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Genetic Analysis of the *Rhizobium meliloti nifH* Promoter, Using the P22 Challenge Phage System

SHOVON I. ASHRAF,† MARY T. KELLY, YING-KAI WANG, AND TIMOTHY R. HOOVER*

Department of Microbiology, University of Georgia, Athens, Georgia 30602

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In several genera of bacteria, the σ^{54}-RNA polymerase holoenzyme ($E\sigma^{54}$) is a minor form of RNA polymerase that is responsible for transcribing genes whose products are involved in diverse metabolic processes. $E\sigma^{54}$ binds to the promoters of these genes to form a closed promoter complex. An activator protein is required for the transition of this closed promoter complex to an open complex that is transcriptionally competent. In this study, the P22-based challenge phage system was used to investigate interactions between $E\sigma^{54}$ and the *Rhizobium meliloti nifH* promoter. Challenge phages were constructed in which the *R. meliloti nifH* promoter replaced the binding site for the Mnt protein, a repressor of the phage P22 *ant* gene. When a *Salmonella typhimurium* strain that overexpressed σ^{54} was infected with these challenge phages, $E\sigma^{54}$ bound to the *nifH* promoter and repressed transcription of the *ant* gene as seen by the increased frequency of lysogeny. Following mutagenesis of challenge phages that carried the *R. meliloti nifH* promoter, mutant phages that could form plaques on an *S. typhimurium* strain that overexpressed σ^{54} were isolated. These phages had mutations within the *nifH* promoter that decreased the affinity of the promoter for $E\sigma^{54}$. The mutations were clustered in seven highly conserved residues within the −12 and −24 regions of the *nifH* promoter.

Bacterial RNA polymerase holoenzyme has the subunit composition $\alpha_2\beta\beta'\sigma$ and is responsible for the recognition of specific promoter sequences and the subsequent initiation of transcription. Promoter recognition by RNA polymerase is conferred by the σ subunit, which dissociates from core RNA polymerase once transcript elongation begins. Bacteria typically have several sigma factors which participate in gene regulation in response to a variety of environmental, spatial, and temporal stimuli (10, 15).

The σ factor, σ^{54} (also referred to as σ^N), has been found in several genera of bacteria within the genus *Proteobacteria* (19, 24). The σ^{54}-RNA polymerase holoenzyme ($E\sigma^{54}$) was first shown to be required for the expression of genes involved in nitrogen assimilation in enteric bacteria but has since been shown to be required for the expression of several genes whose products are involved in diverse metabolic functions (19, 24).

$E\sigma^{54}$ binds to the promoter to form a closed complex but cannot undergo isomerization to an open complex in the absence of an activator protein or ATP hydrolysis (3, 20, 21, 27, 31, 33). The activator binds to enhancer-like elements located 100 to 200 bp upstream of the transcriptional start site and contacts $E\sigma^{54}$ through DNA looping (32, 35). To catalyze the isomerization of the closed complex to a transcriptionally competent open complex, the activator must hydrolyze ATP (3, 20, 21, 31, 39). This mechanism of transcription initiation with $E\sigma^{54}$ differs from that of other forms of the RNA polymerase holoenzyme and shares some similarities with transcription initiation by the eukaryotic RNA polymerase II (10, 28, 30).

Sequence analysis of σ^{54}-dependent promoters has led to the formulation of the consensus sequence 5′-TGGCACN_4 TTTGCA/T-3′, where the underlined GG and GC doublets are located at approximately −24 and −12 relative to the transcriptional start site (27). Purified σ^{54} can bind to certain promoters in the absence of core RNA polymerase, indicating that the DNA binding determinants of σ^{54} can function in the isolated protein (6). This is in contrast to the major σ factor of enteric bacteria, σ^{70}, in which the DNA binding determinants are masked until the protein binds to core RNA polymerase (12). Mutational analysis of σ^{54} has shown that determinants of specific DNA binding reside in the C terminus of the protein (23, 34, 40). Moreover, DNA binding assays with purified peptides containing various domains of σ^{54} indicated that determinants for specific DNA binding are carried on a C-terminal 16-kDa peptide (9).

To further understand how $E\sigma^{54}$ interacts with the promoter, we mutagenized the *Rhizobium meliloti nifH* promoter and identified mutant promoters that had reduced affinities for $E\sigma^{54}$. We employed a P22-based challenge phage system (4, 22) to analyze binding of $E\sigma^{54}$ to the *R. meliloti nifH* promoter. The challenge phage system takes advantage of the *immI* region of phage P22, which can determine the lysis-lysogeny decision of the phage under certain conditions. Within the *immI* region is the *ant* gene, which encodes an antirepressor protein that alleviates repression of genes required for lytic growth of the phage by binding to the c2 repressor. Two other genes in the *immI* region, *arc* and *mnt*, encode proteins that repress transcription of the *ant* gene. In the P22 challenge phage, *mnt* and *arc* have been inactivated, and the operator to which the Mnt protein binds (O_{mnt}) is replaced with a binding site of another DNA-binding protein. This second protein can then act as a genetic repressor of the *ant* gene, and interactions between the protein and its cognate DNA binding site can be monitored by determining whether phage lysis or lysogeny occurs (4, 22).

We replaced O_{mnt} with the *R. meliloti nifH* promoter, which allowed $E\sigma^{54}$ to act as a repressor of *ant* in the challenge phage system. Following mutagenesis of this phage, we selected for mutations in the *R. meliloti nifH* promoter that decreased the affinity of the promoter for $E\sigma^{54}$ by isolating phages which could grow lytically in a *Salmonella typhimurium* strain that

* Corresponding author. Mailing address: Department of Microbiology, 527 Biological Sciences Bldg., University of Georgia, Athens, GA 30602. Phone: (706) 542-2675. Fax: (706) 542-2674. E-mail: hoover@bscr.uga.edu.

† Present address: Department of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, Worcester, MA 01605.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source or reference
Strains		
S. typhimurium		
MS1389	Δ(*prt*) *proAB-ataP*::[P22 *int3* (*c2-ts29*)*sieA44*]	J. Gardner (41)
MS1582	*leu414*(Am) Fels$^-$ *hsdSB*(r$^-$ m$^+$) *supE40 ataA*::[P22 *sieA44 16*(Am)H1455 *tpfr49*]	S. Maloy (14)
MS1868	*leuA414*(Am) Fels$^-$ *hsdSB*(r$^-$ m$^+$)	S. Maloy (14)
MS1883	*leu414*(Am) Fels$^-$ *hsdSB* (r$^-$ m$^+$) *supE40*	S. Maloy (14)
TSM216	*leu414*(Am) Fels$^-$ *supE40 mutS121* Tn*10*	S. Maloy
TH564	*leuA414*(Am) Fels$^-$ *supE40 ataA*::[P22 *sieA44 Ap7 tpfr184* Δ(*mnt-a1*) (Amp^s)]	S. Maloy (18)
SK284	*hisF645 ntrA209*::Tn*10*	S. Kustu
TRH100	*leu414*(Am) Fels$^-$ *hsdSB*(r$^-$ m$^+$) *ntrA209*::Tn*10*	This study
TRH102	Δ(*prt*) *proAB-ataP*::[P22 *int3* (*c2-ts29*)*sieA44 mnt*::Kan9 P_{nifH} *arc*(Am)H1605 *ant′-′lacZYA* Δ*9 a1*]	This study
TRH107	TRH102 *ntrA209*::Tn*10*	This study
E. coli PY13579	[*araD139* del (*ara-lac*)*U169* del *lac174 galU galK hsdR2 mcrB rpsL*/pJS28 (P22 *9*$^+$, Amp^r)]	J. Gardner (22)
Plasmids		
pPY190	pBR322 *mnt* P_{ant} *arc′*	S. Maloy (22)
pCyt-3	pBR322 *lacI*^{q} P_{lac}	E. Altman
pMS580	pBR322 P_{lac} *arc*(Am)H1605 *ant′-′lacZYA* Δ*9 a1*	J. Gardner (13)
pJES82	pBR322 *ntrA orf95* (*S. typhimurium*)	S. Kustu
pSA2f	pPY190 P_{nifH} (orientation I)	This study
pSA2r	pPY190 P_{nifH} (orientation II)	This study
pSA4	pCyt-3 P_{lac}-*ntrA* (*S. typhimurium*)	This study

overexpressed σ^{54}. The DNA sequences of 12 mutant phages were analyzed, and all of the mutant phages carried single base substitutions at one of seven highly conserved residues within the *R. meliloti nifH* promoter. For selected mutant *nifH* promoters, we confirmed that the mutations reduced the affinity of the promoter for Eσ^{54} in a gel mobility shift assay. To further investigate interactions between Eσ^{54} and the *nifH* promoter mutants in vivo, we examined expression from an *ant′-′lacZ* fusion system. In this system, Eσ^{54} functions as a repressor of the *ant′-′lacZ* fusion. We confirmed that mutations in the *nifH* promoter that reduced the affinity of the promoter for Eσ^{54} interfered with the ability of Eσ^{54} to repress expression from the *ant′-′lacZ* fusion.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are indicated in Table 1. All *S. typhimurium* strains were derivatives of LT2.

Media, chemicals, and enzymes. For routine growth of bacterial cultures, Luria broth (LB) was used unless indicated otherwise. Ampicillin, tetracycline, and kanamycin (Sigma Chemical Co.) were added to final concentrations of 100, 50, and 50 μg/ml, respectively. Isopropyl-β-thiogalactoside (IPTG), purchased from Gold Biotechnology, Inc., was added as indicated. T4 DNA ligase, restriction endonucleases, and *Taq* DNA polymerase were purchased from either New England Biolabs or Promega. *Escherichia coli* core RNA polymerase and *S. typhimurium* σ^{54} were purified as described previously (21). For preparation of phage lysates, cells were grown in LBEDO broth, which was prepared by adding 2 ml of 50× E salts ([per liter] 10 g of magnesium sulfate heptahydrate, 100 g of citric acid monohydrate, 500 g of dipotassium hydrogen phosphate, and 175 g of sodium dihydrogen phosphate dihydrate) and 1 ml of 1 M glucose to 100 ml of LB. Top agar used for the plaque selection contained, per liter, 10 g of tryptone, 5 g of sodium chloride, and 7 g of agar. Selection of lysogens bearing *ant′-′lac* fusions was done as described previously (16), by growing cultures in NCE-lactose medium, which contained, per liter, 2 g of lactose, 0.25 g of magnesium sulfate heptahydrate, 3.94 g of potassium phosphate monobasic, 5 g of dipotassium hydrogen phosphate, and 3.5 g of sodium ammonium phosphate tetrahydrate.

Plasmid constructions. Plasmid pPY190 carries an ~500-bp DNA fragment from the *immI* region of phage P22 cloned into the *Eco*RI-*Hin*dIII sites of pBR322 (22). A DNA fragment bearing the *R. meliloti nifH* promoter was inserted into the *Sma*I site of pPY190, which placed the *nifH* promoter at −3 relative to the transcriptional start site of the *ant* promoter (P_{ant}). This was done by allowing the complementary oligonucleotides 5′-ACGGCTGGCACGACTT TTGCACGt-3′ and 5′-aCGTGCAAAAGTCGTGCCAGCCGT-3′ to anneal and then cloning this DNA fragment, which corresponds to the *R. meliloti nifH* promoter sequence from −33 to −9, into the *Sma*I site of pPY190. The lowercase letters indicate a change in the promoter sequence from A to T on the noncoding strand at position −9. This change was introduced to create a unique *Eco*72I restriction site upon cloning of the oligonucleotide into the *Sma*I site of pPY190. The orientation of the insert was determined by digesting the resulting plasmids with *Eco*72I and *Hin*dIII. Both orientations were chosen for construction of challenge phages. These plasmids were designated pSA2f, in which the *R. meliloti nifH* promoter and P_{ant} were in the same direction (orientation I), and pSA2r, in which the promoters were in the opposite directions (orientation II).

Plasmid pSA4, which carries the *S. typhimurium ntrA* gene under control of the *lac* promoter/operator and *lacI*^{q}, was constructed as follows. The *ntrA* gene was amplified from plasmid pJES82 by PCR, with the primers 5′-CATGAGGATC CTATGAAGCAAGGTTTG-3′ and 5′-CAAATTCACGCAAAGCTTCAGTA A-3′. These primers created unique *Bam*HI and *Hin*dIII sites immediately upstream of the initiation codon of *ntrA* and in *orf95*, respectively. Amplified DNA was digested with *Bam*HI and *Hin*dIII, and the resulting DNA fragment was cloned into the *Bgl*II and *Hin*dIII sites of pCyt-3 to create plasmid pSA4.

Construction of P22 challenge phages carrying the *R. meliloti nifH* promoter. Plasmids pSA2f and pSA2r were transformed by electroporation into *S. typhimurium* MS1883, and phage lysates of P22 *mnt*::Kan9 *arc*(Am) (22) (provided by S. Maloy) were prepared from these strains. These phage lysates were mixed with *S. typhimurium* MS1582 along with top agar and poured onto LB agar plates. The Mnt protein expressed from the prophage in MS1582 prevented replication of phages that retained O_{mnt}, whereas phages that had acquired the *R. meliloti nifH* promoter following recombination between the phage DNA and plasmid formed plaques. Recombinant phages with the *arc*$^+$ allele formed cloudy plaques, while recombinant phages with the *arc*(Am) allele formed large, clear plaques. Phages from several large, clear plaques were purified twice on a lawn of strain MS1582 and then once on a lawn of strain MS1883. High-titer phage lysates were prepared following growth of the purified recombinant phage on strain MS1883. Correct constructs were confirmed by DNA sequencing and in the challenge phage assay.

Challenge phage assay. Challenge phage assays were carried out essentially as described previously (22). High-titer phage lysates of challenge phages carrying the *R. meliloti nifH* promoter (hereafter referred to as *R. meliloti nifH* promoter challenge phages) were used to infect strain MS1868 bearing plasmid pSA4. Cells were grown overnight in LB medium containing ampicillin, subcultured in fresh medium the next day, and grown to mid-log phase. Cells were diluted into LB medium containing various concentrations of IPTG and grown for 2 h at 37°C to induce the expression of σ^{54}. Phage lysates were mixed with the cells to give a multiplicity of infection of ~25. After 1 h of incubation at room temperature, infected cells were diluted serially in LB medium, and samples from dilutions were spotted onto LB agar containing the corresponding concentration of IPTG, ampicillin, and kanamycin to select for lysogens that carried the *mnt*::Kan9 fusion. Plates were incubated overnight at 37°C, and lysogens were counted the next day. Viable cell counts were determined by using uninfected cells from each culture. The percent lysogeny was calculated by dividing the number of lysogens by the viable cell count and multiplying the quotient by 100%.

Isolation of promoter mutations with reduced affinity for Eσ^{54}. *R. meliloti nifH* promoter challenge phages were subjected to in vitro mutagenesis with hydroxylamine essentially as described previously (11). Phages were exposed to hydroxylamine until phage titers had decreased 2 to 3 orders of magnitude. Alternatively, spontaneous mutations arising within the *R. meliloti nifH* promoter challenge phages were isolated following growth of these phages on strain TSM216, which has a mutation in the *mutS* gene.

To isolate phages with mutations in the *R. meliloti nifH* promoter with reduced affinity for Eσ^{54}, strain TH564 carrying either plasmid pJES82 or pSA4 was infected with the mutagenized phage at a multiplicity of infection of ~25, mixed with 3 ml of top agar, and then overlayed on LB agar containing ampicillin. When pSA4 was used, the expression of σ^{54} was induced with 5 μM IPTG prior to infection with the mutagenized phage and the same concentration of IPTG was included in the LB agar. Plates were incubated at 37°C for several hours, and phages from clear plaques were purified by streaking them on a lawn of strain TH564 that carried either pJES82 or pSA4. Phages were then purified on a lawn of strain MS1883. High-titer phage lysates were prepared for several of the

mutants, and phage DNA was purified for DNA sequencing from these lysates with DEAE-cellulose as described previously (22).

DNA sequencing. Specific base changes within mutant promoters were identified by directly sequencing purified phage DNA in cycle sequencing reactions with the TAQuence Cycle Sequencing kit (U.S. Biochemicals) according to the supplier's recommendations. The primer for sequencing, 5′-GATCATCTCTA GCCATGC-3′, corresponded to the region from −75 to −58 relative to the transcriptional start site of the *ant* promoter and was end labelled with polynucleotide kinase and [γ-^{32}P]ATP (Amersham). Samples from each cycle sequencing reaction mixture were analyzed on denaturing urea gels, and X-ray film was exposed to the gels overnight.

Construction of *ant′-′lacZ* phages. Phages containing *ant′-′lacZ* fusions were constructed following recombination between *R. meliloti nifH* promoter challenge phages bearing either wild-type or mutant *nifH* promoters and plasmid pMS580 as described previously (13). Plasmid pMS580 carries an *ant′-′lacZ* gene fusion and a deletion of gene *9*, which encodes the P22 tail protein. Recombination produced tail-dependent phages that carried the *ant′-′lacZ* fusion and the *R. meliloti nifH* promoter in place of O_{mnt}. The recombinant phages yielded blue, fuzzy (tail-dependent) plaques when plated on a lawn of strain MS1883 along with the P22 tail protein on LB agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The P22 tail protein was purified from *E. coli* PY13579, which overexpressed the P22 tail protein, as described previously (22). Recombinant *ant′-′lacZ* phages were purified twice by streaking on a lawn of MS1883 in the presence of the P22 tail protein. Lysates were then prepared by growing the recombinant phages on strain MS1868 in the presence of the P22 tail protein. Lysogens were generated by transducing strain MS1389 with these phage lysates along with the P22 tail protein. MS1389 is a *pro* mutant prophage deletion lysogen in which the deletion removes all of the P22 prophage to the left of gene *12* and also removes the adjacent host genes *proAB* and *prt* (38, 41). Lysogens were selected on NCE-lactose medium supplemented with proline and then scored on MacConkey agar (Difco) containing lactose and kanamycin. Isogenic strains of these lysogens that were deficient in σ^{54} were constructed by P22-mediated transduction of the *ntrA209*::Tn*10* allele from strain SK284.

β-Galactosidase assays. Whole-cell β-galactosidase activities were determined for lysogens carrying the *ant′-′lacZ* fusions. Determination of β-galactosidase activities for lysogens in which σ^{54} was overexpressed from plasmid pSA4 was done as follows. Lysogens were grown overnight in LB medium containing ampicillin. Cells were subcultured the next day in fresh medium and grown to an optical density at 600 nm of ~0.5. Cells were subcultured a second time in fresh medium that contained 100 μM IPTG and were grown to mid-log phase. β-Galactosidase activities were then determined for these cells as described previously (26). Each assay was performed in triplicate in least two independent experiments. Mean values for β-galactosidase activity were determined for each sample, and 95% confidence intervals for these mean values were estimated by Student's *t* test.

Gel mobility shift assays. A 154-bp DNA fragment containing either the wild type or one of the mutant forms of the *R. meliloti nifH* promoter was amplified by PCR from purified phage DNA, with oligonucleotides 5′-GATCATCTCTA GCCATGC-3′ and 5′-CGGCATTTTGCTCATTCC-3′ as primers. The former primer was end labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP prior to PCR.

DNA binding assays were performed with purified σ^{54} and core RNA polymerase by using a modification of a previously described method (36). The buffer used for these binding assays was 40 mM *N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid (EPPS) (pH 8.0) containing 10 mM magnesium acetate, 100 mM potassium acetate, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mg of bovine serum albumin per ml, and 5% (wt/vol) glycerol. Proteins were incubated with the labelled DNA probe (<0.1 pmol) in 10 μl of buffer, along with sonicated calf thymus DNA (1 μg), which was included as competitor DNA. Binding reaction mixtures were incubated at 30°C for 10 min and then loaded onto a 5% polyacrylamide gel. Protein-DNA complexes were separated from free DNA by electrophoresis for 1 to 2 h at 250 V in a Hoeffer SE600 vertical slab gel unit. The unit was cooled with a circulating water bath set at 20°C. Following electrophoresis, X-ray film was exposed to the gel overnight.

RESULTS

Binding of Eσ^{54} to the *R. meliloti nifH* promoter in the challenge phage system. The P22-based challenge phage system provides a powerful system for studying protein-DNA interactions in vivo. To study the protein-DNA interaction between Eσ^{54} and σ^{54}-dependent promoters, challenge phages that carried the *R. meliloti nifH* promoter in either orientation were constructed (Fig. 1). This promoter was chosen because it closely resembles the consensus sequence for σ^{54}-dependent promoters and is a strong binding site for Eσ^{54} (6, 27).

We wished to overlap the *R. meliloti nifH* promoter with the *ant* promoter (P_{ant}) as much as possible to maximize repression of P_{ant} by Eσ^{54}. The DNA sequence that was introduced into the challenge phage was located at −3 relative to the transcriptional start site of P_{ant} and corresponded to the −33 to −9 region of the *R. meliloti nifH* promoter. Footprinting analysis of closed complexes between Eσ^{54} and the *R. meliloti nifH* promoter indicated that close contacts between the promoter and Eσ^{54} extend from −31 to −5 (7). Sequence-specific contacts, however, are restricted to the −27 to −12 regions of the promoter, and contacts downstream of −12 are thought to be primarily sequence independent (7). Therefore, we reasoned that omitting the region downstream of the −9 region was not likely to significantly reduce the affinity of the *R. meliloti nifH* promoter for Eσ^{54}.

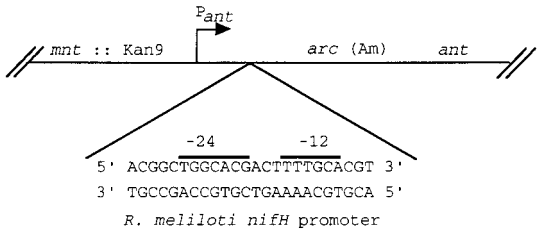

FIG. 1. Organization of *immI* region in *R. meliloti nifH* promoter challenge phages. The indicated DNA sequence bears the *R. meliloti nifH* promoter and replaced the sequence encoding O_{mnt} in the *R. meliloti nifH* promoter challenge phage. The consensus promoter elements (overscored) are 5′-TGGCAC-3′ from −27 to −22 and 5′-TTTGCA-3′ from −17 to −12. These promoter elements contain the GG and GC doubles that are characteristic of σ^{54}-dependent promoters (27). The inserted DNA sequence was located −3 relative to the transcriptional start site of P_{ant}. In the construction shown here, P_{ant} and the *R. meliloti nifH* promoter were in the same direction (orientation I). A challenge phage was also constructed in which the *R. meliloti nifH* promoter and P_{ant} were in opposite directions (orientation II). The challenge phages also carried a kanamycin cassette inserted in *mnt* and an amber mutation in *arc*.

To evaluate the effect of σ^{54} concentrations on the frequency of lysogeny for the *R. meliloti nifH* promoter challenge phages, the *S. typhimurium ntrA* gene was placed under the control of the *lac* promoter and the *lacI*^{q} gene in plasmid pSA4, which allowed us to modulate the levels σ^{54} inside the cell by the addition of IPTG to the medium. Western blot analysis showed that the level of σ^{54} expression from pSA4 was dependent on the IPTG concentration and was maximal at ~50 μM IPTG (Fig. 2A). When cells carrying pSA4 were exposed to different concentrations of IPTG and then infected with the *R. meliloti nifH* promoter challenge phages, the frequency of lysogeny was dependent on IPTG concentration (Fig. 2B). For cells that had not been exposed to IPTG, the frequency of lysogeny was background (<0.00001%). For cells that had been exposed to IPTG, the frequency of lysogeny increased with increasing IPTG concentrations, reaching a maximum of ~75% lysogeny at 100 μM IPTG. When cells that overexpressed σ^{54} were infected with phages that lacked the *R. meliloti nifH* promoter, the frequency of lysogeny was equal to background levels, indicating that the *nifH* promoter was required for Eσ^{54} to channel the *R. meliloti nifH* promoter challenge phages towards lysogeny. We infer from these data that when σ^{54} was overexpressed, Eσ^{54} repressed transcription from P_{ant} by binding to the *R. meliloti nifH* promoter in the challenge phage, thereby channelling the phage towards lysogeny.

Isolation of mutations in the *R. meliloti nifH* promoter. *S. typhimurium* TH564 carries a prophage that expresses the c2 repressor. When this strain carried plasmid pJES82, which gives a constitutively high level of σ^{54} expression, it was resistant to superinfection by the *R. meliloti nifH* promoter challenge phages due to repression of *ant* by Eσ^{54}. Likewise, when TH564 carried plasmid pSA4 and expression of σ^{54} in this strain was induced with IPTG, it was resistant to superinfection

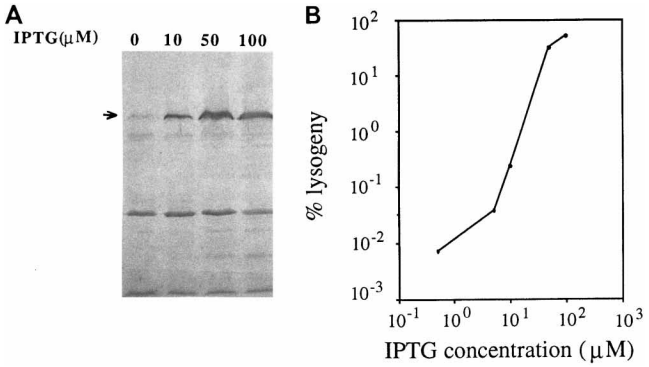

FIG. 2. Effect of σ^{54} concentration on frequency of lysogeny for *R. meliloti nifH* promoter challenge phage. (A) Overexpression of σ^{54} from plasmid pSA4 is indicated. *S. typhimurium* TRH100 bearing plasmid pSA4 was grown overnight in LB medium containing ampicillin. Cells were subcultured the next day in fresh LB medium containing ampicillin and grown to mid-log phase. Cells were subcultured a second time in fresh LB medium that contained ampicillin and various concentrations of IPTG as indicated above each lane. After a 2-h induction, whole-cell extracts were analyzed by Western blotting with rabbit polyclonal antiserum directed against *S. typhimurium* σ^{54} (provided by S. Kustu). The σ^{54} band is indicated by the arrow. Note that the intensity of this band increased with increasing IPTG concentrations while the level of a second protein that cross-reacted with the antiserum remained unchanged. (B) The frequency of lysogeny for the *R. meliloti nifH* promoter challenge phage as a function of the σ^{54} concentration is shown. Strain MS1868 bearing plasmid pSA4 was grown overnight in LB medium containing ampicillin. Cells were subcultured the next day in LB medium containing ampicillin and various concentrations of IPTG to induce the synthesis of σ^{54} to different levels. After the cells were infected with the *R. meliloti nifH* promoter challenge phage, stable lysogens were selected on LB agar containing ampicillin, kanamycin, and the corresponding concentration of IPTG. The percent lysogeny was calculated as described in Materials and Methods.

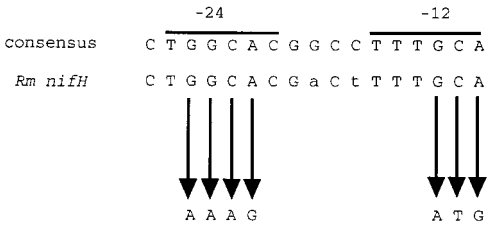

FIG. 3. Summary of mutations in *R. meliloti nifH* promoter that decrease the affinity of the promoter for $E\sigma^{54}$. The sequence of the *R. meliloti nifH* promoter is compared with a consensus sequence (27). Promoter elements within the *R. meliloti nifH* promoter (5′-TGGCAC-3′ and 5′-TTTGCA-3′) are overscored, and the mutations are indicated below the *R. meliloti nifH* promoter sequence.

by the *R. meliloti nifH* promoter challenge phages. Phages that form plaques under these conditions may be mutants that synthesize enough antirepressor to allow lytic growth. Many such mutant phages are likely to have mutations within the *R. meliloti nifH* promoter that decrease the affinity of the promoter for $E\sigma^{54}$.

Mutagenized phages were used to infect TH564 carrying either plasmid pJES82 or pSA4. Cells with plasmid pSA4 were grown in the presence of 5 μM IPTG prior to infection, which gave a low level of expression of σ^{54} (Fig. 2A). We reasoned that phages with mutations in the *nifH* promoter that only modestly reduced the affinity of the promoter for $E\sigma^{54}$ might be able to grow lytically under these conditions. Mutant phages were purified from clear plaques and analyzed by DNA sequencing.

From 12 independent mutant phages, seven different mutations in highly conserved residues within the *R. meliloti nifH* promoter were identified (Fig. 3). All 12 mutant phages had single base changes within the *R. meliloti nifH* promoter, with four of the mutations occurring more than once (Table 2).

The challenge phage system can be used to estimate the relative affinities of mutant binding sites for the repressor. For phages with mutations in the binding site that severely decrease the affinity of the site for the repressor, higher levels of protein are needed to repress transcription from P_{ant} and channel the phage towards lysogeny than for phages bearing mutations that less severely affect the affinity of the site for the repressor. When cells carrying pSA4 were exposed to 100 μM IPTG to fully induce expression of σ^{54} and then infected with the mutant *R. meliloti nifH* promoter challenge phages, background levels of lysogeny were observed for all of the mutants. This finding indicated that the challenge phage system could not be used to compare the relative affinities of the mutant *nifH* promoters for $E\sigma^{54}$. Therefore, the following approach was taken to estimate the relative affinities of the mutant *nifH* promoters for $E\sigma^{54}$ in vivo.

Repression of *ant′-′lacZ* fusion genes by $E\sigma^{54}$. Wild-type and mutant *R. meliloti nifH* promoter challenge phages were used to construct phages that carried *ant′-′lacZ* fusions. Lysogens were generated from these recombinant phages, and the ability of $E\sigma^{54}$ to repress transcription from P_{ant} in these lysogens was assessed by measuring β-galactosidase activities. Plasmid pSA4 was introduced into these lysogens, and cells were grown in either the absence or the presence of 100 μM IPTG prior to measuring β-galactosidase activities. For these experiments, we used lysogens that had the wild-type *ntrA* allele to avoid polar effects on genes downstream of the chromosomal copy of *ntrA*, as these genes have been suggested to influence the activity of σ^{54} at some σ^{54}-dependent promoters (25).

In the absence of IPTG, the levels of β-galactosidase activity in lysogens with the wild-type *nifH* promoter were three- to sevenfold lower than those for lysogens with mutant *nifH* promoters (Fig. 4). Following overexpression of σ^{54} by the addition of IPTG to the medium, expression from P_{ant} was repressed an additional three- to eightfold in lysogens with the wild-type *nifH* promoter. For some of the lysogens with mutant *nifH* promoters, overexpression of σ^{54} had little or no effect on repression of P_{ant} (G to A at −25, G to A at −14, and C to T at −13), while in the other lysogens, overexpression of σ^{54} repressed expression from P_{ant} an addition four- to fivefold (Fig. 4). In general, mutations in the conserved GG and GC

TABLE 2. *R. meliloti nifH* promoter challenge phages with mutations in the *nifH* promoter

Phage strain no.[a]	Mutation (position)	Type of mutagenesis
3.1-108	G to A (−25)	Hydroxylamine
3.1-110	G to A (−25)	Hydroxylamine
3.1-801	C to T (−13)	Spontaneous
3.1-802	C to A (−24)	Spontaneous
3.1-803	A to G (−23)	Spontaneous
3.1-805	A to G (−12)	Spontaneous
3.1-806	A to G (−23)	Spontaneous
3.7-108	G to A (−14)	Hydroxylamine
3.7-109	G to A (−26)	Hydroxylamine
3.7-112	C to T (−13)	Hydroxylamine
3.7-903	A to G (−12)	Spontaneous
3.7-905	A to G (−12)	Spontaneous

[a] The 3.1 and 3.7 designations refer to the orientation of the *R. meliloti nifH* promoter in the challenge phages. In phages with the 3.1 designation, the *nifH* promoter is in orientation II, while in phages with the 3.7 designation, the *nifH* promoter is in orientation I.

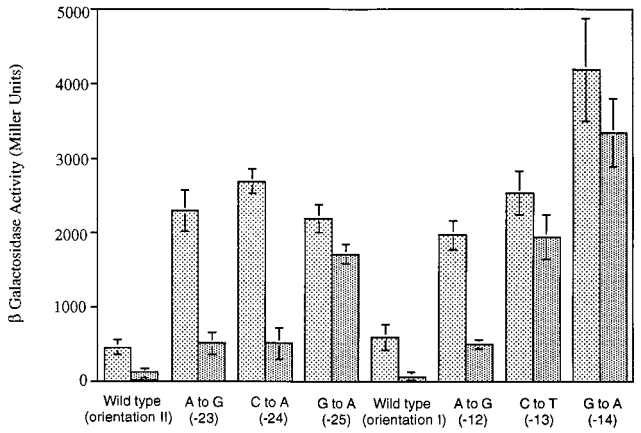

FIG. 4. Repression of *ant'-'lacZ* fusion gene by $E\sigma^{54}$ in lysogens bearing either the wild-type or mutant *R. meliloti nifH* promoters. Lysogens that carried the *ant'-'lacZ* fusion gene and either wild-type or mutant *R. meliloti nifH* promoters in place of O_{mnt} were transformed with plasmid pSA4. Transformants were grown overnight in LB medium that contained ampicillin and subcultured the next day in fresh LB medium that contained either ampicillin or ampicillin plus 100 μM IPTG. After 2 to 3 h, samples were taken from these cultures and assayed for β-galactosidase activity as described previously (26). The lightly stippled bars indicate the activities for cells that were not exposed to IPTG, while the more heavily stippled bars indicate the activities for cells in which σ^{54} expression was induced with IPTG. For each lysogen, β-galactosidase assays were done in triplicate in two or three separate experiments. The results are the mean values for these assays, and the error bars show the 95% confidence intervals for the means.

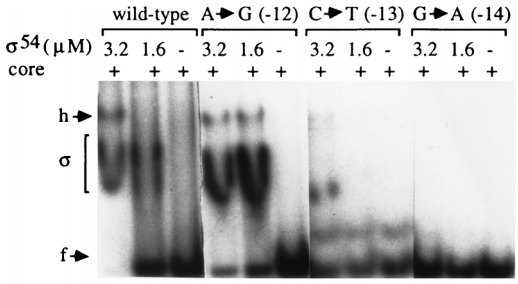

FIG. 5. Gel mobility shift assays with wild-type and mutant *R. meliloti nifH* promoters. DNA fragments bearing either wild-type or mutant *R. meliloti nifH* promoters were amplified from phage DNA by PCR as described in Materials and Methods. End-labelled DNA probes were incubated with purified *S. typhimurium* σ^{54} (0 to 3.2 μM) and *E. coli* core RNA polymerase (100 nM) as indicated. Following incubation of the DNA and proteins, DNA-protein complexes were resolved from free DNA (f) by electrophoresis and visualized by exposing X-ray film to the gels. Complexes between σ^{54} and the promoter (σ) gave a diffuse band, whereas complexes between $E\sigma^{54}$ and the promoter (h) gave a sharper band that migrated more slowly through the gel. This more slowly migrating species is referred to as a supershifted species (36). Incubating the DNA probes with core RNA polymerase alone did not give a mobility shift of the probes.

doublets decreased the affinity of the promoter for $E\sigma^{54}$ more severely than mutations in other conserved positions within the *nifH* promoter. (An *ant'-'lacZ* phage was not constructed for the challenge phage that carried the G to A transition at −26.)

We verified that the mutations in the *R. meliloti nifH* promoter did not directly affect expression from P_{ant} by measuring the β-galactosidase activity for each of the lysogens in the *ntrA209*::Tn*10* genetic background (Table 3). None of the mutations directly affected expression from P_{ant}. We did observe, however, that the orientation of the *nifH* promoter influenced expression from P_{ant}, as the levels of β-galactosidase activity for lysogens in which the *nifH* promoter and P_{ant} were in the same direction (orientation I) were slightly higher than the levels of activity for the lysogens in which the *nifH* promoter and P_{ant} were in opposite directions (orientation II).

Finally, we determined if the levels of σ^{54} expressed from the chromosomal copy of *ntrA* were sufficient to repress transcription from P_{ant}. This determination was made by measuring β-galactosidase activities in lysogens that had either the wild-type *ntrA* allele (strain TRH102) or the *ntrA209*::Tn*10* allele (strain TRH107). Strain TRH102 was constructed by transducing strain MS1389 with a recombinant phage that carried the *ant'-'lacZ* fusion and the *R. meliloti nifH* promoter in place of O_{mnt} (*nifH* promoter in orientation II). Strain TRH107, an isogenic strain of TRH102, was constructed by transducing the *ntrA209*::Tn*10* allele from strain SK284 to TRH102. The levels of β-galactosidase activity in the lysogen that lacked σ^{54} (2,680 ± 248 Miller units) were approximately fourfold higher than the levels observed in an isogenic $ntrA^+$ strain (564 ± 128 Miller units), indicating that the amount of σ^{54} expressed from the chromosomal copy of *ntrA* was high enough to modestly repress transcription from P_{ant}.

TABLE 3. Effect of mutations in *R. meliloti nifH* promoter on expression from P_{ant}

Orientation of *nifH* promoter	Mutation (position)	β-Galactosidase activity (Miller units)
II	None (wild type)	2,680 ± 248
	A to G (−23)	2,578 ± 198
	C to A (−24)	3,258 ± 304
	G to A (−25)	2,894 ± 677
I	None (wild type)	4,238 ± 147
	A to G (−12)	4,042 ± 595
	C to T (−13)	4,174 ± 179
	G to A (−14)	4,319 ± 556

Gel mobility shift assays with wild-type and mutant *R. meliloti nifH* promoters. To verify that the relative affinities of the mutant *nifH* promoters for $E\sigma^{54}$ were the same in vitro and in vivo, gel mobility shift assays were performed with purified σ^{54} and core RNA polymerase. DNA fragments bearing either wild-type or mutant *nifH* promoters were amplified from *R. meliloti nifH* promoter challenge phage DNA by PCR for these assays. The gel mobility shift assay was adapted from the procedure described by Tintut and coworkers (36), which allowed for the detection of complexes between σ^{54} and the *nifH* promoters, as well as complexes between $E\sigma^{54}$ and the promoters.

For these gel mobility shift assays, we compared the wild-type *nifH* promoter with mutant *nifH* promoters that had the A-to-G transition at −12, the C-to-T transition at −13, or the G-to-A transition at −14. These mutant promoters were selected because they were in the same orientation in the phage and displayed a wide range of affinities for $E\sigma^{54}$ in the in vivo studies (Fig. 4). These mutant *nifH* promoters displayed a similar range of affinities for $E\sigma^{54}$ in the gel mobility shift assays (Fig. 5). For the mutant promoter with the A-to-G transition at −12, complexes between either σ^{54} and the promoter or $E\sigma^{54}$ and the promoter were readily detected. In the in vivo assay, $E\sigma^{54}$ moderately repressed expression from P_{ant} in the lysogen bearing this mutant *nifH* promoter. For the mutant promoter with the C-to-T transition at −13, complexes between either σ^{54} and the promoter or $E\sigma^{54}$ and the promoter were detected only at the highest concentration of σ^{54}, and $E\sigma^{54}$ weakly repressed expression from P_{ant} in the lysogen

with this mutant *nifH* promoter in the in vivo assay. Finally, no protein-DNA complexes were observed in the gel mobility shift assay with the mutant *nifH* promoter that had the G-to-A transition at −14, and $E\sigma^{54}$ did not repress expression from P_{ant} in the lysogen with this mutant *nifH* promoter in the in vivo assay.

DISCUSSION

Because transcription by $E\sigma^{54}$ occurs by an unique mechanism that requires an activator protein and ATP hydrolysis for open complex formation, stable, closed complexes between $E\sigma^{54}$ and the promoter can be readily observed in the absence of these components (10, 19, 24). The transition from a closed complex between $E\sigma^{54}$ and the promoter to an open complex in the absence of either activator or ATP hydrolysis is negligible due to both a strong kinetic barrier and a thermodynamic barrier to open complex formation (37). This finding is in contrast to transcription with the σ^{70}-RNA polymerase holoenzyme, the major form of the RNA polymerase holoenzyme in enteric bacteria, which generally undergoes the transition to the open complex without the aid of auxiliary factors once it forms a closed complex with the promoter (10).

We have taken advantage of the ability of $E\sigma^{54}$ to form stable, closed complexes to study interactions between $E\sigma^{54}$ and the *R. meliloti nifH* promoter in vivo. Specifically, we have shown that $E\sigma^{54}$ can function as a genetic repressor of P_{ant} in the P22-based challenge phage system. Overexpression of σ^{54} allowed $E\sigma^{54}$ to repress transcription from P_{ant}, thereby channelling the phage toward lysogeny. The *R. meliloti nifH* promoter closely resembles the consensus sequence for σ^{54}-dependent promoters and is a strong binding site for $E\sigma^{54}$ (6, 27). We have also constructed a challenge phage in which the *K. pneumoniae nifH* promoter, a much weaker binding site for $E\sigma^{54}$, replaced O_{mnt}. As expected from its lower affinity for $E\sigma^{54}$, the level of lysogeny that we observed when cells that overexpressed σ^{54} were infected with the challenge phage which carried the *K. pneumoniae nifH* promoter was much less than the levels that we observed for the *R. meliloti nifH* promoter challenge phages (2).

We further demonstrated that in lysogens carrying an *ant'-'lacZ* fusion and the *R. meliloti nifH* promoter in place of O_{mnt}, expression from P_{ant} was repressed by $E\sigma^{54}$. Repression of this *ant'-'lacZ* fusion by $E\sigma^{54}$ was observed at physiologically relevant concentrations of σ^{54}, as the level of σ^{54} expressed from the chromosomal copy of *ntrA* was sufficient to repress transcription approximately fourfold. These data indicate that there is significant occupancy of strong σ^{54}-dependent promoters by $E\sigma^{54}$ in vivo at concentrations of σ^{54} that normally occur within the cell.

Given that $E\sigma^{54}$ can function as a repressor in this synthetic promoter region, $E\sigma^{54}$ may possibly function as a repressor at certain genes. A likely candidate is the *Caulobacter crescentus rpoN* (*ntrA*) gene, which appears to be negatively autoregulated (1). The DNA sequence 5′-CcGGCcCGATTaTTGCA-3′, which extends from −58 to −42 in the *C. crescentus rpoN* promoter (1), closely resembles the consensus sequence of σ^{54}-dependent promoters (consensus promoter elements are underlined and lowercase letters indicate bases that deviate from those in the consensus sequence) and appears to be properly positioned within the promoter to allow repression by $E\sigma^{54}$. From the results reported here, it seems likely that $E\sigma^{54}$ may bind to this sequence to repress transcription from the *rpoN* promoter in *C. crescentus.*

We used the P22-based challenge phage system to isolate mutant forms of the *R. meliloti nifH* promoter that had decreased affinities for $E\sigma^{54}$. While an exhaustive search for such mutant promoters was not made, we were able to identify seven different mutations that lowered the affinity of the *nifH* promoter for $E\sigma^{54}$. As expected, all of these mutations occurred at highly conserved positions within the *nifH* promoter. Based on dimethyl sulfate footprinting experiments (6, 8, 27, 29) and 5-bromouridine footprints (7) of closed complexes between $E\sigma^{54}$ and the *R. meliloti nifH* promoter, σ^{54} is believed to make specific contacts within the major groove at bases −27 to −22, −18, −16, and −14 to −12. All seven of the mutant *nifH* promoters that we isolated had base changes at 1 of these 11 positions, suggesting that these bases are probably important for sequence-specific contacts. The region around −12 is distorted on binding of $E\sigma^{54}$, but not σ^{54} alone, to the *R. meliloti nifH* promoter (6, 29). From the in vivo repression assays (Fig. 4) and the gel mobility shift assays with the mutant *nifH* promoters (Fig. 5), the G-to-A mutation at −12 appeared to have the least serious effect on the affinity of the promoter for $E\sigma^{54}$. Consistent with this observation, the distorted region around −12 does not appear to be tightly contacted by $E\sigma^{54}$ as determined from hydroxyl radical and dimethyl sulfate footprinting experiments (6–8, 27, 29).

Notably, we did not isolate any mutant promoters that had substitutions for any of the T residues within the −15 to −18 region. The presence of T residues within this region has been shown to be an important determinant in the affinity of the promoter for $E\sigma^{54}$ in other σ^{54}-dependent promoters (5, 17). Our failure to isolate mutations in the −15 to −18 region of the *R. meliloti nifH* promoter may be because multiple substitutions are required in this region to significantly reduce the affinity of the promoter for $E\sigma^{54}$.

The experiments presented here point out the utility of the P22-based challenge phage system for examining interactions between $E\sigma^{54}$ and σ^{54}-dependent promoters. In addition to providing a convenient method for isolating mutations that decrease the affinity of the σ^{54}-dependent promoters for $E\sigma^{54}$, the genetic tools that we have developed here should also be useful for identifying amino acid residues within σ^{54} that are involved in specific interactions with the promoter.

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