MtrR Control of a Transcriptional Regulatory Pathway in Neisseria meningitidis That Influences Expression of a Gene (nadA) Encoding a Vaccine Candidate

Jason M. Cloward
William M Shafer, Emory University

Journal Title: PLoS ONE
Volume: Volume 8, Number 2
Publisher: Public Library of Science | 2013-02-08, Pages e56097-e56097
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1371/journal.pone.0056097
Permanent URL: http://pid.emory.edu/ark:/25593/f7hts

Final published version: http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0056097

Copyright information:
This is an Open Access article distributed under the terms of the Creative Commons Universal : Public Domain Dedication License (http://creativecommons.org/publicdomain/zero/1.0/), which permits making multiple copies, distribution of derivative works, distribution, public display, and publicly performance, provided the original work is properly cited.

Accessed December 29, 2018 5:55 PM EST
MtrR Control of a Transcriptional Regulatory Pathway in Neisseria meningitidis That Influences Expression of a Gene (nadA) Encoding a Vaccine Candidate

Jason M. Cloward¹,²* and William M. Shafer¹,²*

¹ Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia, United States of America; ² Laboratories of Bacterial Pathogenesis, Veterans Affairs Medical Center (Atlanta), Decatur, Georgia, United States of America

Abstract

The surface-exposed NadA adhesin produced by a subset of capsular serogroup B strains of Neisseria meningitidis is currently being considered as a vaccine candidate to prevent invasive disease caused by a hypervirulent lineage of meningococci. Levels of NadA are known to be controlled by both transcriptional regulatory factors and a component of human saliva, 4-hydroxyphenylacetic acid. Herein, we confirmed the capacity of a DNA-binding protein termed FarR to negatively control nadA expression. We also found that a known transcriptional regulator of farR in N. gonorrhoeae termed MtrR can have a negative regulatory impact on farR and nadA expression, especially when over-expressed. MtrR-mediated repression of nadA was found to be direct, and its binding to a target DNA sequence containing the nadA promoter influenced formation and/or stability of FarR:nadA complexes. The complexity of the multi-layered regulation of nadA uncovered during this investigation suggests that N. meningitidis modulates NadA adhesin protein levels for the purpose of interacting with host cells yet avoiding antibody directed against surface exposed epitopes.

Introduction

Neisseria meningitidis is a Gram-negative obligate human pathogen that colonizes the nasopharynx in 10–35% of adults [1]. For reasons not currently understood, commensal meningococcal (MC) colonization develops into an invasive disease causing septicemia and meningitis in 0.5 per 100,000 persons in the United States and up to 1,000 per 100,000 persons in sub-Saharan African epidemics [2]. The speed of disease progression results in up to 10–15% mortality even with antibiotic therapy [3], while often leaving survivors with permanent neurological complications [4]. Vaccines against the capsular polysaccharide of the most common disease-associated serotypes (A, C, W135, and Y) are available, leaving the hypervirulent and immune-evasive serotype B as a current focus for vaccine research [5].

Adhesion to the mucosal surface of the nasopharynx is the first step in successful colonization, mediated by a variety of factors, with type IV pili [6,7,8] and Opa and Opc proteins [9,10] produced in the greatest abundance. Recently, a non-fimbrial “Oca” family (Oligomeric coiled-coil adhesin) Neisseria adhesin termed NadA was identified in 50% of hypervirulent MC capsular serogroup B lineages [11], but not in other capsular serogroup strains. Comprised of a leader peptide, globular “head” domain, α-helix intermediate region, and a C-terminal membrane anchor, NadA forms highly stable multimeric coiled-coil structures along the helical stalk, positioning the globular “head” for host cell interaction [12]. Importantly for consideration as a vaccine candidate, recombinant NadA lacking the C-terminal anchor elicits a bactericidal antibody response with epitopes accessible in encapsulated MC. Although nadA allele sequences differ between strains, varied antigen expression, not diversity, influences immune sera titer levels and protection [11]. Accordingly, the identification of factors influencing NadA levels at the gene expression level is critical for optimizing the efficacy of a NadA-targeted vaccine. Furthermore, understanding nadA expression may offer clues into the signals involved in converting a passive co-inhabitant of the human mucosal lining into an invasive and fatal septic infection.

MC uses a multi-tiered approach to control nadA expression. Maximum levels of the NadA protein are observed in stationary-phase in a growth-dependent manner [11], with expression of nadA varying widely among MC strains [13,14]. Upstream from the promoter are multiple tetranucleotide (TAAA) repeats whose number corresponds with varied nadA expression [13,15]. These repeats are phase variable, likely caused by slipped-strand mispairings during replication [16]. Several regulatory proteins bind to the nadA promoter (Figure 1), including integration host factor (IHF) and ferric uptake regulatory protein (Fur), though nadA expression is unchanged in a Fur null mutant [14]. Recently, a MarR-family transcriptional regulator, termed FarR and NadR...
in separate publications [14,17], was identified as a repressor of \( \text{nadA} \), further expanding the list of \( \text{nadA} \) regulatory factors. This DNA-binding protein was first identified in the gonococcus (GC) and was shown to repress expression of the \( \text{farAB} \)-encoded efflux pump that is responsible for high levels of fatty acid resistance [18]. In contrast, MC FarR does not affect fatty acid resistance through FarAB, perhaps due to naturally high fatty acid resistance expressed by this pathogen [19]. Interestingly, however, MC FarR does bind to its \( \text{farAB} \) promoter region with relatively high affinity and represses \( \text{farAB} \) expression as shown by RT-PCR [20]. Because FarR regulates expression of \( \text{farAB} \) in both MC and GC, while \( \text{nadA} \) is present only in a subset of MC populations, we will continue to use the nomenclature of FarR for the repressor of \( \text{nadA} \) based on its more universal activity on \( \text{farAB} \) in both GC and MC.

The small molecule 4-hydroxyphenylacetic acid (4HPA) was identified as an inducer or de-repressor of \( \text{nadA} \) by relieving the DNA-binding activity of FarR [14]. Being a colonizer of the oropharynx, MC is washed in saliva, in which 4HPA is a common metabolite [21], possibly leading to increased expression of \( \text{nadA} \) and subsequent invasive disease. Curiously, FarR-controlled targets in GC are directly and indirectly regulated by the TetR family regulator MtrR. Repression of \( \text{farR} \) by MtrR indirectly up-regulates \( \text{farAB} \) [18], while the gene encoding glutamine synthetase (\( \text{glnA} \)) is directly regulated by both FarR and MtrR [22]. Therefore, we questioned whether MtrR similarly affects \( \text{nadA} \) expression in MC, adding to the growing list of regulatory factors targeting \( \text{nadA} \). Here we confirm that FarR is the primary repressor of \( \text{nadA} \), yet MtrR, when expressed at elevated levels, directly represses \( \text{nadA} \) as well. Furthermore, DNA-binding and DNase I protection assays suggest that MtrR influences FarR binding at the \( \text{nadA} \) promoter similar to the phenomenon seen in \( \text{glnA} \) expression in GC [22], suggesting a higher complexity to Neisserial regulatory schemes that is conserved across species.

### Results and Discussion

#### Control of \( \text{nadA} \), \( \text{farR} \), and \( \text{mtrR} \) expression in MC strain M7

Co-regulation and competitive regulation between FarR and MtrR has been shown previously for multiple targets in GC [18,22] but not MC. Given previous observations that in MC FarR can regulate \( \text{nadA} \), we asked if MC MtrR can control \( \text{farR} \) expression. We also tested if MC MtrR could control \( \text{nadA} \) directly.

To investigate possible influences of MtrR on \( \text{nadA} \) and/or \( \text{farR} \) expression in MC, the expression profiles for these genes were determined using a promoterless \( \text{lacZ} \)-fusion expression system (Figure 1) employed previously to monitor gene expression in GC [23]. Using translational \( \text{lacZ} \)-fusions to each gene’s promoter (ranging from 427 to 524 bp; Figure 1), \( \text{mtrR} \) expression was compared to \( \text{farR} \) and \( \text{nadA} \) across multiple growth phases of broth–grown cultures or from overnight, agar-grown cultures (Figure 1) of MC strain M7, which is a capsule-deficient mutant of...
strain NMB that is used for biosafety purposes [24]. This was done because earlier work [25] reported that farR was maximally expressed between late-log and stationary phase, while nadA expression peaks at stationary phase [11]. Importantly, the lacZ fusions did not significantly impact growth rates in broth for the M7-derived strains (Figure 2A), suggesting that any expression profile differences are not growth rate-dependent. Aliquots from different growth phases (Figure 2A; boxed A, B, and C) were assessed for β-galactosidase activity and compared against the activity of overnight cultures grown on agar plates (Figure 2B). The results showed that agar-grown MC had higher levels of expression for all three genes compared to broth-grown strains, and this was especially true for nadA. With respect to agar-grown cultures, we noted that nadA expression was considerably greater than farR or mtrR with the latter being the most poorly expressed gene (Figure 2B). Based on these results, all subsequent

![Figure 2. Growth phase-dependent expression of nadA, farR, and mtrR in N. meningitidis M7.](image)

(A) Growth curve of strain M7 expressing lacZ fused to nadA (solid line; circle timepoints), farR (dotted line; triangle timepoints), and mtrR (dashed line; square timepoints) promoter regions measured by OD600 optical density. Boxed A, B, and C; timepoints for sample harvest. (B) Specific activity of β-galactosidase activity of lacZ fusions as indicated. Samples harvested from liquid culture at various growth phases (A, B, C) were compared with O/N growth on agar plates. Inset; magnified view of mtrR-lacZ expression. NS, not significant; **, P<0.01.

doi:10.1371/journal.pone.0056097.g002

MtrR Repression of nadA
gene expression studies were performed on cultures grown overnight on GCB agar plates.

Having established that the lacZ fusion technology could be employed to monitor gene expression in strain M7, we next asked if MtrR can regulate farR as it does in GC and if, in turn, it can modulate nadA expression. We first asked if loss of MtrR impacts MC farR expression levels and for this purpose constructed an M7 farR-lacZ fusion strain (427 bp promoter region; Figure 1) in an mtrR null mutant that carries the non-polar aphA-3 cassette within the mtrR coding sequence. With this fusion strain, we noted a small but significant (p<0.01; Figure 3) increase in farR-lacZ expression, which was reversed by complementation when the M7 mtrR allele was expressed ectopically under its own promoter or an IPTG-inducible promoter. Having observed a significant, albeit modest, influence of MtrR on farR-lacZ expression, we next asked if nadA expression, known to be negatively controlled by FarR [17], would influence the mtrR on

Figure 3. MtrR regulation of farR. Specific activity of β-galactosidase activity of farR-lacZ in the strains M7 wild-type, ΔmtrR, and ΔmtrR complemented with the native and inducible-promoter alleles (super-script asterisk), respectively. NS, not significant; *, p<0.05; **, p<0.01. doi:10.1371/journal.pone.0056097.g003

FarR and MtrR binding to a target DNA sequence upstream of nadA

The data obtained using translational fusion strains bearing an over-expressed wild-type mtrR allele in an mtrR null mutant background indicated that elevated levels of MtrR can exert negative regulatory influences on nadA expression in MC strain M7. Based on this hypothesis, we asked if MtrR can bind target DNA sequences upstream of nadA by employing electrophoretic mobility shift assays (EMSA) using FarR or MtrR fused to maltose-binding protein (MBP) at their C-terminus maps describing the various DNA probes used are shown in Figure 1. As described previously [14], FarR was found to bind the target nadA sequence (457 bp) in a specific manner with at least three DNA-protein complexes observed (Figure 5A, arrows). Furthermore, the results from binding specificity assays suggested that FarR has greater affinity for the nadA-promoter than to its own (443 bp) or farR promoter-bearing sequences (497 bp and shown in Figure 5B; importantly, a probe lacking a FarR-binding site (mbpB; 609 bp) was unable to compete with the labeled nadA probe for complexing with FarR.

Having verified that the nadA probe could be recognized in a specific manner with an MC DNA-binding protein (FarR), we next asked if nadA could bind MtrR. First, we confirmed by EMSA the DNA-binding capacity of the MtrR-MBP fusion protein by evaluating its ability to bind a known target DNA sequence, namely the promoter-bearing region upstream of MC mtrCDE

Figure 4A). Furthermore, with the wild-type mtrR sequence under the control of an IPTG-inducible promoter significantly [p<0.01] reduced nadA-lacZ expression by 26% (strain JC4AZ; Figure 4B). Based on these results, we concluded that FarR-mediated repression of nadA-lacZ was greater than that mediated by MtrR, but that MtrR could impact nadA expression by a FarR-independent mechanism. In order to further test this, we compared nadA-lacZ expression in a ΔfarR/ΔmtrR double mutant (strain JC7AZ) against the single ΔfarR mutant (strain JC5AZ). Here, no significant impact on nadA-lacZ expression was observed, further supporting a dominant repression of nadA by FarR. However, IPTG-induction of mtrR in the absence of FarR (strain JC9AZ) did result in a significant reduction in nadA expression levels, with restoration of nadA expression in a stra...
In GC, MtrR is a repressor of the mtrCDE-encoded antimicrobial efflux pump by virtue of its binding between the -210 and -235 promoter elements [26,27]. We found that as little as 1.0 μg of MC MtrR-MBP incubated with MC mtrCDE promoter-bearing probe resulted in virtually a complete shift (Figure 6A; arrow) of the target sequence. Importantly, a similar shifting of the nadA probe by MtrR was observed (Figure 6B; arrow). Specificity of MtrR-binding to the nadA probe was confirmed by use of competitive EMSA. Although the heterologous unlabeled rnpB promoter-bearing probe did to some extent compete for binding, especially at a high concentration (100×), the mtrCDE and nadA unlabeled probes proved to be more effective competitors at a lower concentration (25×) with the mtrCDE probe appearing to be superior (Figure 6C).

The ability of both FarR and MtrR to bind the nadA promoter-bearing region in a specific manner was reminiscent of their ability to bind the DNA sequence upstream of glnA of GC (22). As the binding of either protein to the glnA target can impact binding of the other we asked if a similar situation might exist for the MC-derived nadA target. To test this possibility, we pre-incubated the nadA probe with a fixed concentration of one protein and then introduced increasing amounts of the second protein. In the absence of a competing protein, both MtrR and FarR exhibited a distinct shift of the probe (Figure 7; arrowhead and arrows, respectively). As FarR-MBP concentrations increased following

Figure 4. Expression of nadA by FarR and MtrR. (A–C) Specific activity of β-galactosidase activity of nadA-lacZ in various M7 backgrounds as indicated. Strains over-expressing MtrR marked with a superscript asterisk. NS, not significant; *, P<0.05; **, P<0.01. (D) Western immunoblot analysis of NadA, FarR, and MtrR levels. Protein samples grown overnight on GC agar plates, collected, and analyzed by electrophoresis through 6% (NadA) or 12% (FarR and MtrR) SDS-PAGE gels followed by immunoblot with the respective antisera. Molecular weight standards are listed to the left. Coomassie-stained gel provided for protein level comparison. Arrows represent minor immunoreactive bands used to determine NadA steady-state differences across strains.

doi:10.1371/journal.pone.0056097.g004
Figure 5. DNA binding properties of FarR-MBP. (A) Successive increases of FarR-MBP incubated with 10 ng nadA promoter region to assess binding by gel-shift analysis. Arrows; various complexes of DNA and FarR-MBP. (B) Competition assays. 32P-labeled nadA promoter (384 bp) was incubated with 0.5 μg FarR-MBP and competed with unlabeled nadA, farR (333 bp), farAB (435 bp), and rnpB (354 bp) at 25, 50, and 100 times molar excess of labeled probe (lanes 3 through 14). The competing probe used is listed below each panel. Arrow; 32P-labeled probe competed away from FarR-MBP by unlabeled probe. Lane 1, labeled probe alone; lane 2, labeled probe and 0.5 μg FarR-MBP without competitor.
doi:10.1371/journal.pone.0056097.g005
pre-incubation of nadA target DNA with MtrR-MBP, the MtrR shift remained relatively unchanged, suggesting that FarR-MBP does not compete with MtrR-MBP. In contrast, increasing amounts of MtrR-MBP changed the electrophoretic mobility of FarR-MBP:DNA complexes, suggesting that MtrR-MBP can significantly influence the formation or stability of FarR::nadA complexes.

Figure 6. DNA binding properties of MtrR-MBP. Successive increases of MtrR-MBP incubated with 10 ng mtrCDE (A) or nadA (B) promoter regions to assess binding by gel-shift analysis. Arrow: primary complex of DNA and MtrR-MBP. (C) Competition assays. 32P-labeled nadA promoter (384 bp) was incubated with 1.0 μg MtrR-MBP and competed with unlabeled nadA, mtrCDE (552 bp), and rnpB (354 bp) at 25, 50, and 100 times molar excess of labeled probe (lanes 3 through 14). The competing probe used is listed below each panel. Arrow: 32P-labeled probe competed away from MtrR-MBP by unlabeled probe. Lane 1, labeled probe alone; lane 2, labeled probe and 0.5 μg MtrR-MBP without competitor. doi:10.1371/journal.pone.0056097.g006
In order to learn the mechanism by which MtrR could influence the formation of FarR::nadA complexes, we used DNase I protection assays to determine if their respective binding sites might be in close proximity. We confirmed FarR-MBP-binding to the three sites (data not shown) described by Metruccio et al. [14], which include the –10 promoter and TAAA phase-variable regions (Figure 8B). In repeated DNase I protection assays, clear evidence for a sequence(s) capable of recognizing MtrR could not be obtained. However, in these experiments a DNase I hypersensitive site was identified positioned at the end of the phase-variable TAAA repeats (Figure 8B; asterisk at nucleotide position 209). The presence of this suggests an interaction of MtrR with a sequence upstream of nadA that could influence binding of FarR.

While N. meningitidis colonizes up to 35% of humans [1], fewer than 1% of the population develops an invasive infection [2], suggesting that the bacterium focuses on a more commensal lifecycle. The ability to effectively transition between passive residence and active infection relies on tight transcriptional regulation involving an array of external and internal control systems. In GC, the transcriptional regulators FarR and MtrR have been well-characterized for their role in antimicrobial resistance, allowing for host persistence [28]. FarR represses expression of the fatty-acid efflux pump FarAB [29]; MtrR represses expression of the mtrCDE, which encodes an antimicrobial efflux pump [26,30]. Recently, MC FarR was shown to repress expression of nadA [17], whose gene product is a highly immunogenic adhesin and invasin associated with hypervirulent strains of serotype B MC [11,12]. Interestingly, GC MtrR has also been shown to repress GC FarR, thereby influencing transcription of farAB [18]. As this regulation of a regulator is not unique to GC [22], we explored whether MtrR likewise modulates farR in MC, thus affecting nadA expression.

Our results suggest that MC employs a dual-repressor approach to control nadA expression. Using lacZ translational fusions, EMSA, and DNase I protection assays, we confirmed earlier work [14,17,25] that FarR is a negative regulator of nadA due to its ability to bind target DNA upstream of the coding region (Figure 7B) and affect subsequent expression (Figure 4A). Complicating this regulatory scheme, our results indicate that MtrR can exert an influence on nadA directly by interacting with the upstream DNA sequence and indirectly through its ability to reduce farR expression (Figure 3). With respect to the first mode of MtrR regulation over nadA, our DNA-binding studies indicate that MtrR can bind upstream of this gene in a specific manner (Figure 6C) and can impact the formation and/or stability of FarR::DNA complexes (Figure 7) when its level exceeded that of FarR. The stronger influence of FarR on nadA expression is likely due to its ability to recognize three target sites (Figure 7B) while under the conditions employed in the DNase I protection assay. A possible site for MtrR binding could only be surmised by the presence of a DNase I hypersensitive site (Figure 7A). Interestingly, this site is positioned within a tract of tetranucleotide repeats and a FarR-binding site (Figure 7B).

Under what conditions might MtrR-mediated regulation of nadA have biologic relevance given the strong regulatory action of FarR? We propose several potential mechanisms: The develop-
ment of mutations impacting FarR regulation of \( \text{nadA} \) would require alternative mechanisms of transcriptional regulation that could in part be fulfilled by MtrR. Thus, mutations in FarR that reduce its DNA-binding activity or mutations in FarR-binding sites could enhance \( \text{nadA} \) expression unless other regulatory processes are available. Alternatively, mutations that enhance MtrR levels or interactions with \( \text{nadA} \)-binding sites might repress \( \text{nadA} \) expression above that seen by FarR alone. Precedent for clinical isolates of \( \text{Neisseria} \) bearing regulatory mutations impacting gene expression exists in that gonococcal strains isolated from patients frequently contain mutations in \( \text{mtrR} \) and these can cause dysregulation of the \( \text{mtrCDE} \)-encoded efflux pump operon. Furthermore, cis-acting regulatory mutations can influence transcription of \( \text{mtrR} \) and/or \( \text{mtrCDE} \) or directly enhance expression of the \( \text{mtrCDE} \)-encoded efflux pump [28,31,32,33]. In M7, \( \text{mtrR} \) expression is typically low, yet overexpression of \( \text{mtrR} \) results in almost 30% repression of \( \text{nadA} \) (Figure 4B and D). Accordingly, it will be important to evaluate MC clinical isolates to determine if they may develop mutations impacting \( \text{nadA} \) expression directly or indirectly; the latter being due to mutations in \( \text{farR} \) or \( \text{mtrR} \). With the expression of \( \text{nadA} \) varying significantly among MC strains [13,14], mutations affecting \( \text{farR} \) or \( \text{mtrR} \) expression may have more profound effects on \( \text{nadA} \) expression than those observed here. Deletion of the Correia element or IHF binding site upstream from \( \text{mtrR} \) affects expression of \( \text{mtrCDE} \), which is an MtrR target [32]; Enriquez et al. observed several MC isolates with Correia element deletions upstream from \( \text{mtrR} \), including one serotype B, suggesting that these mutations are not an exception [34]. We propose that this multi-layered regulation of \( \text{nadA} \), which now includes direct regulation by MtrR, reflects an effort by MC to balance levels of the NadA adhesion important for interacting with host cells yet avoiding potentially protective antibody responses.

**Materials and Methods**

**Bacterial strains and growth conditions**

All \( \text{N. meningitidis} \) strains listed in Table 1 are derivatives of strain M7 constructed for this study; M7 is a stable capsule-negative variant of strain NMB and was used for biosafety purposes. MC were cultured on GCB agar (Difco Laboratories, Detroit, MI) with defined supplements I and II [35] at 37°C under 3.8% (vol/vol) CO2. For growth-phase analysis, MC were grown in a shaking incubator at 37°C in GCB broth with sodium bicarbonate and defined supplements I and II as previously described [35].

**Strain construction and verification**

For construction of strain JC2, overlapping PCR products were generated to replace \( \text{mtrR} \) with \( \text{aphA-3} \) [36] by allelic exchange at the native \( \text{N. meningitidis} \) locus, conferring kanamycin resistance. Specifically, primers \( \text{mtrC_R_out}_5' \) and \( \text{mtrR_R_Kan}_5' \) ovhg generated product A; \( \text{kan_F_mtrR}_5' \) ovhg and \( \text{kan_R_mtrR}_3' \) ovhg generated product B; and \( \text{mtrR_F_Kan}_5' \) ovhg and \( \text{NMB1718_FWD} \) generated product C (Table 2). Products A, B,
and C were used as template with flanking primers mtrC_R_out_5' and NMB1718_FWD (Table 2) to generate the final PCR product used for transformation of wild-type M7 with selection on kanamycin at 50 \( \mu \text{g mL}^{-1} \). The substitution of mtrR with aphA-3 was verified by PCR (data not shown) and Western blot (Figure 4). For construction of JC5, PCR products generated by FarR_prom_F and FarR_Sma_R (Table 2) were subcloned into vector pCR\textsuperscript{H}2.1 (Invitrogen) generating pJC5\textsubscript{a} with selection on ampicillin at 100 \( \mu \text{g mL}^{-1} \). PCR products from FarR_Sma_F & FarR_pmalC_Xba_R (Table 2) were purified, digested with Smal, and ligated into pJC5\textsubscript{b} at the Smal site, generating pJC5\textsubscript{b} used to transform M7 with selection on spectinomycin at 60 \( \mu \text{g mL}^{-1} \). The interruption of farR with spc was verified by PCR (data not shown) and Western blot (Figure 4).
Complementation of JC2 and JC5 was accomplished by delivering the parent gene ectopically using vector pGCC3 or pGCC4 [37]. Briefly, primers mtrR_F_Pac_GC3 and mtrR_R_Pme or mtrR_F_Pac_GC4 and mtrR_R_Pme (Table 2) were used to amplify the mtrR allele then digested with appropriate restriction enzymes and ligated into pGCC3 and pGCC4, respectively. Similarly, farR_F_Pac_GC3 and farR_R_Pme (Table 2) were used to amplify the farR allele for subsequent digestion and ligation into pGCC3. All constructs were verified by sequencing prior to transformation. Transformants were selected on erythromycin at 1 m\(\text{g} \text{ml}^{-1}\).

Construction of lacZ reporter fusions, \(\beta\)-galactosidase assay and immunoblot analysis

All lacZ fusions used in this study were prepared in pLES94 and performed as described previously [18,23] using appropriate primers. For nadA-lacZ \(\beta\)-galactosidase assays, we used: for farR-lacZ \(\beta\)-galactosidase assays, farR_F_Bam and farR_lacZ_R were used (Table 2); and for mtrR-lacZ \(\beta\)-galactosidase assays, mtrR_F_Bam and mtrR_lacZ_R were used (Table 2). Constructs encoding a lacZ fusion were grown overnight on GCB agar with supplements, 1 mM IPTG, and 5 mM 4-hydroxyphenylacetic acid, when appropriate. Cells were harvested directly from plates or used to inoculate GCB broth with appropriate supplements and 1 mM IPTG then grown through stationary phase for growth-phase analysis of protein expression. Cells collected from overnight plates were resuspended in phosphate-buffered saline pH 7.2 (PBS), centrifuged for 2 min at 13,000 rpm, and stored overnight at 2\(\text{°C}\) after the supernatant was removed. From liquid cultures, 5 mL aliquots were removed at the indicated growth phases (Figure 1) and centrifuged for 15 min at 5,000 rpm. To determine \(\beta\)-galactosidase specific activities, cell pellets were exposed to 1 mM IPTG, and 5 mM 4-hydroxyphenylacetic acid, when appropriate. Cells were harvested directly from plates or used to inoculate GCB broth with appropriate supplements and 1 mM IPTG then grown through stationary phase for growth-phase analysis of protein expression. Cells collected from overnight plates were resuspended in phosphate-buffered saline pH 7.2 (PBS), centrifuged for 2 min at 13,000 rpm, and stored overnight at 2\(\text{°C}\). To determine \(\beta\)-galactosidase specific activities, cell pellets were used.

Table 2. Oligonucleotides used.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5' → 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>kan_F_mtrR_5_ovhg</td>
<td>AAA CGG CAT TAT GGC TAA AAT GAG AAT ATC ACC</td>
</tr>
<tr>
<td>kan_R_mtrR_3_ovhg</td>
<td>CAA GGC TTG ACT AAA ACA ATT CAT CCA GTA AAA TA</td>
</tr>
<tr>
<td>mtrR_Kan_5_ovhg</td>
<td>CAT TTT AGC CAT AAT GGC GTT TTC TGG TCG G</td>
</tr>
<tr>
<td>mtrR_F_Kan_3_ovhg</td>
<td>ATT GTT TTA GTC AAG CCT TGG TAG CAA TGC</td>
</tr>
<tr>
<td>mtrC_R_out_5'</td>
<td>GAA CAG GCG TTT TTT GAT GAT GC</td>
</tr>
<tr>
<td>NMB1718_FWD</td>
<td>GCC CAC ATC GTT ATT CTC ATA AAG GC</td>
</tr>
<tr>
<td>mtrR_Pme</td>
<td>GGG TTT AAA CT TTT TTC GTC GCA GGT CGG</td>
</tr>
<tr>
<td>mtrR_F_Pac_GC3</td>
<td>GCC ATT AAT TAA CCT ATC GTG TGG TCA TGT AAA GG</td>
</tr>
<tr>
<td>mtrR_F_Pac_GC4</td>
<td>GGG TAA TTA ACC GCC CTC ATC AAA CCG ACC</td>
</tr>
<tr>
<td>farR_Sma_F</td>
<td>CTG ATA CAG GCC CCG GAA GCC CCG ATG</td>
</tr>
<tr>
<td>farR_Sma_R</td>
<td>CAT CAG GTC TTC CCG GGC GTG TAG CAG</td>
</tr>
<tr>
<td>farR_P_F_Pac_GC3</td>
<td>GGT TAA TTA AGA TGC GGC GGC GTT TTC TTT TCT GG</td>
</tr>
<tr>
<td>farR_P_Pme</td>
<td>GGG TTT AAA CT TTT AGC TGC TCA AG CAT</td>
</tr>
<tr>
<td>nada_F_Bam</td>
<td>ATA TGG ATC CTT CCT CTA TCA AAA CCG</td>
</tr>
<tr>
<td>nadaA_lacZ_R</td>
<td>ATA TGG ATC GTG TTT CAT GCT CAT TAC C</td>
</tr>
<tr>
<td>mtrR_F_Bam</td>
<td>CGG GAT CCC GAG CCA TTA TIT ATC CTA TCT GTC</td>
</tr>
<tr>
<td>mtrR_lacZ_R</td>
<td>GGT TGG ATC CAT AAT GGC GTT TTC TGG G</td>
</tr>
<tr>
<td>farR_F_Bam</td>
<td>ATA TGG ATC CCG CGG CTT TGT TTT TTT CTT G</td>
</tr>
<tr>
<td>farR_lacZ_R</td>
<td>CGG AGG ATC CTA GTG GTG AGG CAT TGT C</td>
</tr>
<tr>
<td>farR_pmalC_F</td>
<td>ATG CCT ACC AAA CAA TCA AAA CCG</td>
</tr>
<tr>
<td>farR_pmalC_Xba_R</td>
<td>TTA CTC TAG ATT AGC AGT TCA AG CAT C</td>
</tr>
<tr>
<td>mtrR_pmalC_F</td>
<td>ATG AGA AAA ACC AAA ACC GAA GCC</td>
</tr>
<tr>
<td>mtrR_pmalC_Xba_R</td>
<td>CAA GTC TAG ATT ATT TCC GCC GCA GGT C</td>
</tr>
<tr>
<td>mtrR_pmalC_Xba_R</td>
<td>CGG GAC GGG CAG ACA GTC GC</td>
</tr>
<tr>
<td>mtrR_pmalC_Xba_R</td>
<td>GGA CAG GCC GTA AGC CGG GTT C</td>
</tr>
<tr>
<td>farAB_prom_F</td>
<td>ATG TGG GAG GTT TTC GAA CCA CCG</td>
</tr>
<tr>
<td>farAB_prom_R</td>
<td>CGT GTG CGT ATC CAT AAG ATT GGG</td>
</tr>
<tr>
<td>farR_prom_F</td>
<td>CCG TTA TGT AGA GAA TCA ACG GG</td>
</tr>
<tr>
<td>farR_prom_R</td>
<td>TGG GTG AGG CAT TGT TTA AGT CTC C</td>
</tr>
<tr>
<td>nada_prom_F</td>
<td>GTC GAC GTC CTC GAT TAC GAA GG</td>
</tr>
<tr>
<td>nada_prom_R</td>
<td>ATG CAT GCT CAT TAC CTT TGT GAG TGG</td>
</tr>
<tr>
<td>K9H_3</td>
<td>AGA CGA CAG TGC CCA TGC AAG G</td>
</tr>
<tr>
<td>mtrR_out_5'</td>
<td>TTG CGG TAA AAG GTT TTC CCA AAC AG</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0056097.t002

PLOS ONE | www.plosone.org | February 2013 | Volume 8 | Issue 2 | e56097
were resuspended in 50/50 PBS and Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgCl₂, 50 mM β-Mercaptoethanol; pH 7.0). After addition of 20 μL 0.1% SDS and 40 μL chloroform, samples were vortexed and incubated at room temperature for 5 min. Protein concentrations were quantified by Nanodrop1000 (Nanodrop Technologies, Wilmington, DE). To 200 μL of Z-buffer, 30 μL of each cell lysate and 70 μL of ONPG (2-Nitrophenyl-β-D-galactopyranoside; Sigma, St. Louis, MO) at a concentration of 4 mg mL⁻¹ in Z-buffer was added. Following a color reaction, the change was stopped with 500 μL of 1 M Na₂CO₃ solution. The reactions were centrifuged at 3000 g for 5 min at 13,000 rpm to remove cell debris and 200 μL of supernatant was transferred to a 96-well microtiter plate and analyzed at 420 nM by a PerkinElmer Victor X3 microplate reader. For data analysis, specific activity was calculated using the formula: {[(OD₄₂₀ * 2)/4500 nL mmol⁻¹ cm⁻¹ x mL cm⁻²]/mg protein} with v being the volume used and t being the reaction time. All reactions were performed in triplicate and repeated at least 3 times. Statistical analysis was performed using multivariate ANOVA followed by Tukey HSD post-hoc pairwise comparison using SAS 9.2 software (The SAS Institute, Cary, NC).

Verification of protein absence, overexpression, and comparison between strains were assessed by immunoblot. Total protein was quantified by Nanodrop1000 (Nanodrop Technologies) prior to SDS-PAGE separation [38] and Western immunoblotting [39]. Rabbit alkaline phosphatase-conjugated secondary antibody (Bio-NadA, 1:2,000; anti-FarR, 1:5,000; and anti-MtrR, 1:1,000. Anti-PAGE) separation [38] and Western immunoblotting [39]. Rabbit sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed similarly, but unlabeled probes generated from the DNA-binding studies

DNA-binding studies

Electrophoretic mobility shift assays (EMSA) and competitive EMSAs using FarR-MBP and MtrR-MBP were performed essentially as described previously [22,27,40] with some modifications. Briefly, unlabeled and 32P-labeled probes were generated with the following primers: nadA, nadA_prom_F and nadA_prom_R; mtrCDE, KH9_3 and mtrR_R_out_5; farR, farR_prom_F and farR_prom_R; farAB, farAB_prom_F and farAB_prom_R; and mtrF, mtrF and mtrF. Competitive PCR products were end labeled with [γ-32P] dATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The labeled products (10 ng) were each incubated with purified FarR-MBP or MtrR-MBP in a 30 μL reaction volume [10 mM Tris-HCl (pH 7.5), 0.5 mM dithiothreitol, 0.5 mM EDTA, 1 mM MgCl₂, 50 mM NaCl, 0.05 μg/mL poly(dI-dC)] at room temperature for 30 min. Loading buffer (Epicentre, Madison, WI) was added to each sample then separated by 6% polyacrylamide gel at 4°C, followed by autoradiography. Competitive EMSAs were performed similarly, but unlabeled probes generated from the same primer sets as labeled probes were included.

DNase I protection assays were performed as described previously [22,27,40] with slight modifications. Target DNA probes were generated with PCR primers nadA_prom_F and nadA_prom_R (Table 2). The 5′ end was labeled with T4 polynucleotide kinase as described above for EMSA probes. Purified MtrR-MBP was incubated with target DNA for 15 min at 37°C. DNase I in loading buffer [10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 4 mM CaCl₂, 1 mM dithiothreitol, 40% glycerol] was added to each reaction for 1 min at 37°C. The reactions were stopped with DNase I stop buffer (95% ethanol and 7.5 mM ammonium acetate), plunged in an ethanol dry-ice bath for 15 min, and precipitated overnight at −80°C. Pellets were washed in 70% (vol/vol) ethanol, dried, and resuspended in loading buffer (Epicentre). Resuspended reaction mixtures were loaded on 6% denaturing polyacrylamide gel and resolved by autoradiography.

Acknowledgments

We thank Virginia Stringer for excellent technical assistance, Lane Pucko for help with manuscript preparation, and Oliver Kurzai for the gift of antisera to FarR and NadA.

Author Contributions

Conceived and designed the experiments: JMC WMS. Performed the experiments: JMC. Analyzed the data: JMC WMS. Contributed reagents/materials/analysis tools: JMC WMS. Wrote the paper: JMC WMS.

References


