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NOTE

P22-Based Challenge Phage Constructs to Study DNA-Protein Interactions between the σ^{54}-Dependent Promoter, *dctA*, and Its Transcriptional Regulators

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A challenge phage system was used to study the DNA-protein interaction between C_4-dicarboylic acid transport protein D (DCTD) or σ^{54}, and a σ^{54}-dependent promoter, *dctA*p. *R. meliloti dctA* promoter regulatory region replaced the O_{mnt} site on the phage. *S. typhimurium* strains overproducing either DCTD or σ^{54} directed this challenge phage towards lysogeny, indicating that DCTD or Eσ^{54} recognized the *dctA* promoter on the phage and repressed transcription of the *ant* gene. These challenge phage constructs will be useful for examining interactions between DCTD (or σ^{54}) and the *dctA* promoter region.

Key words: P22-based challenge phage, σ^{54}, *dctA*, C_4-dicarboxylic acid transport protein D (DCTD)

Bacterial RNA polymerase holoenzyme consists of a core enzyme ($\alpha_2\beta\beta'$), which is responsible for transcript elongation, and one of the σ factors, which allows the holoenzyme to recognize promoter sequences. For example, σ^{54} is an alternative factor that is required for the expression of many genes whose products are involved in diverse metabolic functions including nitrogen fixation and carbon starvation (1, 4, 10). In contrast to the σ^{70}-RNA polymerase holoenzyme (Eσ^{70}), which is the major form of RNA polymerase in *Escherichia coli*, σ^{54}-RNA polymerase holoenzyme (Eσ^{54}) always requires an activator protein, which binds to sites about 100 base pairs upstream of the transcription initiation site to initiate transcription.

C_4-dicarboxylic acid transport protein D (DCTD) is a σ^{54}-dependent activator that activates transcription from *dctA*, which encodes a C_4-dicarboxylic acid transport protein in *Rhizobium*. Studies with the *E. coli glnA*p2 promoter and *Rhizobium meliloti nifH* promoter showed that Eσ^{54} forms a closed promoter complex with these promoters in the absence of an activator protein (6, 7, 9). In contrast, Eσ^{54} does not form a stable closed complex with the *Klebsiella pneumoniae nifLA* promoter in the absence of a nitrogen regulatory protein C (NtrC), which binds approximately 130 base pairs upstream of the transcriptional start site at the *nifL* promoter regulatory region (5). These data suggest that Eσ^{54} has a low affinity for the *nifL* promoter, and NtrC functions to stabilize the closed complex at this promoter in addition to its role in catalyzing open complex formation (2). The consensus sequence for σ^{54}-dependent promoters is TGGCACN$_5$TTGCA, with the spacing between the dinucleotides GG and GC being invariant (4). In the *glnA*p2 and *nifH* promoters, the sequence at the region adjacent to the GC doublet is TTT while in the *K. pneumoniae nifLA* promoter the sequence of the same region is GTT. Changing this sequence to TTT in the *K. pneumoniae nifLA* promoter increased the affinity of Eσ^{54} for the promoter (5). Since *R. meliloti dctA* contains TGT in this region, it is also possible for DCTD to stabilize the closed complex of Eσ^{54} and the *dctA* promoter.

P22, a temperate phage of *Salmonella typhimurium*, contains two regions, *immC* and *immI* that control the decision between lysis and lysogeny (11). The *immC* region encodes the c2 repressor, which establishes and maintains lysogeny by regulating transcription from P_L and P_R. The *immI* region contains three genes; *ant* (antirepressor), *arc* (antirepressor control), and *mnt* (maintenance of lysogeny) and modulates the activity of the c2

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Table 1. Plasmids used in this study.

Plasmid	Relative phenotype/genotype	Source
pJES82	pBR322 with *S. typhimurium* 2.7 kbp *Cla*I fragment bearing *ntrA* and *orf*95	8
pPY190	pBR322 *mnt* P*ant arc*$^+$ Amp^r Tet^r	3
pSA04	*S. typhimurium ntrA* in p*lacI*^{q}PO Amp^r Tet^r	this study
pJHLC1	P22*dctA*p-12,-24 in pPY190 Amp^r Tet^r(reverse)	this study
pJHLC2	P22*dctA*pUAS in pPY190 Amp^r Tet^r(forward)	this study
pJHLC3	pACYC184 with 1.7 kb *Eco*RI/*Sal*I fragment bearing *dctD*	this study

repressor. The Ant protein binds to the c2 repressor and prevents it from repressing transcription from P_L and P_R (11). Both the Arc and Mnt proteins repress transcription of *ant*. The *arc* gene is transcribed along with the *ant* gene from the P_{ant} promoter in the early stage of infection. Arc binds to an operator site (O_{arc}), which overlaps P_{ant}, preventing further transcription. The Mnt protein binds to a second operator that overlaps P_{ant} (O_{mnt}) to further prevent the expression of the *ant* gene (11). In the present work, we sought to construct an *in vivo* assay system to study the DNA-protein interactions exploiting the *immI* regulatory region of phage P22. The plasmids used in this study are listed in Table 1.

For cloning the *R. meliloti dctA* promoter regulatory region (*dctA*p) into pPY190, the following oligonucleotides were used: 5'-CATGGTGCATGTTTTCGC-3' (5' primer) and 5'-CAGCAACATGCGTGCCAG-3' (3' primer). The 5' and 3' primers correspond to the regions from -152 to -136 and from -26 to 10 of the *dctA*p, respectively. These primers were used to amplify the entire *dctA*p by polymerase chain reaction (PCR). The amplified DNA fragments (109 base pairs) were cloned into the *Sma*I site of pPY190 to create plasmids pJHLC1 (P_{dctA} overlaps P_{ant}) and pJHLC2 (The *dctA* UAS overlaps P_{ant}).

The P22 challenge phage, a derivative of P22 in which *mnt* is disrupted with a kanamycin cassette and *arc*, which bears an amber mutation, was utilized to analyze DNA-protein interactions (Fig. 1). Both orientations were chosen for recombination with the P22 challenge phage as follows. Plasmids pJHLC1 and pJHLC2 were transformed into *S. typhimurium* strain MS1883 [*leuA414*(Am) *hsdL*(r$^-$m$^+$)*Fels2*$^-$*supE*40]. Overnight cultures of these transformants were infected with phage P22 *mnt*::Kn9 *arc*(Am) at a multiplicity of infection of 1. After adsorption, 5 ml of phage broth were added, and the cultures were grown at 37°C until lysis occurred. The phage lysates were mixed with *S. typhimurium* strain MS1582 and plated on LB agar. The *sieA* mutation in the prophage in *S. typhimurium* strain MS1582 allows for superinfection of phage. Mnt expressed from the prophage in this strain prevented replication of phage that retained the O_{mnt}. Recombinant phages that are *arc*$^+$ form cloudy plaques on

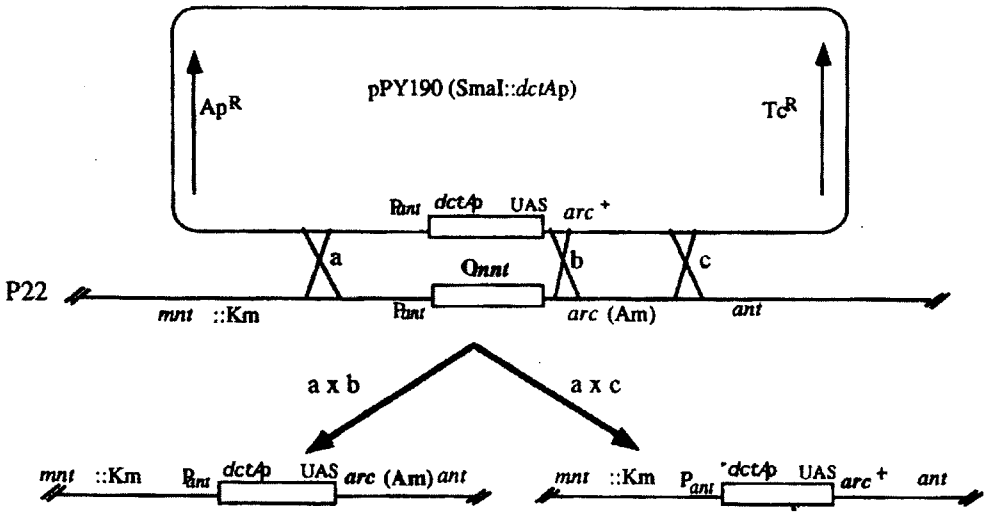

Fig. 1. Construction of challenge phage with the *R. meliloti dctA* promoter regulatory region. Plasmid pPY190 bearing *dctA* promoter regulatory region was used for recombination with phage P22 *mnt::km9 arc*(Am) in *S. typhimurium* strain with the P22 *c2*$^+$*mnt*$^+$*sie*$^-$ prophage. Homologous recombination between the prophage and plasmid generated two different recombinant phages that bear different alleles of *arc*. Recombination, indicated as a x b, resulted in challenge phage with *arc*(Am), which makes large clear plaques on a lawn of *S. typhimurium* strain MS1852 {*leuA414*(Am) *hsdL*(r$^-$m$^+$)*Fels2*$^-$*supE*40 *attP*::[P22*sieA44 16*(Am) *H1455*Tpfr49]}. Recombination involving a x c generated challenge phage with a wild-type allele of arc, which produced smaller cloudy plaques on a lawn of *S. typhimurium* strain MS1852. This difference in plaque morphology allowed us to screen for challenge phage with the *arc*(Am) allele. The *dctA* promoter regulatory region was cloned into pPY190 in both orientations (only one shown).

this strain because Arc can repress the expression of *ant* to a certain extent. Recombinant phages that have the amber mutation in *arc* generated large, clear plaques (Fig. 1). Clear plaques were picked and purified two times on a lawn of *S. typhimurium* strain MS1582, then *S. typhimurium* strain MS1883.

To test whether the challenge phage bearing the *dctA*p had the correct construction, high-titer phage lysates were prepared and used to infect *S. typhimurium* strain MS1868 [*leuA414*(Am) *hsdL*(r^-m^+)F*els2*$^-$] bearing plasmids that allow the overexpression of ^{54} (from pSA4) or DCTD (from pJHLC3). Correct constructs of challenge phage in which *dctA*p overlapped P_{ant} were predicted to favor lysogeny when σ^{54} was overexpressed, and favor the lytic pathway in the absence of σ^{54}. On the other hand, correct constructs of challenge phage in which *dctA*UAS overlaps the P_{ant} were predicted to favor lysogeny when DCTD was overexpressed, and favor the lytic pathway in the absence of DCTD. *S. typhimurium* strain MS1868 carrying pJHLC3 or pSA4 were used to test the challenge phage. Cultures of strains carrying the corresponding plasmid were grown overnight, subcultured in the same medium and grown to an OD_{650} of 0.5. For the strains with pSA4 and pJHLC3, cells were subcultured a second time containing various concentrations of IPTG (from 0 to 100 μM) at 37°C for 2 h to induce σ^{54} or DCTD expression, respectively. Challenge phages were added to a multiplicity of infection of 25, and the mixture was incubated at 37°C for 1 h. Dilutions of the mixtures were plated on LB agar containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml) for strains bearing pSA4 and chloramphenicol (50 μg/ml) and kanamycin (50 μg/ml) for strains bearing pJHLC3 to select for stable lysogens. Plates were incubated at 37°C overnight. The frequency of lysogeny was calculated from the following equation: (the number of lysogens/the number of infected cells) × 100. The number of infected cells was assumed to be equal to the number of colony forming units in the culture infected with the challenge phage.

R. meliloti dctA promoter regulatory region includes a conserved σ^{54}-dependent promoter region and an upstream activating sequence (UAS) that is recognized by DCTD and is approximately 100 base pairs upstream of the transcriptional start site. We constructed a P22 challenge phage in which O_{mnt} was replaced with the *dctA* promoter regulatory region. Challenge phages were constructed in both directions: with the promoter region overlapping P_{ant} (P22*dctA*p) (Fig. 2A) or the UAS overlapping P_{ant} (P22*dctA*UAS)(Fig. 3A).

Occupancy of a binding site for a DNA-binding protein is determined by the amount of the protein in the cell and the affinity of the protein for its site. Therefore, to study protein-DNA interactions between DCTD (or σ^{54}) and the *dctA* promoter using the challenge phage system, we needed to control the expression of DCTD and σ^{54}. Plasmid pSA4 contained the *ntrA* gene under the control of the *lac* promoter, and the level of σ^{54} in the cell could be

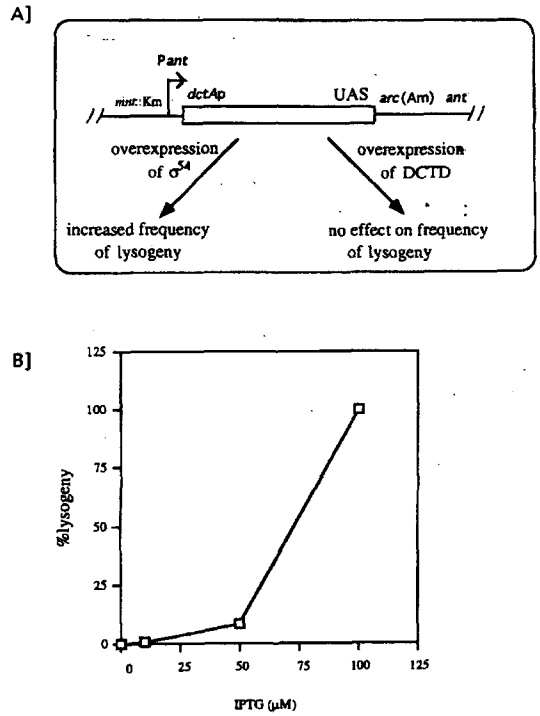

Fig. 2. Effect of σ^{54} overexpression on the frequency of lysogeny in cells infected with challenge phage P22*dctA*p. A. The *imml* region of a P22 challenge phage in which O_{mnt} has been replaced with the dctA promoter regulatory region. In the orientation shown, the *dctA* promoter overlapped P_{ant}. Overexpression in *S. typhimurium* resulted in an increase of frequency of lysogeny upon infection with this challenge phage. Overexpression of DCTD had no effect on the frequency of lysogeny, presumably due to the distance of the DCTD-binding sites (UAS) from P_{ant}. B. The expression of the *ntrA* gene carried on plasmid pSA4 was under the control of P_{lac}, and was inducible with IPTG. The level of overexpression of σ^{54} in *S. typhimurium* strain 1868 was controlled by adding different concentrations of IPTG to the medium. Cells were then infected with challenge phage P22 *dctA*p, and stable lysogens were selected on LB agar containing kanamycin and the corresponding concentration of IPTG. The frequency of lysogeny was calculated as described in the text.

regulated by the concentration of IPTG added to the medium. To evaluate the IPTG-dependent expression of *ntrA* from the plasmid pSA4 and its effect on the frequency of lysogeny, challenge phage assays were performed with different concentrations of IPTG. The frequency of lysogeny increased with increasing IPTG concentration when *S. typhimurium* cells carrying pSA4 were infected with the challenge phage P22*dctA*p (Fig. 2B). These results demonstrated that σ^{54} can repress *ant* expression in challenge phage P22*dctA*p in a concentration dependent manner. Overexpression of σ^{54} did not affect the frequency of lysogeny in cells infected with the challenge phage P22*dctA*UAS (data not shown), indicating that binding of this protein to a site which is positioned approximately 100 base pairs from P_{ant} does not repress transcription of the *ant* gene in this challenge phage.

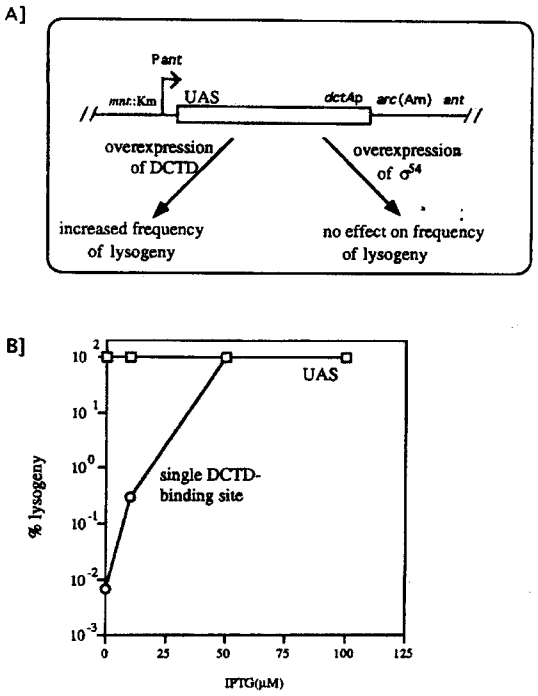

Fig. 3. Effect of overexpression of DCTD on the frequency of lysogeny in cells infected with P22 challenge phage with a single DCTD-binding site. A. The immI region of a P22 challenge phage in which O_{mnt} has been replaced with the *dctA* promoter regulatory region. In the orientation shown, one of the DCTD-binding sites in the UAS (the one distal to the *dctA* promoter) overlapped P_{ant}. Expression of DCTD in *S. typhimurium* cells infected with this challenge phage resulted in increased frequency of lysogeny, while overexpression of σ^{54} had no effect on the frequency of lysogeny. The distance of the *dctA* promoter from P_{ant} (~100 bp) apparently does not allow E^{54} to repress transcription of the *ant* gene. B. Expression of the *dctD* gene carried on plasmid pJHLC3 was under the control of P_{lac}, and was inducible with IPTG. When cells harboring pJHLC3 were infected with challenge phage P22 *dct*AUAS, the frequency of lysogeny was ~100%, even without IPTG-induction of *dctD*. When cells harboring pJHLC3 were infected with challenge phage in which a single DCTD-binding site replaced O_{mnt}, the frequency of lysogeny increased with increasing IPTG concentration.

Plasmid pJHLC3 is a derivative of pACYC184 that carries *dctD* under the control of the *lac* promoter, which allows the level of DCTD within the cell to be regulated with IPTG. When cells harboring this plasmid were infected with challenge phage P22 *dct*AUAS, the frequency of lysogeny was almost 100% (Fig. 3B). This high level of lysogeny was observed even without expressing DCTD. In contrast, when cells harboring pJHLC3 were infected with a challenge phage in which a single high affinity DCTD-binding site replaced O_{mnt}, the frequency of lysogeny was proportional to the IPTG concentration (Fig. 3B). These data suggest that either DCTD has a higher affinity for the tandem DCTD-binding site in the UAS than it does for a single DCTD-binding site, or the stability of the DCTD-DNA complex is higher with the UAS compared to the single site. These challenge phages seemed suitable for examining interactions between DCTD (or σ^{54}) and the *dctA* promoter regulatory region. This system will be used for further genetic analysis of the *dctA* promoter and its regulatory protein, DCTD (or σ^{54}).

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