to a glass tube and allowed to settle for 24 h at 4°C. A 0.2 ml aliquot of the upper portion of suspension was carefully transferred to a 96-well microplate and the OD_{600} was measured (OD_{Final}). A control tube was vortexed for 30 s, and the OD_{600} was determined ($OD_{initial}$). The percentage of aggregation was calculated as $100[1-(OD_{final}/OD_{inital}]$.

2.10. PA analysis by high-performance thin-layer chromatography

Free, intracellular PAs were extracted, derivatized with dansyl chloride and analyzed by high performance thin layer chromatography (HPTLC) as described previously [31].

2.11. Bioinformatics

Phylogenetic analysis was done by separate blastP searches of the *S. meliloti* and *V. cholerae* NspS-MbaA protein region against 16,076 complete proteobacterial genomes from GenBank that had identical GCA (GenBank assemblies) and GCF (RefSeq assemblies) annotations. Hits to NspS-MbaA homologs with at least 60 % sequence coverage and 30 % amino acid identity to the query sequences and in which *nspS* and *mbaA* were separated by ≤ 20 bp were included in the phylogeny. The phylogenetic analysis was done with PhyML 3.3.20190321 [40]. Multiple sequence alignments were made with Clustal Omega at the EMBL's European Bioinformatics Institute website (https://www.ebi.ac.uk/Tools/msa/clustalo/). Operon prediction for the *S. meliloti* 1021 *nspS-mbaA* was made using the Operon and Regulon feature with *smc00991* as query at http://www.microbesonline.org. The presence of putative transmembrane segments in protein sequences was determined with DeepTMHMM ([41]; https://dtu.biolib.com/DeepTMHMM)

3. Results

3.1. Sequence and phylogenetic analysis of the S. meliloti nspS-mbaA

The start codon of the *S. meliloti* 1021 *nspS and mbaA* genes *(smc00991* and *smc00992*, respectively*)* overlap by 3 bp and the two genes are predicted to form an operon (see Methods). These *S. meliloti* protein

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products are henceforth referred to as Sm NspS and Sm MbaA. The *V. cholerae* MO10 *nspS* (*vc0704*) overlaps the downstream *mbaA* gene (*vc07003*) by 16 bp and the genes were experimentally shown to be cotranscribed [42]. The *V. cholerae* NspS and MbaA proteins are henceforth referred to as Vc NspS and Vc MbaA.

The Sm NspS and Vc NspS have nearly 32 % amino acid identity and 52% similarity (Fig. 1) and both proteins have significant similarity to bacterial spermidine/putrescine ABC transporter substrate binding proteins PotD and PotF. Homology modeling of the Vc NspS using the *Pseudomonas aeruginosa* PotD as template predicted that 8 residues directly contact NSpd in the ligand binding pocket (W41, D70, D90, F131, Y233, D236,W261 D263) [43]. These residues are all identical in the Sm NspS while only 5 of the 8

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Vc NspS      MTNFCNEWVSYSQMIKRFLSLMVLNTVCYQAS-ALELNVYLWEDTIAPSVVEAWHKKTGV 59
Sm NspS      ------------MKARGVISVFLATLLCASASRAETLNLLIWEDYIDEGLIDRWTEKTGV 48
                        : .:*::: . :* .** *  **: :*** *  .::: * :****

Vc NspS      SVNLFHFDNDDERSLLMLKSVQLPFDIMVLDNVSAFIFSRQNVFEDL--TALPNRANNDP 117
Sm NspS      SIRQINFDSDDARDEILADPGRN-IDLVIVDENGATLFGRKGIIEPLSETNLPALEDYAP 107
             *:. ::**.** *. :: .. :  :*::::*: .* :*.*:.::* *  * **   :  *

Vc NspS      MWLQACGTHAVPYFWGSVGIAYRKSLFDKPPTQWSEVVDIAPAHRGRVGMLKDSVETLLP 177
Sm NspS      EWRKSCAGYGLPYFWGTVGILYRSDMVTPPPTSWRDMMRPAPALRKHIAMFADHSEIFVP 167
              * ::*. :.:*****:*** **..:.  ***.* :::  *** * ::.*: *  * ::*

Vc NspS      ALYMLNASPITDSIDTLRQAYRLLDAANPHILTYEYVLSYVRSHPQTDNLHMAVSYSGDH 237
Sm NspS      PLMLLGASVNADDTPTLKAAFALLKTQAPFVLTYDYVVTSIQDPALAGNIYLALGYSGDQ 227
             .* :*.**  :*.  **: *: **.:  *.:***:**:: ::. . :.*:::*:.****:

Vc NspS      YSLN-RFFNTQDWDFSVPEGRPYLWVDCMAVNSVSPNTVQAKAFLDFLMKPDIAAINAEY 296
Sm NspS      HVLNSKAGKAGLWRYSVPKEGTLSWLDCFSVTAASPRKQRALEFLNFIGSPEAAAANAIA 287
             : ** :  ::  * :***:  .  *:**::*.:.**.. :*  **:*: .*: ** **

Vc NspS      IRAASPNYKARALLPVEHREDLSIYLPEQRLAEGIIDSELSAKNLSLRAKIISSVTYQYE 356
Sm NspS      LNMPTASNAALKLLPDTMRSDPEIYPPTEIMAKNQHHQELSVRSIEARRRIINTLANFQ- 346
             :. .:..  *  ***   *.* .** * : :*:.  ..***.:.:. * :**.:::

Vc NspS      AKP 359
Sm NspS      ---
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Fig. 1. Clustal Omega alignment of NspS proteins from *V. cholerae* O1 (Vc NspS) and *S. meliloti* 1021 (Sm NspS). Identical residues, conserved substitutions and semi-conserved substitutions are indicated by asterisks, colons, and periods, respectively. Residues outlined in gray in the Vc NspS are those predicted to make direct contact with NSpd and blue-shaded residues cause biofilm phenotypes that are non-responsive to NSpd if mutated [43]. The percentage of identity/similarity was 31.95/52.06 calculated by the BLOSSUM62 matrix. The alignment was done with ClustalW.

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(W41, Y233, D236, W261, D263) are identical or conservatively substituted in 6 other representative PotD homologs analyzed by Young et al. [43], including PotD and PotF from *E. coli.* Of 7 amino acids shown by random mutagenesis to be required for the Vc NspS-mediated biofilm response to NSpd, only those corresponding to L89, S216, D263 are conserved or similar in the Sm NspS. [43] (Fig. 1).

The Sm MbaA and Vc MbaA amino acid sequences are 30.9 % identical (50.1 % similar) and both proteins have an alternative DGC motif (SGDEF) in place of the canonocal GG[D/E]EF (Fig. 2). DGC motifs in which a different amino acid replaces the first G are relatively common and are functional in c-di-GMP synthesis [44,45]. Both the Vc and Sm MbaAs lack a nearby allosteric inhibition site (RXXD), which is also absent in the majority of the putative DGCs in *S. meliloti* [14]. A putative HAMP domain, found in many signal tranducing regulatory proteins, occurs upstream of the SGDEF motif in both MbaA proteins (underlined in Fig. 2). The Sm MbaA has a canonical PDE motif (EAL) (Fig. 2) while the Vc MbaA has EVL. The 4 residues involved in binding Mg^{2+} in the *P. aeruginosa* RocR PDE (D295, N233, E265 and E175; [46]) are conserved in the *S. meliloti* and *V. cholerae* MbaAs (as D674, N611, E643 and E552 in the Vc MbaA). A number of other RocR catalytic residues that are conserved in Sm MbaA are described in Fig. 2

The predicted secondary structure of the Sm MbaA consists of C-terminal amino acids 1-6 localized in the cytoplasm, a transmembrane domain spanning residues 7-23, periplasmic residues 24-256, a second transmembrane segment from residues 257-277 and cytoplasmic residues 278-787. The latter cytoplasmic segment of MbaA contains the HAMP, cyclase and PDE domains (Fig. 2). The Vc MbaA predicted secondary structure is very similar [43].

BlastP analysis of 16,076 complete proteobacterial genomes using the *S. meliloti* 1021 NspS-MbaA proteins as query returned 222 genomes that encoded the proteins. The same search using the *V. cholerae* NspS-MbaA as query returned just 8 fewer (216 total) genomes, which demonstrates very similar results with either query. Thus, among proteobacteria, NspS-MbaA paralogs occur in less than 1.4 % of the genomes analyzed. Phylogenetic analysis of the potential NspS-MbaA operons resolved major clades into which *Vibrio*, *Pseudomonas* and *Sinorhizobium/Ensifer* species were grouped (Fig. 3). Smaller clades including *Marinomonas*, *Schewanella*, and *Shinella* spp., among others, were also present. These latter genera have few complete genome sequences deposited in the database. Many of the organisms

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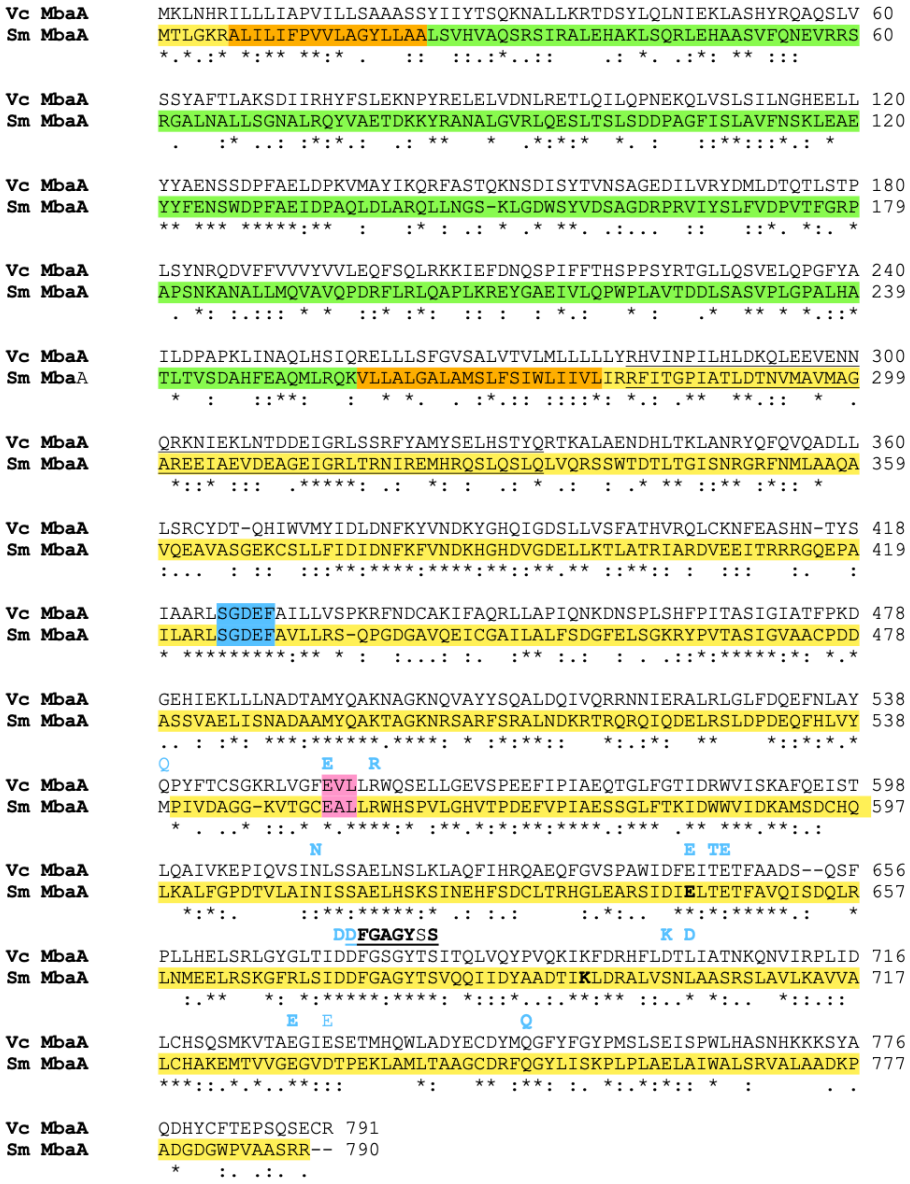

Figure 2. Clustal Omega sequence alignment of the *V. cholerae* O1 (Vc MbaA) and *S. meliloti* 1021 (Sm MbaA) MbaA proteins. Identical residues, conserved substitutions and semi-conserved substitutions are indicated by asterisks, colons, or periods. Color shading of residues in the Sm MbaA denotes protein topology predicted with DeepTMHMM [41]: yellow, cell interior; orange, membrane, green, outside of membrane (periplasm). The cyclase and phosphodiesterase motifs are indicated in blue and pink, respectively. A putative HAMP motif present in both proteins first reported by Schäper et al. [14] was localized using Prosite and is underlined. The 14 key amino acid residues required for PDE

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activity in the *P. aeruginosa* RocR protein [46] appear in light blue above the sequences: the 12 residues in bold are present in Sm MbaA. The domain consisting of 8 amino acids is shown above the sequences and underlined is the functionally important loop 6 identified in RocR [47], and residues identical to those in the Sm MbaA are in bold.

present in the phylogeny are either aquatic and associated with animal hosts (*Vibrio* spp.) or are found in soil and associated with plants (*Sinorhizobium*/*Ensifer*). Among rhizobia, NspS-MbaA occurred in 24 *S. meliloti* strains, all isolated from *Medicago* spp. Four *Sinorhizobium fredii* strains isolated from *Phaseolus* or *Glycine* contained the proteins. Most *Ensifer* species having NspS-MbaA were isolated from legume nodules, while *Ensifer adherens* strains were mostly from soil. There is a 3 bp overlap of nspS and *mbaA* in all of these rhizobia.

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Over 180 strains of environmental (mostly water) and clinical isolates of *V. cholerae* contain NspS-MbaA. Other *Vibrio* species in the phylogeny were mostly isolated from fresh- or salt water animals. *nspS* and *mbaA* overlap by 16 bp in all *Vibrio* species.

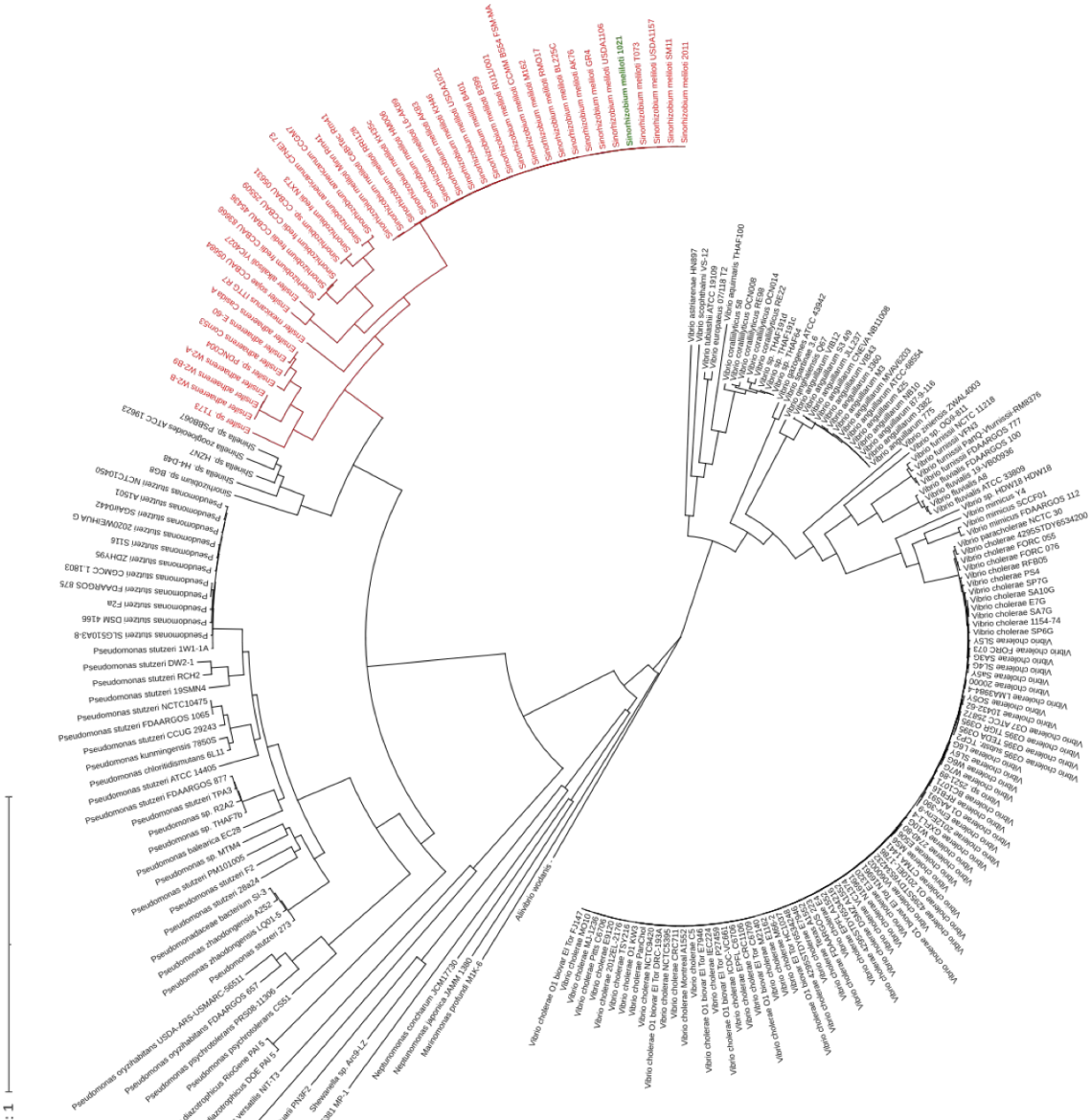

Figure 3. Phylogenetic analysis based on 222 proteobacterial genomes that contain neighboring genes encoding NspS-MbaA. Hits were returned by blastP search using the *S. meliloti* NspS-MbaA proteins as query against 16,076 complete proteobacterial genome sequences from NCBI. Sinorhizobium and Ensifer species are in red text, S. meliloti 1021 is in green.

Most of the 24 strains of *Pseudomonas stutzeri* formed a cluster and the species as a whole was represented by isolates from sediments, sludge, soil and rhizospheres, and seawater. Other *Pseudomonas* species were represented by both environmental and clinical isolates. Absent from the phylogeny were commonly plant-associated *Pseudomonas* species like *P. fluorescens*, *P. syringae* and *P. putida*. A plant growth-promoting

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rhizobacterium isolated from sugarcane roots, *Gluconacetobacter diazotrophicus* PA1 5, is also in this group. In *Pseudomonas* species and *G. diazotrophicus*, *nspS* and *mbaA* overlap by 13 bp.

The synteny of genes encoded near *nspS-mbaA* was perfectly conserved in 41 of the 42 *S. meliloti* strains and conserved to a large degree in other *Sinorhizobium* and *Ensifer* species (Fig. S3, supplemental material). Genes encoded near *nspS-mbaA* did not bear any apparent relation to polyamine or c-di-GMP metabolism. A similar analysis done with the *V. cholerae* strains showed that all had a largely syntenic set of genes with no obvious relation to NspS-MbaA function, and these genes were unrelated to those neighboring *nspS-mbaA* in *S. meliloti*.

In summary, the proteobacteria that encode *nspS* and *mbaA* include a restricted subset of aquatic or soil dwelling species that often have pathogenic or mutualistic interactions with eukaryotes.

3.2. Growth and polyamine profiles in S. meliloti *wild types and* nspS *mutants*

For the phenotypic characterization experiments described below, cultures of the 1021 and Rm8530 wild types and *nspS* mutants were grown in MMSN without or with an exogenous polyamine (0.1 mM). There were no significant differences in the growth of the strains with or without polyamines (Figs S1 and S2, Supplemental material). In both the 1021 and Rm8530 wild types and *nspS* mutants, the addition of an exogenous polyamine to a culture caused a two- to several-fold increase in the intracellular concentration of the added polyamine, without affecting the levels of other polyamines (results not shown).

3.3. Effect of exogenous PAs on biofilm formation, EPS production and autoaggregation in QS-deficient strain 1021

To determine the effect of NspS on biofilm formation in *S. meliloti* 1021, which lacks a functional QS system, we grew the wild type and 1021 nspS in MMSN without (control conditions) or with an exogenous polyamine and determined biofilm formation after 3 days (Fig. 4A). In comparison to wild type 1021 grown under control conditions, cultures with exogenous Spm, Put, DAP and Cad had 59, 47, 28 and 13 % less biofilm, respectively. More biofilm was formed by the 1021 nspS in comparison to the wild type under all conditions, with increases ranging from 1.1 fold (cultures with Spd) to 2.6 fold (cultures with Spm). In cultures with added Put or DAP, the mutant produced 2.2 and 1.6-fold more biofilm, respectively, than wild type, while the presence of other polyamines caused more modest increases in biofilm formation in the mutant. Under control conditions, 1021 nspS made 1.4-fold more biofilm than the wild type (Fig. 4A). In comparison to the control, exogenous Spd, NSpd and HSpd increased biofilm formation by the 1021 wild type by a modest 1.1-1.2-fold. In 1021 nspS, added NSpd and DAP did not change the high level of biofilm formation seen under control conditions. Biofilm formation in 1021 nspS cultures containing Put, Cad, Spd,

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Spm or HSpd decreased 11-26 % relative to the control culture. In summary, when *nspS* is inactivated in strain 1021, biofilm levels show less change in response to exogenous polyamines.

To relate total EPS production with biofilm formation, we measured the hexose content of culture supernatants obtained from the cultures used for the biofilm assays. EPS levels in wild type 1021 were only significantly affected in cultures containing exogenous Put, Spm and DAP, which reduced EPS levels 53, 47 and 43 % relative to the control culture (Fig. 4B). Compared to control conditions, EPS production by 1021 nspS was most affected in cultures containing Spm (1.6-fold increase). EPS production by the wild type in comparison to the *nspS* mutant increased in the mutant grown with Put, DAP and Spm (1.9 to 2.4-fold), and decreased in the control and with exogenous HSpd (19 % decreases for each). In summary, the levels of biofilm formation and EPS production correlated with the significant changes seen in these parameters for both 1021 wild type and *nspS* mutant in cultures containing Put, DAP and Spm (Fig. 4A and 4B).

We measured autoaggregation in 1021 wild type and 1021 nspS cultures grown in the absence or presence of an exogenous polyamine (Fig. 4c). Under control conditions without added PAs, 1021 nspS aggregated 1.8-fold more than the wild type (Fig. 4C), consistent with a 1.4-fold increase in biofilm formation (Fig. 4A) but contrasted by a 19 % decrease in EPS production (Fig. 4B). In cultures with added Put versus control conditions, aggregation increased 1.8-fold in both the wild type and 1021 nspS (Fig. 4c), which did not reflect the significantly increased biofilm and EPS production in the mutant over wild type when grown with Put (Figs 4A and 4B). However, 1.7 and 1.4-fold increases in aggregation in the mutant versus wild type grown with DAP and Spm, respectively (Fig. 4C) did reflect the mutant's increased biofilm and EPS production found under these conditions (Fig. 4A and 4B).

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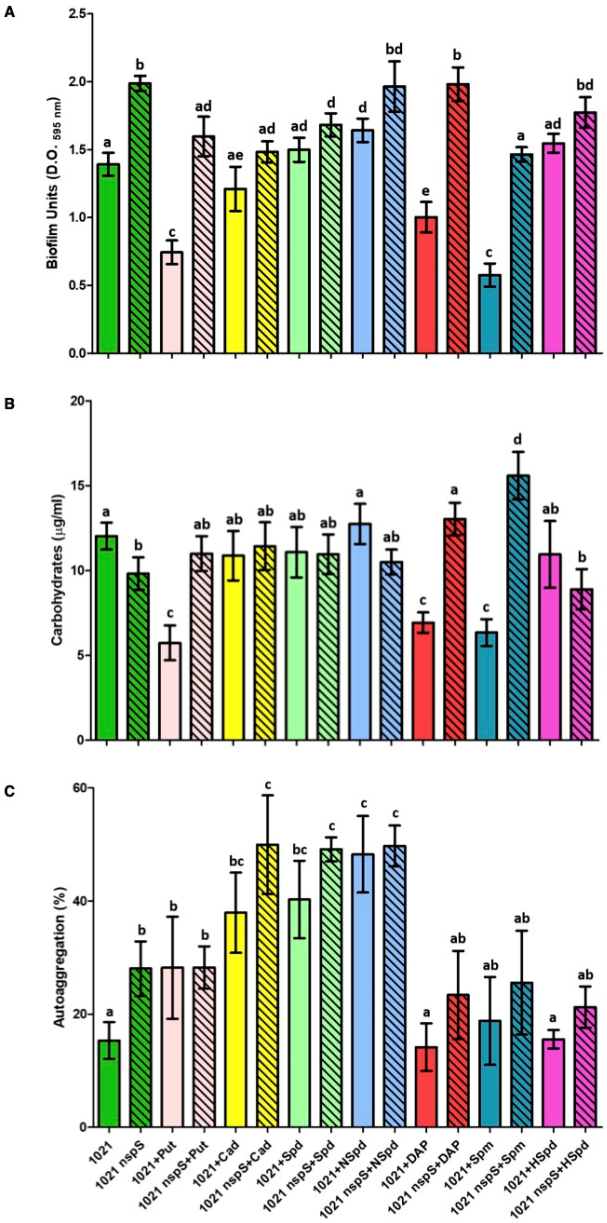

Figure 4. Biofilm formation (A), culture supernatant carbohydrates (B) and autoaggregation (C) of *S. meliloti* 1021 and 1021 nspS grown in the absence or presence of exogenous (0.1 mM) polyamines. Biofilm was determined by crystal violet staining of cultures grown in borosilicate glass tubes. Carbohydrate (hexose) concentrations of culture supernatants from biofilm assay cultures were determined by the Anthrone method and represent total EPS production. Autoaggregation assay show the mean percentage aggregation for each strain. The experiments was performed in quadruplicate and repeated three times. Error bars represent SD. One-way analysis of variance with a Tukey post test were performed. Values for columns with the same letter are not statistically significant.

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In comparison to control conditions, Cad, Spd and NSpd each increased autoaggregation by 1.7 to 1.8-fold in the mutant and by 2.4, to 3.1-fold in the wild type. Exogenous DAP or Spm caused no significant changes in aggregation for either strain compared to control conditions (Fig. 4C), with the mutant having higher autoaggregation than the wild type under these conditions (Fig. 4B).

3.4. Effect of exogenous PAs on biofilm formation, EPS production and aggregation in QS-competent strain Rm8530

To determine how QS affected the biofilm, EPS and aggregation responses of *S. meliloti* to polyamines and the NspS-MbaA system, we repeated the phenotypic assays described above with strains Rm8530 and Rm8530 nspS (Fig. 5). Under control conditions and in cultures with exogenous Put, Cad, Spm or HSpd, biofilm formed at a similar level and there were no large differences between strains Rm8530 and Rm8530 nspS. In cultures with added Spd, biofilm formation was 1.9 and 1.5-fold higher than the control level in the wild type and mutant, respectively, while NSpd caused the biggest changes in biofilm formation with 3.3 and 2.2-fold increases in Rm8530 and Rm8530 nspS, respectively, versus controls. Added DAP caused 1.5 and 1.8-fold increases in the Rm8530 and Rm8530 nspS, respectively (Fig. 5A).

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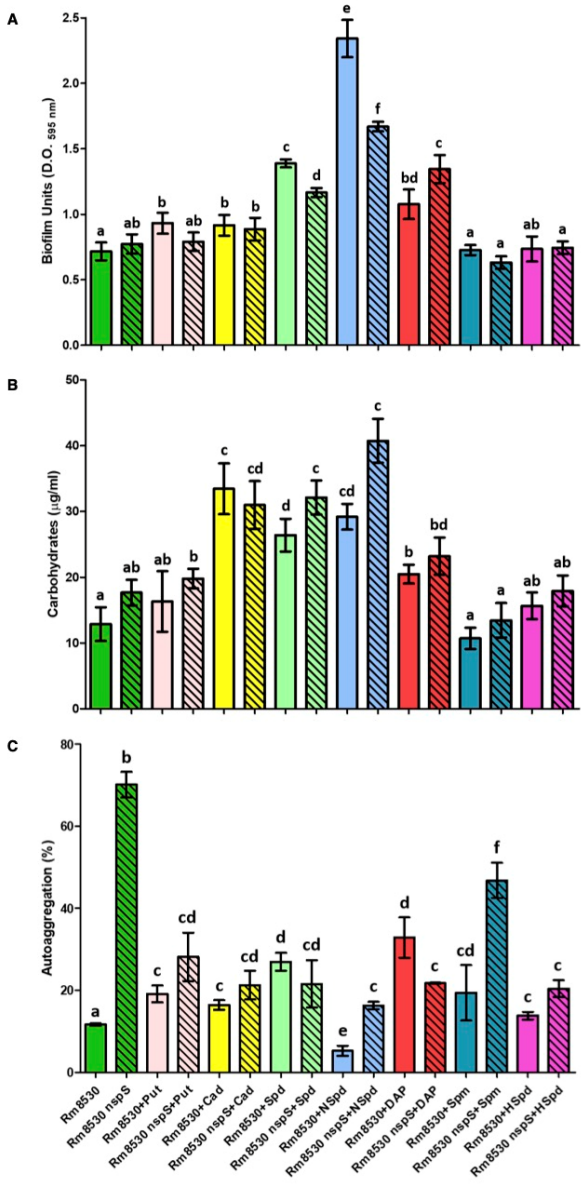

Figure 5. Biofilm formation (A), culture supernatant carbohydrates (B) and autoaggregation (C) of *S. meliloti* Rm8530 and Rm8530 nspS grown in the absence or presence of exogenous (0.1 mM) polyamines.. Biofilm was determined by crystal violet staining of cultures grown in borosilicate glass tubes. Carbohydrate (hexose) concentrations of culture supernatants from biofilm assay cultures were determined by the Anthrone method and represent total EPS production.. Autoaggregation assay show the mean percentage aggregation for each strain. The experiments was performed in quadruplicate and repeated three times. Error bars represent SD. One-way analysis of variance with a Tukey post test were performed. Values for columns with the same letter are not statistically significant.

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Hexose assays showed that under control conditions Rm8530 nspS produced 1.4-fold more EPS than the wild type (Fig. 5B), which corresponded to a 1.1 fold increase in biofilm (Fig. 6A). Relative to control conditions, EPS production in the mutant and wild type changed 2.3 and 2.3-fold with NSpd, 1.8 and 2.7-fold with Cad, 1.8 and 2.1-fold with Spd, 1.3 and 1.6-fold with DAP, 1.1 and 1.3-fold with Put, 1.0 and 1.2-fold with HSpd, and 0.8 and 0.9-fold with Spm, respectively (Fig. 5B). Cultures with exogenous Spd or NSpd showed an inverse correlation between biofilm and EPS. Put, DAP, Spm and HSpd caused little change in either biofilm or EPS production between the mutant and wild type (Figs 5A and 5B).

Cell aggregation increased 2.4 to 5.8-fold in Rm8530 nspS versus the wild type in control cultures or with exogenous NSpd or Spm (Fig. 5C). These large differences between wild type and mutant were not reflected in similar changes in biofilm formation. Exogenous NSpd greatly decreased autoaggregation in both strains while substantially increasing EPS production and biofilm formation. Cultures grown with Put, Cad, Spd or DAP showed a maximum of 1.5-fold increases or 1/3 decreases in autoaggregation between Rm8530 and Rm8530 nspS (Fig. 5C), generally with no corresponding changes in biofilm and EPS production.

3.5. The NspS-MbaA system alters motility in response to exogenous PAs

Swarming is a social spreading phenomenon involving cell elongation and hyperflagellation that occurrs on solid or semisolid surfaces such as the culture medium containing 0.5 % agar used in our assays. In representative assays (Fig. 6A), swarming by wild type 1021 increased or decreased by no more than 12 % with any of the added polyamines. In comparison to 1021 nspS assayed under control conditions, exogenous Put, NSpd and DAP increased swarming by 1.2, 1.7 and 1.6-fold. In comparing the wild type and 1021 nspS, swarming in the mutant was 78 % that of the wild type under control conditions. No significant change in swarming between the wild type and mutant occurred in assays with Cad and Spd, while DAP and NSpd increased swarming 1.2 and 1.5-fold, respectively in the mutant. Other polyamines reduced swarming by the mutant from 12-16 % in comparison to the wild type (Fig 6A).

In comparison to control conditions, swarming in Rm8530 increased 1.2 to 1.4-fold with exogenous Put, Spd NSpd or DAP, and decreased 44 % with Spm (Fig. 6B). For Rm8530 nspS, all of the polyamines either caused little change (Put, Spd, Spm) or increased swarming about 1.5-fold (Cad, NSpd and DAP).

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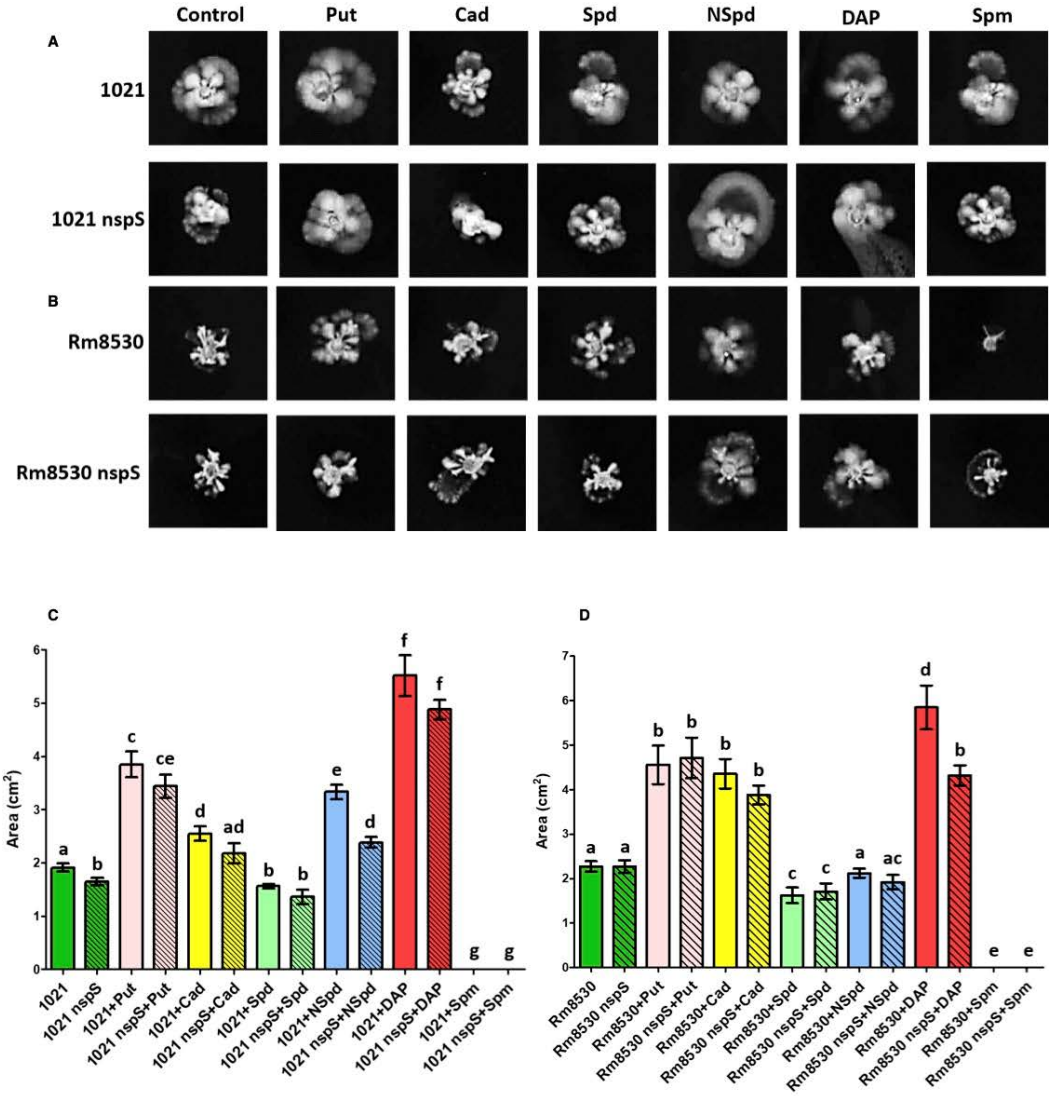

Figure 6. Swarming motility of *S. meliloti* 1021 and 1021 nspS. (Panel A) and Rm8530 and Rm8530 nspS (Panel B) grown without or with exogenous (0.1 mM) polyamines. The experiment was performed in triplicate and repeated three times. Results of a representative experiment are shown. Swimming motility of *S. meliloti* 1021 and 1021 nspS. (Panel C) and Rm8530 and Rm8530 nspS (Panel D) grown without or with exogenous (0.1 mM) polyamines. The experiment was performed in triplicate and repeated three times. Error bars represent SD. One-way analysis of variance with a Tukey post test were performed. Values for columns with the same letter are not statistically significant.

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Swarming in the mutant versus wild type was very similar under control conditions but decreased nearly 30 % with exogenous Put and Spd. Other polyamines caused the mutant to swarm 1.2 to 1.7-fold more than the wild type (Fig. 6B).

Swimming motility involves the movement of individual cells in liquid or semisolid media such as one with 0.3 % agar used in our assays. In 1021 and 1021 nspS, exogenous PAs with the exception of Spd increased swimming 1.4 to 3.1-fold. Spd decreased swimming by about 20 % in both strains (Fig. 6C). The main difference between the two strains occurred with exogenous NSpd, which decreased swimming in the mutant by nearly 30 % in comparison to the wild type. Exogenous Spm completely prevented swimming in both the wild type and mutant strains (Fig 6C).

Swimming in Rm8530 and Rm8530 nspS was identical under control conditions and increased up to 2-fold in both strains grown with Put or Cad (Fig 6D). Swimming in cultures with Spd or NSpd were similar to control conditions for both strains. The most notable difference in swimming between the Rm8530 wt and *nspS* mutant was with added DAP, where swimming was reduced almost 25 % in the mutant. Swimming was completely prevented in both strains by added Spm (Fig 6D).

In summary, 1021 and 1021 nspS show an increase in both swarming and swimming only in cultures with added Put. For Rm8530 and Rm8530 nspS, similar increases or decreases in swarming and swimming occurred in both wild type and mutant with any of the polyamines except Spm, which decreased swarming in the wild type Rm8530.

3.6. Exogenous Put, DAP and Spm increase nspS *transcription*

The the effect of exogenous polyamines on the transcription of *nspS* was measured in strain 1021 with a plasmid containing the *nspS* promoter region fused to the *gusA* reporter gene. Growth of the fusion strain with exogenous DAP, Put and Spm caused 1.7. 2.5 and 3.9-fold increases in *nspS* transcription, respectively. No changes in *nspS* expression occurred when Cad, Spd, or NSpd were added to the cultures (Fig 7).

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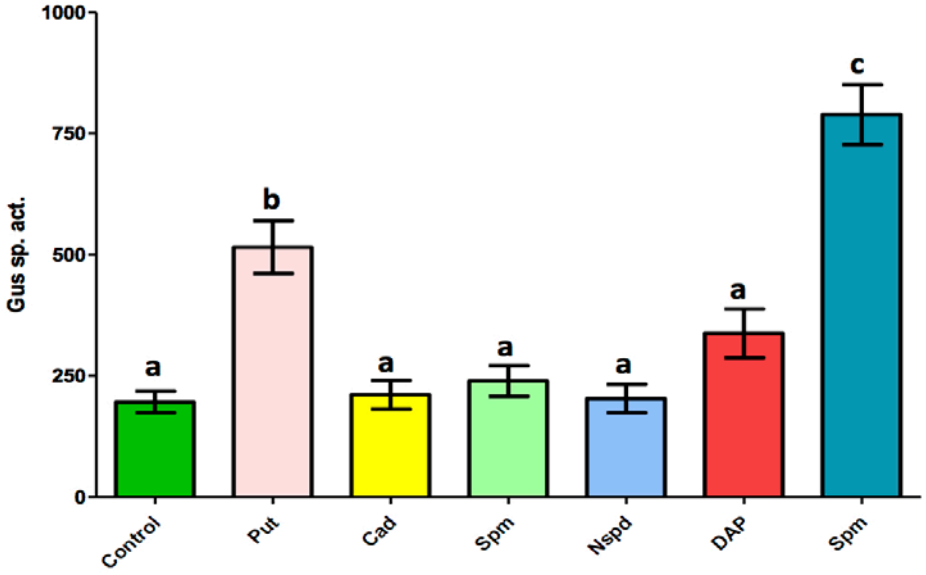

Figure 7. β-glucuronidase (Gus) activities produced by *S. meliloti* 1021 (pBBR_nspS::gusA) with exogenous PA added. Cultures were grown 18 h at 30 °C in MMSN minimal medium with the indicated polyamine at a 0.1 mM. Values are the mean ±SD for two independent experiments with two technical replicates. 1 U=nmol product min^{-1} mg $protein^{-1}$. One-way analysis of variance with a Tukey post test were performed. Values for columns with the same letter are not statistically significant.

4. Discussion

We previously reported that exogenous polyamines affect biofilm formation, EPS production and motility in *S. meliloti* Rm8530 [36]. We hypothesized that these effects were at least partly due to the *S. meliloti* NspS-MbaA homologs acting as a polyamine sensing-transducing system to modulate biofilm formation in a manner analogous to the *V. cholerae* system [23,24]. We show here that the inactivation of the *S. meliloti* NspS-MbaA system does indeed affect the ability of specific exogenous polyamines to alter biofilm formation in *S. meliloti* 1021 and Rm8530 and that the phenotypic responses are also conditioned by QS.

The role of the *S. meliloti nspS* has not been previously studied, but *mbaA* null mutants have been characterized. Schäper et al. [14] found that a *S. meliloti* Rm2011 *mbaA* mutant made 1.17-fold more biofilm and had 0.84-fold the swimming motility as the wild type. Rm2011 is an *expR*⁻ strain closely related to 1021. Wang et al. [16] found that a *S. meliloti* 1021 *mbaA* mutant swarmed significantly less and produced several

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fold more EPS than the wild type. Biofilm formation was not reported [16]. In strain 1021, we found a similar trend of more biofilm and/or EPS and lower motility when *nspS* was inactivated (Figs. 4A and 4B). As mentioned, the insertional inactivation of *nspS* almost certainly prevent *mbaA* expression, effectively making our *S. meliloti nspS* mutants *nspS mbaA* double mutants.

The overlap of *nspS* and *mbaA* might allow translational coupling of the two genes [48]. In *V. cholerae*, *nspS* overlaps *mbaA* by 17 bp and the genes are co-transcribed [42]. Virtually all of the *nspS* and *mbaA* gene pairs obtained in our phylogenetic analysis (Fig. 3) overlap and are perhaps co-regulated, thus allowing balanced production of the two proteins forming the system.

In the QS-deficient strain 1021 wild type, exogenous Put, DAP and Spm all greatly reduce biofilm formation, while 1021 nspS showed little response to these polyamines and maintained a high level of biofilm formation in their presence (Fig. 4A). Changes in biofilm formation in response to different exogenous polyamines in 1021 and 1021 nspS correlated with total EPS production. It is notable that Put, DAP and Spm were the only polyamines tested that affected the transcriptional expression of *nspS* in strain 1021, causing 1.7 to 3.9 fold increases (Fig. 7). Based on the *V. cholerae* model, we hypothesize that the *S. meliloti* NspS that is unligated to a polyamine (apo-NspS) or ligated to Put, DAP or Spm does not interact with MbaA and permits MbaA to have full PDE activity, thus lowering c-di-GMP levels, EPS production and biofilm formation. Having more copies of NspS-MbaA due to the increased transcription of *nspS* (and presumably the overlapping mbaA) would result higher total PDE activitiy and c-di-GMP degradation in the presence of these polyamines.

Cell autoaggregation was markedly affected by exogenous polyamines but did not greatly differ between the 1021 wild type and *nspS* mutant (Fig. 4C). Aggregation did not correlate with biofilm formation (Fig. 4A), contrary to previously reported interdependence of these phenotypes in *S. meliloti* 1021 [4,15]. The discrepancy could be due to methodological differences between these experiments.

In the QS-proficient Rm8530 genetic background, exogenous polyamines affected biofilm formation to a lesser degree than in strain 1021. Exogenous NSpd caused a marked increase in biofilm formation in both strains, with a larger increase in the wild type (3.2 fold) versus the Rm8530 nspS mutant (2.2 fold) (Fig. 5A). Increased biofilm formation in response to NSpd resembles the biofilm-enhancing effect of this polyamine in *V. cholerae*. NSpd production is relatively rare in bacteria, occurring mostly in *Vibrio* species and, among rhizobia, in *Sinorhizobium* species [24,49,50]. As in *V. cholerae*, its present in the environment could act as a signal of "self" and thus promote biofilm formation by *Sinorhizobium* spp.

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The S. meliloti *nspS* mutants are expected to also lack *mbaA* expression (see above). In *V. cholerae*, *nspS mbaA* double mutants make more biofilm than the wild type, similar to the higher biofilm formation in both the Rm8530 wild type and nspS mutant grown with NSpd. Total EPS production did not correlate with biofilm levels in the Rm8530 wild type and mutant strains (Fig. 5B). While this contrasts the EPS-biofilm correlation observed in strain 1021, it is not wholly unexpected given the complex effects that different mixtures of EPS I, EPS II, arabinose-containing polysaccharide and ß-glucan have on biofilm formation [11]. The production of all these polymers is affected by changes in c-di-GMP levels. For example, high c-di-GMP levels promote EPS I biosynthesis in *S. meliloti* Rm2011 (*expR*$^-$) but not in Rm8530 (*expR*$^+$). EPS I generally has a negative effect on biofilm and cell aggregation [11]. In comparison to the wild type, Rm8530 nspS showed very high autoaggregation in cultures without exogenous polyamines (Fig. 5C). NSpd, which increased biofilm formation in both the wild type and mutant, greatly decreased autoaggregation in both strains. As measured in our assays, biofilm formation and autoaggregation were not correlated in either 1021 or Rm8530 wild types or nspS mutants, with each wild type and mutant showing a distinct patterns. The contrasting results regarding exogenous polyamines affecting c-di-GMP dependent phenotypes in strains 1021 and Rm8530 could reflect QS being placed higher in the regulatory hierarchy than NspS-MbaA and overriding the latter [19].

Within the 1021 and Rm8530 genetic backgrounds, swarming motility was differently affected in the wild types versus *nspS* mutants only with selected polyamines. In 1021, NSpd and DAP caused the largest differential between wild type and mutant, while Rm8530 the difference was greatest with Spd and Spm (Figs 6A and 6B). Changes in swarming motility did not correlate with biofilm, EPS or autoaggregation in any of the strains. Swimming motility showed no large *nspS*-dependent differences in either 1021 or Rm8530, although significant changes in swimming were caused by some polyamines (eg, DAP caused notable increases in both of the wild types and *nspS* mutants) (Figs. 6C and 6D). In summary, changes in swimming and swarming motility caused by exogenous polyamines were generally similar in the 1021 and Rm8530 wild types and their corresponding *nspS* mutants. The is consistent with previous results indicating that c-di-GMP has less effect on motility than biofilm in *S. meliloti* [11].

We did not find any polyamines that reciprocally regulated biofilm formation or other phenotypes in *S. meliloti*. This contrasts *V. cholerae*, where Spd or Spm and NSpd significantly decrease and increase, respectively, the formation of biofilm and expression of EPS biosynthesis genes. [20,21,51]. The modulation of c-di-GMP levels and biofilm formation by the NspS-MbaA system in *V. cholerae* is exerted through the absolute and proportional extracellular concentrations of the signal polyamines NSpd and Spd [21]. It is

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possible that changes in biofilm formation dependent on the NspS-MbaA system in S. meliloti would differ with higher or lower concentrations of polyamines than those used in our experiments or with mixtures of different polyamines.

We found that many of the amino acid residues required for the function of PotD/PotF homologs and PDE and/or DGC catalyzing proteins were conserved in the homologous *S. meliloti* NspS or MbaA proteins. The *S. meliloti* proteins also conserved many residues specific to the function of the *V. cholerae* NspS and MbaA (Figs. 1 and 2). These sequence similarities between the two systems support that the *S. meliloti* NspS-MbaA is functional sensor-transducer system, while the differences in some key residues could explain the different phenotypic responses of the *S. meliloti* and *V. cholerae* systems. NspS proteins have their greatest sequence similarity to periplasmic binding proteins of the ABC transporters for Spd and Put, PotD and PotF, respectively [52]. We found no differences in the ability of the wild type or *nspS* mutants to accumulate exogenous polyamines in either the 1021 or Rm8530 genomic backgrounds, indicating that NspS is not required for their transport. This agrees with the lack of transport function found for the V. cholerae NspS [42].

The taxonomic distribution of NspS-MbaA proteins in proteobacterial genomes indicates that it is present in relatively few predominantly water and soil inhabiting species. Most of these species interact symbiotically or pathogenically with eukaryotes, and their NspS-MbaA systems might respond to environmental polyamines released by these organisms in order to modulate the interaction.

In conclusion, the *S. meliloti* NspS-MbaA does affect several c-di-GMP-dependent phenotypes in response to exogenous polyamines. We are currently determining how global gene expression is affected by polyamines in the *S. meliloti* wild types and *nspS* mutants. We will also determine if the *S. meliloti* NspS-MbaA system responds to polyamine cues and tailors biofilm formation and other c-di-GMP phenotypes to specific environments.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/xxx. Fig. S1: Growth of *S. meliloti* 1021 and 1021 nspS in liquid MMSN minimal medium with or without 0.1 mM of the indicated polyamine. Fig. S2: Growth of *S. meliloti* Rm8530 and Rm8530 nspS in liquid MMSN minimal medium with or without 0.1 mM of the indicated polyamine. Fig. S3: Genomic context of *nspS-mbaA* in *Sinorhizobium* and *Ensifer* species, showing synteny in the arrangement of the genes in the region 10 Kb upstream and 10 Kb downstream of the operon.

Author Contributions: Concept, M. F. D.; methodology, analysis and investigation, V. M. C.-J., M.F D., G. G and V. A. B.-R.; writing-original draft, V. M. C.-J.; writing- editing and final draft, M. F. D. and V. M. C.-J.; project administration and funding, M. F. D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded in part by grants IN206317 and IN207620 from DGAPA-UNAM to M. F. Dunn. V. M. Chávez-Jacobo was supported by a DGAPA-UNAM post-doctoral fellowship.

Data Availability Statement: Not applicable.

Acknowledgments: We thank Alejandro Aguilar Vera for additional bioinformatic analysis and Catalina Ortíz Ortíz for laboratory assistance.

Conflicts of Interest: The authors declare no conflict of interest.

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