

DNA binding by DctD and DctD-NifA chimeras

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SUMMARY

DctD is a σ^N - or σ^{54} -dependent transcriptional activator of *Rhizobium meliloti* and *Rhizobium leguminosarum* that regulates expression of DctA, a C₄-dicarboxylate transport protein. Based on sequence analysis, DctD was predicted to possess three separate functional domains. Prior results described an N-terminal regulatory domain, homologous to two-component regulators, that inhibits transcriptional activation but not sequence-specific DNA binding. Here we confirm that C-terminal deletions eliminate both UAS-directed transcriptional activation and sequence specific DNA binding. Such deletion proteins retained some ability to activate the *dctA* promoter, but this activity was UAS-independent. One mutant protein lacking the HtH region was purified and found to still bind DNA nonspecifically. Positive evidence for a sequence-specific, C-terminal DNA binding module was observed in chimeric DctD-NifA proteins, in which various C-terminal deletions of *R. leguminosarum* DctD were fused to C-terminal fragments of *R. meliloti* NifA. While *in vivo* activity levels were very low for these chimeric proteins, two were regulated by the *dct* pathway, preferentially stimulating transcription of *nifH* rather than that of *dctA* in response to succinate as the sole carbon source. Fusions of selected DctD-NifA chimeras and maltose binding protein were made, purified and used in gel-shift assays to provide direct evidence for altered DNA binding specificities. These results confirm predictions of sequence specific DNA binding by the HtH motif regions of DctD and NifA, and raise the possibility that during activation the central domain of DctD binds DNA nonspecifically.

INTRODUCTION

The C₄-dicarboxylates succinate, fumarate, and malate are carbon and energy sources for the free-living soil bacteria *Rhizobium leguminosarum* and *Rhizobium meliloti* and are thought to be the primary carbon and energy sources provided to symbiotic bacteroids by their respective legume host plants (Ronson et al., 1981; Finan et al., 1983; Arwas et al., 1985; Ronson and Astwood, 1985; Bolton et al., 1986; Engelke et al., 1987, 1989; Finan, 1988; Watson et al., 1988; Wang et al., 1989; Jiang et al., 1989; Yarosh et al., 1989; Watson, 1990). In both *R. meliloti* and *R. leguminosarum*, the *dctA* gene encodes a transport protein responsible for the uptake of C₄ dicarboxylates (Ronson et al., 1987a). Expression of *dctA* is controlled by a two-component regulatory system composed of the *dctB* and *dctD* gene products (Nixon et al., 1986; Ronson et al., 1987a; 1987b). In the current model, DctB is a transmembrane protein which, in response to the presence of environmental C₄-dicarboxylates, positively regulates the transcriptional activator DctD by phosphorylation. Activated DctD then interacts with RNA polymerase to stimulate transcription from the *dctA* promoter.

Recognition of the *dctA* promoter by RNA polymerase requires the alternative sigma factor σ^{54} (σ^N). In general, transcription of σ^{54} -dependent promoters involves the action of trans-acting factors such as DctD, NtrC, and NifA (reviewed by Gussin et al., 1986; Kustu et al., 1989; Thony and Hennecke, 1989). These proteins stimulate transcription through a common mechanism in which the regulator protein binds to one or more sites contained within an upstream activation sequence (UAS) located approximately 100 base pairs upstream of the target promoter (Buck et al., 1986; Reitzer and Magasanik, 1986; Ledebur et al., 1990; de Lorenzo et al., 1991). Direct contact between the activator and σ^{54} -holoenzyme (Lee and Hoover, 1995) is achieved via a DNA loop (Buck et al., 1987; Minchin et al., 1989; Su et al., 1990; Wedel et al., 1990; Santero et al., 1992). This interaction results in isomerization of the closed polymerase-promoter complex to an open complex, in which a region of DNA around the start site is denatured (Sasse-Dwight and Gralla, 1988; Morett and Buck, 1989; Popham et al., 1989; Buck and Cannon, 1992; Cannon et al., 1994). Nucleotide hydrolysis, catalyzed by the activator, accompanies isomerization (Popham et al., 1989; Weiss et al., 1991; Lee et al., 1993; Lee et al., 1994).

Deletion of portions of the 5'-end of the *dctD* gene showed that removal of up to 143 residues from the amino terminus produces a constitutive activator that also hydrolyses purine nucleotides (Ronson, 1988; Huala and Ausubel, 1992; Gu et al., 1994; Lee et al., 1994). Within the deleted region lies a 125-residue segment that is similar to other two-component receiver domains. Adjacent to this N-terminal regulatory domain is a central domain that includes a nucleotide-binding motif. This central domain alone, when expressed in *E. coli* or *R. meliloti* cells, is transcriptionally competent (Ronson, 1988; Huala and Ausubel, 1992; this work). The C-terminal domain of DctD includes a sequence predicted to form a helix-turn-helix (HtH) motif that is, by analogy to similar motifs in NifA, NtrC and FlbD proteins, strongly implicated in site-specific DNA binding (Drummond et al., 1986; Contreras and Drummond, 1988; Morett and Buck, 1988; Morett et al., 1988; Buck and Cannon, 1989; Cannon et al., 1991; Mullin and Mullin, 1993).

Given the apparent physical separation of functional domains in σ^{54} -dependent activators, attempts have been made to construct functional chimeras. Exchanging the N-terminal domains of *Klebsiella pneumoniae* NtrC, NifA, and *E. coli* OmpR failed to produce detectable hybrid activity (Drummond et al., 1990). Drummond and co-workers reported successful exchange of C-terminal DNA-binding functions for the *Azotobacter vinelandii* σ^{54} activators VnfA and AnfA, measuring promoter specificity with an *in vivo* repression assay on a synthetic promoter (Jacob and Drummond, 1993). Here we precisely define the functional boundary between transcription activation and sequence specific DNA-binding domains of DctD, and report for the first time *in vitro* evidence that the DNA-binding function of one σ^{54} -dependent activator can replace that of another. The chimeric proteins functioned poorly, however, suggesting that the functional domains in these two component transcriptional activators have diverged to an extent that does not allow simply recombining modules to make novel proteins. We also observed

nonspecific DNA binding in a purified mutant protein lacking the HtH domain, which may indicate that the central domains of σ^{54} -dependent activators approach DNA closely during activation.

RESULTS

Removing the C-terminal 56 residues from *R. meliloti* DctD₍₁₋₁₄₂₎, making DctD_(1-142,406-460), rendered it incapable of sequence specific binding to the *dctA* UAS *in vitro*.

DctD and DctD₍₁₋₁₄₂₎, which lacks the first 142 residues of DctD, were previously shown to bind to tandem, dyad-symmetric sequence elements present in the *dctA* UAS region (Ledebur et al., 1990; Ledebur and Nixon, 1992; Gu et al., 1994; Scholl and Nixon, unpublished results). Such binding can be qualitatively demonstrated using whole cell extract of *E. coli* containing DctD₍₁₋₁₄₂₎ (Fig. 1A), which is found to retard the electrophoretic mobility of 1 ng of a radiolabelled DNA fragment carrying the *dctA* UAS (Fig. 1B, lane 1). Unlabeled probe DNA (100 ng) effectively competes for the protein (Fig. 1B, lane 2) even in the presence of 2 μ g polydI/dC, demonstrating but not fully quantifying sequence specificity in the binding reaction. The putative HtH DNA binding element is contained in residues V417 to Q436 of DctD (Fig. 2). The portion of DNA encoding the last 56 residues of DctD, I405 to T460, was deleted from pDctD₍₁₋₁₄₂₎, creating pDctD_(1-142,405-460). SDS-PAGE analysis of extracts of *E. coli* cells carrying pDctD₍₁₋₁₄₂₎ or pDctD_(1-142,405-460) showed stable products of the expected molecular masses (~41 kD and ~36 kD, respectively), which accumulated to similar amounts (Fig. 1A). Extracts from *E. coli* expressing DctD_(1-142,405-460), and from the host *E. coli* cells, failed to shift the *dctA* UAS probe (Fig. 1B, lanes 3-4). Similar results were obtained from gel retardation assays using extracts prepared from cells of strain *Rhizobium meliloti* 6000 which carried plasmids constructed by joining the pRK290-derived broad host range plasmid pBG Ω with pDctD₍₁₋₁₄₂₎ or pDctD_(1-142,405-460) (Fig. 1C). To address the possibility that DctD₍₁₋₁₄₂₎ binds to the *dctA* UAS, but the resulting protein-DNA complex dissociates during initial entry into the gel matrix, we performed filter-binding assays. When extract prepared from cells synthesizing DctD₍₁₋₁₄₂₎ was incubated with ~1 ng of DNA, 7,900 cpm of radioactivity was retained on the filters. A 100-fold molar excess of unlabeled plasmid pUCN, which carries a single copy of the *dctA* UAS, prevented 80% of the labeled DNA from binding the filters. On the other hand, when similar competition assays were done with an equal amount of pUC lacking the *dctA* UAS region, none of this radioactivity was prevented from binding to the filter. In contrast to these results, when an equal concentration of extract protein made from cells synthesizing DctD_(1-142,405-460) was used, only background radioactivity (250 cpm) was retained on the filters. We were not able to purify DctD_(1-142,406-460) for further analysis.

A second C-terminal deletion mutant of DctD₍₁₋₁₄₂₎, in which residues I405 to R437 were removed yielding DctD_(1-142,405-437), was purifiable. It was thus possible to study this mutant protein at concentrations higher than present in the extracts described above. Purified DctD_(1-142,405-437) showed no evidence of sequence specific DNA binding, but did display nonspecific DNA binding activity in two assays. First, the mutant protein interfered with DNaseI digestion of UAS-containing DNA in a nonspecific manner (Fig. 3A). Adding equal aliquots of the protein buffer had no effect on the DNaseI digestion (Fig 3A). Second, gel-shift assays showed equivalent retardation of DNA containing or lacking the *dctA* UAS (Fig. 3B). Concentrations of presumed dimers of DctD_(1-142,405-437) that interfered with DNaseI digestions were 30 nM and higher, while somewhat lower amounts of protein retarded electrophoretic migration of the DNA fragments (Fig. 3C). The difference observed for these two assays might be expected, given that DNaseI will generate fragments even with protein bound to many of the nonspecific sites, while all binding events would generate a shift in the gel mobility assay. Filter binding assays were not conducted using DctD_(1-142,405-437).

Deleting a portion of the C-terminal domain of *R. meliloti* DctD_{L143} destroyed its ability to utilize the *dctA* UAS, but not to activate transcription.

Failure to recognize the *dctA* UAS should diminish or eliminate the ability to activate transcription. The abilities of DctDA₍₁₋₁₄₂₎ and DctDA_(1-142,405-460) to activate transcription in *E. coli* cells carrying $\Phi(dctA'-lacZ)$ (Hyb) reporter plasmids that contained or lacked a UAS were compared. No IPTG was added to induce expression of the activator genes, as previous studies of DctDA₍₁₋₁₄₂₎ showed induction was unnecessary, and even toxic for the cells (Gu et al., 1994). DctDA₍₁₋₁₄₂₎ stimulated approximately 10-fold more β -galactosidase activity from the +UAS construction than from the -UAS reporter (44,000 units vs 3,900 units, background less than ten units). In contrast, in the presence of either reporter plasmid, DctDA_(1-142,405-460) induced β -galactosidase activity to levels similar to those supported by DctDA₍₁₋₁₄₂₎ and the reporter gene lacking the UAS (2,900 units and 1,800 units, respectively). After DctDA_(1-142,405-437) was purified, its failure to distinguish between reporter genes having or lacking a UAS was demonstrated *in vitro* using a coupled transcription / translation system. As previously shown, DctDA₍₁₋₁₄₂₎ did discriminate between these reporter genes (Fig. 4, closed and open circles, respectively). DctDA_(1-142,405-437) stimulated the production of less β -galactosidase activity in the coupled assay, but this amount of activity was the same regardless of the presence or absence of the *dctA* UAS (Fig. 4, closed and open triangles, respectively). For both proteins, the amount of β -galactosidase activity observed depended upon the amount of activator present in these assays (Fig. 4). At higher protein concentrations, the activity of DctDA_(1-142,405-437) for both reporter genes approached that of DctDA₍₁₋₁₄₂₎ on the reporter gene lacking the UAS region.

A functional boundary between the C-terminal and central domains of *R. meliloti* DctD was observed between residues G381 and A384.

To determine the boundary between the C-terminal DNA-binding function and the central domain of DctD, the *in vivo* transcription activation assay was used to screen serial 3'-deletions of pDctDA₍₁₋₁₄₂₎ (Fig. 5). Blunt ends generated by digesting the 3'-end of the coding region with nuclease *Bal31* were joined to a small DNA fragment that contained termination codons in all three reading frames. Resulting mutant proteins were then compared with the parental DctDA₍₁₋₁₄₂₎ for ability to support β -galactosidase activity from reporter genes having or lacking the *dctA* UAS. DctDA₍₁₋₁₄₂₎ stimulated 11 times more β -galactosidase production from the +UAS reporter gene than from the -UAS reporter gene. Mutant proteins with deletions up to residue 384 (mutants A384, P395, A403, and D404) were able to induce β -galactosidase activity, but only as well as DctDA₍₁₋₁₄₂₎ acting on the reporter gene lacking a UAS. Mutant proteins bearing deletions that extended beyond residue 382 (mutants G381 to V308) failed to raise β -galactosidase activity above background levels. SDS-PAGE analysis of whole cells showed that these more extensive deletions, while leading to a loss of transcription activation, did not grossly affect expression levels (data not shown).

Some, but not all, swaps of the C-terminal portion of *R. leguminosarum* DctD with that of *R. meliloti* NifA changed the *in vivo* promoter specificity of the activator.

To explore the possibility that the C-terminal and central domains of DctD and other σ^{54} -dependent activators are modularly linked, we attempted to replace the C-terminal DNA-binding domain of *R. leguminosarum* DctD with that of *R. meliloti* NifA. We chose the *R. leguminosarum* *dctD* gene over that of *R. meliloti* to avoid potential complications that might arise from homologous recombination between endogenous *dctD* and the recombinant, plasmid-borne genes when assayed in *R. meliloti*. Various 3'-portions of *dctD* were replaced with analogous pieces of *R. meliloti* *nifA* by using *Bal31* nuclease to create random junctions within the 3'-end of each gene (see Experimental Procedures). Sequence analysis of nearly 200 such fusions identified 33 in-frame fusions, 13 of which were cloned adjacent to *dctB* to allow expression of the mutant $\Phi(dctD'-nifA)$ (Hyb) genes in the normal context of the *dctBD* locus. We had previously shown that the *R. leguminosarum* *dctBD* genes function in *R. meliloti* (Jiang et al., 1989). The *R. meliloti* DctD and NifA and *R. leguminosarum* DctD sequences are aligned

in Fig. 2, and Fig. 6 shows the position of the junction in each of the selected chimeras. The plasmids containing the recombinant operons were joined with pBGΩ and conjugated into *R. meliloti* mutant strain Rm6000. Rm6000 is not able to grow in media containing succinate as the sole carbon source because of the insertion of an *R. leguminosarum* *dctA-lacZ* reporter gene in *dctD*. The *in vivo* activities of the fusion proteins are given in Fig. 6. Ten of the DctD-NifA mutant products lacked the putative HtH motif of DctD. Although none of these activators allowed the mutant Rm6000 strain to grow in succinate defined media, cells containing pBGΩ-D20 had a three- to four-fold increased level of β-galactosidase activity in media containing glucose as the carbon source. Of the three *dctD-nifA* constructs that did contain the putative DctD DNA-binding motif, only D1 enabled Rm6000 to grow in succinate minimal media. When grown in glucose defined minimal media, Rm6000 cells containing pBGΩ-D1 produced two to three times more β-galactosidase than Rm6000 cells containing the control plasmid pBGΩ, and when grown in succinate defined media these cells produced about 50-times more β-galactosidase activity than did the control. Succinate thus caused about a 15-fold increase in the ability of DctD-NifA protein D1 to stimulate transcription of the *dctA-lacZ* reporter gene. In comparison, succinate inducibility of the wild-type *R. leguminosarum* DctD was approximately six-fold, but rose from a significantly higher basal level.

The activation activity of the 13 mutant constructions was also studied in Rm4000, which is a *nifA::Tn5* mutant strain of *R. meliloti* that has a *nifH-lacZ* reporter gene integrated into the *nifH* gene. Low basal levels of β-galactosidase activity (<30 units) were detected from cells that have each of the *dctD-nifA* chimeric genes on pBGΩ-plasmids when cells were grown in glucose defined media. When cells were grown in succinate defined media, only those containing the constructions E96 and D20 showed greater than a 2-fold increase in the level of β-galactosidase activity. These two constructs stimulated the amount of β-galactosidase activity about four- to ten-fold. The time course of this increase in *nifH*-driven β-galactosidase activity is shown in Fig. 7. Given the very low *in vivo* transcription activation activity demonstrated by the E96 and D20 fusion proteins, *in vitro* analysis was not attempted.

Purified MBP-DctD-NifA proteins showed selective specificity for binding to DNA containing the *R. meliloti* *nifH* UAS.

To directly demonstrate that a change in DNA binding specificity had accompanied the altered *in vivo* promoter specificity of the *dctD-nifA* fusion gene products E96 and D20, we cloned portions of seven of the fusion protein constructions into the *E. coli* vector pMAL for overexpression and purification (Fig. 8A). We chose to examine constructions E96, E5, D20, and D1, which contained essentially the same NifA fragment but possessed different apparent *in vivo* activities; C17, which included the predicted HtH motif of both DctD and NifA; D12, which contained an intact HtH motif from DctD and a minimal one from NifA; and the wild-type *R. leguminosarum* DctD. The MBP-DctD-NifA fusion products were used in a two probe gel mobility shift assay containing two radiolabelled fragments, one carrying the *R. meliloti* *nifH* UAS and the other carrying the *R. leguminosarum* *dctA* UAS (Fig. 8B). In this assay the free DNAs had distinct mobilities while the protein DNA complexes could not be uniquely quantified; therefore, the results are reported as the fraction of total DNA in the unbound state rather than that in the bound state (Fig 9). Purified *R. meliloti* DctD and MBP-DctD of *R. leguminosarum* were used as controls for specific binding to the *dctA* UAS. MBP alone did not bind either UAS fragment at concentrations similar to those used for the chimeric proteins (data not shown). MBP-DctD construct D12 bound both DNA fragments, showing a preference for the *dctA* UAS. Construct MBP-C17 also bound both DNA fragments, but preferred the *nifH* UAS. All other constructs showed binding to the DNA fragment containing the *nifH* UAS, but little or no binding to DNA containing the *dctA* UAS. Removing the MBP-domain from the purified fusion proteins was feasible using factor Xa protease. Specific activities of the DNA binding proteins were only marginally increased (data not shown), and thus not thoroughly studied.

DISCUSSION

Activators of the σ^{54} -form of RNA polymerase generally appear to have three separate domains, each of which is responsible for a distinct function. The amino-terminal domain is responsible for regulation, the central domain for transcription activation, and the carboxy-terminal domain for sequence specific DNA binding. In this study, we have focused on the activity of the C-terminal domains of the σ^{54} activators DctD from *R. meliloti* and *R. leguminosarum*, and NifA from *R. meliloti*. We began these studies by making C-terminal deletions of DctD $\Delta_{(1-142)}$, a constitutively active form of *R. meliloti* DctD which lacks the first 142 residues of the wild-type protein. From a comparison of the DNA-binding and transcriptional activation characteristics of these proteins, we confirm that the C-terminus of DctD contains a major determinant for sequence-specific binding to its associated upstream activation sequence, and that this binding activity is separable from transcription activation. Together with previous results (Gu et al., 1994), these define the N-terminal, central, and C-terminal subdomain boundaries of *R. meliloti* DctD to reside within residues 144-150 and 381-384. The results also indicate that the DctD C-terminal DNA binding function can still function with the addition of several residues to the C-terminus, as was present in construct T460.

While it may not be commonly observed that transcription activators can function in the absence of sequence specific DNA binding, this has been previously reported for σ^{54} -dependent activators (Huala and Ausubel, 1989; 1992; Berger et al., 1994). It has never been determined if nonspecific DNA binding activity is needed for activation by σ^{54} -dependent activators in such cases. It is possible that nonspecific DNA binding allows these activators to bind DNA upstream of the promoters where UAS regions are typically located, albeit at reduced efficiency compared to when specific binding activity is present. Alternatively, a nonspecific DNA binding function may be used by σ^{54} -dependent activators to closely approach DNA at the polymerase-promoter complex, joining the complex to promote DNA melting. The concentration dependent, nonspecific binding that we observed for purified DctD $\Delta_{(1-142,405-437)}$ might provide either or both of these hypothetical functions. Future experiments will have to be performed to rule out the artifactual creation of a non-specific DNA binding surface in this recombinant protein, and to demonstrate a functional role for such DNA binding in transcription activation.

We also demonstrated that it is possible to alter the DNA-binding specificity of *R. leguminosarum* DctD by adding to or replacing its C-terminal domain with the corresponding portion of *R. meliloti* NifA. The results for *in vivo* assays are consistent with the following conclusions: 1) the C-terminal region of *R. leguminosarum* DctD is required for recognition of the *R. leguminosarum* *dctA* UAS; 2) the C-terminal 46 residues of *R. meliloti* NifA comprise a sequence-specific DNA-binding module; and 3) this NifA module can be attached to the *R. leguminosarum* DctD N-terminal and central domains to produce a hybrid activator. Some aspects of the *in vivo* data are, however, hard to interpret. Not only was the amount of activity exhibited by the chimeric DctD-NifA proteins very low, but only one of the fusion proteins that retained the DctD HtH was able to support growth on succinate. Failure to support growth on succinate may reflect an incompatibility between the two HtH motifs, or the NifA sequence may have destabilized the other parts of the protein. The feeble ability of these fusion proteins to activate the *nifH* promoter may have arisen from the formation of heterodimers or higher order oligomers between the fusion proteins and the endogenous DctD that remained in the tester strain Rm4000. Such hybrid proteins would be expected to interact inefficiently with *nifH* and *dctA* DNA binding sites.

Given these problems interpreting the *in vivo* data, we turned to an *in vitro* DNA-binding assay to directly study UAS recognition independent of transcriptional activation. Using MBP-fusions and a two probe gel mobility shift assay, we were able to directly visualize recognition of the two UAS fragments in the same binding reaction by purified proteins. Even though the overall binding activities of these protein preparations were very low, and only marginally improved by proteolytic removal of the MBP-domain, the *in vitro* binding specificities correlated well with the presence or absence of each putative HtH motif. This contrasted starkly with the *in vivo* results. MBP-E96 and MBP-E5, which lacked the DctD HtH and included the NifA HtH, clearly preferred the DNA containing the *nifH* UAS. MBP-D20 and MBP-D1, which contained

the same NifA portion as MBP-E96 and MBP-E5 but possessed larger pieces of the DctD C-terminal domain, still demonstrated specificity toward the *nifH* UAS. MBP-D12 and MBP-C17, which included intact HtH motifs of both DctD and NifA, retained the ability to alter the mobility of both *nifH* and *dctA* UAS fragments. They showed, however, 8- to 15-fold contrasting preferences for the two DNA fragments. Finally, MBP-R1 DctD, and wild-type Rm DctD both possessed the entire DctD C-terminus and lacked an intact NifA HtH, and as expected, bound the *dctA* UAS fragment preferentially.

Jacob and Drummond (1993) demonstrated *in vivo* an altered promoter specificity for *Azotobacter vinlandii* AnfA containing an exchange of its HtH plus 36 additional residues to the N-terminal side for the equivalent residues of VnfA. Because the assay failed to show a similar altered specificity after exchanging just the HtH motif, it was concluded that sequence specific binding determinants resided outside of the HtH motif. The results reported here define the location of DNA binding determinants of DctD and NifA and illustrate the modularity of these C-terminal domains. For NifA, this specificity is seen to reside largely in the HtH motif; this would explain binding to the *nifH*-UAS by construct D12 for which the N-terminal residue of the NifA module is predicted to be the second residue of the first helix. Since we did not explicitly show that binding of D12 to DNA containing the *nifH*-UAS was sequence specific, a more certain conclusion can be drawn from the results of constructs D1, D20, E5 and E96. For these constructs, a clear preference was demonstrated for the *nifH*-UAS rather than the *dctA*-UAS, definitively showing that the NifA DNA binding module resides within the last 46 residues. This portion of NifA contains 17 residues N-terminal to the HtH motif. The specific amino acids comprising these additional residues are compatible with the formation of a β -turn followed by an additional α -helix that might be needed for positioning the HtH structure and or providing additional binding surface (see Harrison, 1991, for review of the HtH motif in other proteins).

Even after considering the *in vitro* binding results, it is still unclear why only one *dctD-nifA* chimeric gene, construct D1, was able to replace the requirement for *dctD* to sustain growth on succinate minimal medium. This was true despite the fact that other chimeric genes (C17 and D12) encoded more complete DctD C-terminal regions, and D12 still exhibited a preference for the *dctA* UAS. The amount of transcriptional activation at the *dctA* promoter that was supported by construct D1 was 15-20 times lower than in the presence of wild-type DctD, both for basal and inducing growth conditions. Curiously, construct D1 did not appear to recognize the *nifH-lacZ* reporter gene *in vivo*, but *in vitro* MBP-D1 did bind to DNA containing the *nifH* UAS better than to DNA containing the *dctA* UAS. It also remains unclear why the same portion of NifA appeared to provide higher discrimination between the *nifH* and *dctA* UAS regions when fused to other residues of the DctD C-terminal region. These inconsistencies may reflect a higher degree of structural integration than might be apparent from the amino acid similarities used to model the regulatory, transcription activation, and DNA binding domains shared by eubacterial "two-component" regulation systems.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, and media

The strains and plasmids used in this study are listed in Table 1. *E. coli* cells were cultured at 30°C and 37°C, respectively, in Luria Broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5). The defined minimal medium for culturing *R. meliloti* contained RDM salts, vitamins, and 5 mM NH₄Cl as previously described (Ronson et al., 1981). Glucose minimal medium also contained 0.1% glucose, while succinate minimal medium contained 10 mM sodium succinate and 50 mM MES (morpholineethanesulfonic acid, pH 6.1). Antibiotic concentrations used for selection of *R. meliloti* were as follows: 300 μ g/ml for streptomycin, 3 μ g/ml for tetracycline, 50 g/ml for neomycin, and 100 μ g/ml for spectinomycin. Concentrations of antibiotics for *E. coli* selection were 10 μ g/ml for tetracycline, 50 μ g/ml for ampicillin, 100 μ g/ml for spectinomycin, and 20 μ g/ml for kanamycin.

Reporter Genes

The 8th codon of *lacZ* was fused to codon 12 of *R. meliloti dctA* in reporter genes that were carried on pRK290, or on a derivative of pRK290 which also contained the ColE1 origin of replication. Plasmids containing both origins of replication were maintained at moderate copy level in *E. coli*, but at low copy level in rhizobia; the RK2 replicon alone yields low copy level in both bacterial species. Reporter genes lacking the *dctA* UAS were identical to those containing it except for a 70 bp deletion from position -170 to -100, relative to the start site for *dctA* transcription. In strain Rm4000, codon 29 of *R. meliloti nifH* is fused to codon 8 of *lacZ* (Sundaresan et al., 1983; Huala and Ausubel, 1989).

DNA Biochemistry

Large and small scale plasmid preparations were made by the alkaline lysis method or by the boiling method (Birnboim and Doly, 1979; Holmes and Quigley, 1981). Sequence analysis was performed by the dideoxy method of Sanger et. al. (Sanger et al., 1977) using either Sequenase enzyme (United States Biochemical) or a missense mutant of T7 DNA polymerase which lacks the 3'-5' exonuclease activity (generously provided by K. Johnson, Penn State).

Construction of mutant *dctD* genes containing 3' deletions

The plasmid pDctD $\Delta_{(1-142)}$ contains codons 143-460 of *R. meliloti dctD* fused to codon 11 of *E. coli lacZ* from pUC13 and thereby encodes an N-terminal truncation of DctD referred to as DctD $\Delta_{(1-142)}$ (Gu et al., 1994). A 140 bp *EcoRV-SmaI* fragment encoding the C-terminal 56 amino acids of DctD was excised from pDctD $\Delta_{(1-142)}$, and the two blunt termini of the plasmid were rejoined to produce pDctD $\Delta_{(1-142,405-460)}$. The resulting fusion protein contains the DctD residues L143 to D404, terminating with the residues SRGSP* that result from a frame shift in the end of the *dctD* coding region. The *EcoRV-NcoI* fragment was removed in a second construction to produce an in frame deletion of residues I405 to R437. Plasmids containing various *dctD* 3' deletions were generated by treating *EcoRV*-linearized pDctD $\Delta_{(1-142)}$ with nuclease *Bal31* and cleaving the plasmid at the *HindIII* site upstream of *dctD*. *HindIII*-blunt end DNA fragments containing the *lacZ'*-*dctD'* region were isolated from low-melting agarose gels and ligated into the *HindIII* and *HincII* sites of pUCX to provide translation termination signals. pUCX was constructed by inserting an *XbaI* linker that has nonsense codons in all three reading frames (CTAGTCTAGACTAG) into the *SmaI* site of pUC13. The fusions in all constructs that were further analyzed were sequenced and each was shown to result in abundant protein of the expected size when whole cells were analyzed by SDS-PAGE (Fig. 1A, and data not shown).

β -galactosidase assays

Preparation of cell extracts by sonication and spectrophotometric assay of β -galactosidase activity using 2-nitrophenyl-b-D-galactopyranoside (ONPG) were performed as previously described (Ledebur et al., 1990).

Coupled transcription-translation assay

In vitro assay of coupled transcription and translation was performed as previously described (Gu et al, 1994). Briefly, cells of *E. coli* strain DH5 α were used to overexpress DctD $\Delta_{(1-142)}$, DctD $\Delta_{(1-142,405-460)}$, or DctD $\Delta_{(1-142,405-437)}$. Cells were harvested by centrifugation, lysed in a French pressure cell, and the resulting extracts clarified by centrifugation. DctD $\Delta_{(1-142)}$ was purified as reported, and DctD $\Delta_{(1-142,405-437)}$ was purified by ammonium sulfate precipitation followed by Q-sepharose chromatography. Both proteins were judged to be greater than 95% pure by CoMassie staining of SDS-PAGE gels. Aliquots of each protein were added to a 50 μ l reaction containing S30 extract from *E. coli* strain NCM789, *Salmonella typhimurium* σ^{54} (110

nM), and supercoiled plasmid bearing $\Phi(dctA'-lacZ)$ (Hyb) reporter genes with or without a UAS (~15 nM). β -galactosidase activity was then determined spectroscopically, and reported as nmoles of ONPG hydrolyzed per minute.

Construction of $\Phi(dctD'-nifA)$ (Hyb) chimeric genes

Nested 3' deletions of the *R. leguminosarum* *dctD* coding region were made by digesting *Hind*III-restricted pLA114 DNA with nuclease *Bal*31. Digestion products were cleaved with *Sal*I at a site near the 5'-end of *dctD* to produce blunt-end *Sal*I fragments which were subsequently purified from low-melting agarose gels. Nested 5' deletions of *R. meliloti* *nifA* were created by *Bal*31 digestion of *Eco*RI-restricted pNIF3 DNA. The *nifA* digestion products were then cleaved with *Hind*III and ligated concomitantly with the set of *dctD* deletion fragments into an M13mp19 vector digested with *Sal*I and *Hind*III. The resulting plasmids represented a collection of DNA fusions, varying in their junction sites. Sequence analysis of nearly 200 fusions identified 33 that encoded in-frame fusion proteins; 13 of these 33 junctions were cloned as *Sal*I fragments into the *Sal*I site of pBS13 (pUC13 carrying the *Bam*HI-*Sal*I fragment from pLA114) to put the chimeric $\Phi(dctD'-nifA)$ (Hyb) fusion genes into the otherwise wild-type context of the *dctBD* operon. Plasmids capable of replicating in both *E. coli* and *R. meliloti* were made by joining pBG Ω , a derivative of the broad host-range plasmid pRK290, with each of the pBS13- $\Phi(dctD'-nifA)$ (Hyb) plasmids at their *Hind*III sites. The resultant constructions were conjugated into the mutant *R. meliloti* strain Rm6000, which contains an insertion of the $\Phi(dctA'-lacZ)$ (Hyb) reporter described above into *dctD*, and into the strain Rm4000, which is a *nifA::Tn5* insertion mutant of Rm1021 that also has a $\Phi(nifH'-lacZ)$ (Hyb) fusion inserted in the *nifH* gene (Huala and Ausubel, 1989). Bacterial crosses were conducted using the tri-parental spot mating method with the *E. coli* strain MM294/pRK2013 used as helper cells (Ditta et al., 1980).

pMal plasmid constructions

Maltose-binding protein (MBP)-DctD-NifA fusion proteins were constructed using PCR-amplified portions of *R. leguminosarum* *dctD* and the *dctD-nifA* fusion genes labeled E96, E5, D20, D12, D1 and C17 (see Figs. 4 and 5). *Taq* polymerase (0.5 units; Boehringer Mannheim Biochemicals) was used in 100 μ l reaction volumes containing 5 pg of template DNA (pBS13 derivatives), 10 μ l of the supplied 10x buffer, 50 μ M dNTPs, and 20 pmol of each primer. Thirty cycles (94°C, 1 min; 55°C, 2 min; 72°C, 2 min) were performed using a Perkin-Elmer Cetus Thermal Cycler. The upstream primer (5'-CCGGAATTCCGGCAGGAGAATTTGCCGCTG-3'; Midland Genetics) annealed to codons Q140 to L145 of *dctD*, previously shown to lie outside of the central domain (Huala and Ausubel, 1992), and provided an *Eco*RI site suitable for ligating in frame to *malE*. Downstream primers (Midland Genetics) were designed to overlap the 3' terminus of either the *nifA* coding region (5'-GCTCTAGACTCAGCCGGCAGCTTAGA-3') or the *dctD* coding region (5'-GCTCTAGACAGGTTAATCGTCGTCAC-3') and introduced an *Xba*I site at the extreme 3' end. Amplified, *Xba*I-digested *dctD-nifA* fragments were ligated to generate fragment dimers containing intact *Xba*I recognition sites. Dimers were digested with *Xba*I and *Eco*RI and ligated into the *Xba*I and *Eco*RI sites of pMAL-c2 (New England Biolabs). The products generated in this way encoded in-frame fusions of MBP to the central and C-terminal domains of each DctD-NifA chimera, as confirmed by sequence analysis of the *malE-dctD* junctions.

Purification of DctD and MBP-activator fusion proteins

DctD was expressed in *E. coli* cells, and purified by phosphocellulose chromatography of a redissolved 35% ammonium sulfate precipitate (D. Scholl, J.H. Lee, T.R. Hoover, and B.T. Nixon, unpublished). Cultures of *E. coli* strain TB1 carrying the pMal-derivative expression plasmids were grown in 500 ml of LB medium at 37°C with vigorous shaking. When cultures reached an O.D.₆₀₀ of ~0.5, isopropyl- β -D-thiogalactopyranoside (IPTG) was added to a final

concentration of 0.3 mM, and incubation was continued for 2.5 hr. Cells were harvested by centrifugation, and cell pellets were resuspended in 25 ml of column buffer (25 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) and stored overnight at -20°C. Frozen cells were thawed in cold water and sonicated with the standard tip of a Fisher Sonic Dismembrator (Model 300) for 10 blasts of 15 sec in an ice-water bath, and extracts were cleared by centrifugation at 10,000 x g for 30 min. Column chromatography was performed essentially according to the manufacturer's instruction (Protein Fusion and Purification System, New England Biolabs). Fractions containing the protein of interest were determined by SDS-polyacrylamide gel electrophoresis, performed as described (Ausubel et al., 1987), and pooled. Glycerol was added to the pooled fractions to a final concentration of 7.5%, and samples were frozen in 1 ml aliquots at -80°C at concentrations of ~1 mg/ml.

Protein-DNA binding assays using whole cell extracts

To assess DNA binding by the LacZ-DctD fusion proteins that carried mutant carboxy termini, a *Bam*HI-*Ava*II DNA fragment from pUCN corresponding to base pairs -62 to -225 relative to the transcription start site of *R. leguminosarum dctA* was radiolabelled with [α -³²P]-dATP and Klenow fragment of DNA Polymerase I. Labelled fragments and whole cell protein extracts of *R. meliloti* were prepared as described (Ledebur et al., 1990). Binding reactions included labelled DNA, protein extracts, and 2 μ g poly dI-dC (as nonspecific competitor DNA) in binding buffer A (25 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.9], 10 mM CaCl₂, 1 mM dithiothreitol, 10% glycerol) incubated for 15 min at 30°C. A 100-fold molar excess of unlabelled DNA was used as specific competitor DNA in some reactions. For gel shift assays, binding reactions were loaded onto native polyacrylamide gels (50 mM Tris, pH 8.5, 380 mM glycine, 2.5 mM EDTA, 4% acrylamide, 0.1% bisacrylamide, 2.5% glycerol) and electrophoresed at 4°C (200 V, approximately 5 hr). In filter binding assays, binding reactions were diluted with 1 ml binding buffer and immediately loaded onto nitrocellulose filters (Millipore HA, 0.45 μ) which had previously been boiled in binding buffer A for five minutes. Reactions were drawn through filters at 12.5 mm Hg with a RED-VAC vacuum pump (Hoefer Scientific Instruments) on a filter binding apparatus (Gelman Science). Radioactivity retained on the filter was counted in a Beckman LS 5801 liquid scintillation system in Ecoscint scintillation solution.

DNaseI and Gel Shift Assays using purified DctDA_(1-142,405-437)

DctDA_(1-142,405-437) was dialyzed to binding buffer (20 mM Tris-HCl, pH 7.9, 50 mM KCl, 5% glycerol), in which it retained full transcription activation activity (data not shown). Aliquots of buffer or buffer plus protein were added to end labeled DNA fragments (2,000 cpm, ~40 pM, for gel-shift analysis, or 10,000 cpm, ~200 pM, for DNaseI assays) in a final volume of 30 μ l. After 20 min incubation at 25°C, samples were subject to native PAGE (4.8% acrylamide, 0.17% bis-acrylamide) for gel-shift analysis (Tris-acetate, pH 8.5, 2 mM EDTA, 5% glycerol running buffer), or processed for DNaseI analysis. For the latter, samples were mixed with ~0.01 units of DNaseI in 10 μ l of DNaseI dilution buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM MgCl₂) for 1 min followed by phenol extraction, ethanol precipitation and denaturing PAGE (6% acrylamide, 0.3% bis-acrylamide). For both assays, a 210 base pair *Bam*H1-*Eco*R1 restriction fragment containing the *R. meliloti dctA* UAS was used. The -222 to -27 region of *dctA* was flanked by *Bam*H1 and *Eco*R1 sites and amplified by PCR, and then cloned into pUC13, making pUCD. The fragment was end-labeled by filling in the *Bam*H1 site using Klenow fragment, released from vector by subsequent digestion with *Eco*R1, and eluted after separation from vector DNA in a native 5% polyacrylamide gel. For nonspecific gel-shift assays, a 223 base pair *Hin*DIII-*Xho*I restriction fragment containing *Hin*DIII-*Acc*I of the Blue Script multiple-cloning sequence region adjacent to the *Bst*BI-*Xho*I portion of the *R. meliloti dctB-dctD* junction was labeled at the *Hin*DIII site, released from vector by *Xho*I digestion, and likewise purified. Gels were dried, and quantified using a Molecular Dynamics 445SI

phosphorimager and ImageQuaNT software. For the DNaseI data, total radioactivity in all lanes was normalized to that present in the control for which no DctDA_(1-142,405-437) was present. The amount of free DNA was then determined by measuring all radioactivity clearly below the signal for full length fragment, and plotting $1 - F/F_0$ as a function of protein concentration or buffer equivalent. Gel-shift data was plotted as $1 - F/F_0$ vs protein concentration without normalization of total radioactivity per lane. Figures were prepared using Adobe Photoshop 3.0, by transforming the 16-bit data files to 8-bit ones, and then linearly transforming the pixel values to make full use of the available range (0-255).

Determination of sequence specific binding to DNA containing the *nifH* UAS vs the *dctA* UAS

The *Bam*HI-*Ava*II DNA fragment of pUCN containing the *R. leguminosarum* *dctA* upstream activation sequence was used to assess DNA binding by the Φ (*malE'*-*dctD'*-*nifA*)(Hyb) fusion products. A DNA fragment containing the upstream activation sequence of the *R. meliloti* *nifH* promoter region (base pairs -68 to -166) was isolated from pVSP9 (Sundaresan et al., 1983) as an *Mlu*I-*Bgl*II fragment, and the *Mlu*I terminus blunted. The resulting fragment, smaller than the one described above containing the *dctA* UAS, was cloned into *Hin*DII-*Bam*HI doubly digested pUC19, making pRMHUAS. Binding reactions were incubated at 4°C for 1 hr in 30 μ l volumes and typically contained 1000 cpm of each labeled DNA fragment (1.5 fmol) and protein in binding buffer B (45 mM HEPES, pH 7.9, 50 mM KCl, 2 mM DTT, 20% glycerol). Purified protein was dialyzed against binding buffer B prior to the assay. The electrophoresis buffer contained 40 mM Tris-acetate, pH 8.0, 1 mM EDTA, and gels contained the same buffer plus 4% acrylamide and 0.1% bisacrylamide. Typical electrophoresis conditions were at 4°C for 1.5 hr at 35 mA constant current. To quantify the fraction of labeled DNA that did not bind to protein, radioactive emissions from appropriate regions of the gels were counted using a Betagen Betascope 603 Blot Analyzer. In initial experiments, we titrated protein over a three log-unit range against constant amounts of each probe, and thereby determined a more narrow window (one log unit) of protein concentration over which measurable binding to one or both of the probes occurred. Gel shift experiments for 9 binding reactions within this narrow concentration range were then performed three times to compare binding specificities.

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Table 1: Strains and Plasmids

Bacterial strain or plasmid	Relevant characteristics	Source or reference
<u><i>E. coli</i></u>		
JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)/ F' [traD36 proAB⁺ lacI^q lacZΔ M15]</i>	Yanisch-Peron et al., 1985
DH5α	<i>supE44 ΔlacU169 [Φ80dlacZΔM1] hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahan, 1983
MM294	<i>thiA endA1 hsdR17 supE44</i> ; Sm ^S	Hanahan, 1983
NCM789	<i>glnF213 lacZ::Tn5 himD::Cm^R rna19 zbe::Tn10</i>	Hoover et al, 1990
TB1 (#801A)	F- <i>ara Δ(lac-proAB) rpsL [Φ80dlac Δ(lacZ)M15] hsdR (r - m⁺)</i> , Sm ^r	Johnston et al., 1986
<u><i>R. meliloti</i></u>		
1021	Wild type, Sm ^r Meade et al., 1982	Jiang et al., 1989
6000	<i>dctD'-(Φ(RldctA'-lacZ)(Hyb))-tet'-dctD</i> , Sm ^r , Tc ^r	
4000	<i>nifH'-(Φ(RmnifH'-lacZ)(Hyb))-tet'-nifH</i> , <i>nifA::Tn5</i> , Sm ^r , Tc ^r , Nm ^r	Huala and Ausubel, 1989
<u>Plasmids and Phage</u>		
pUC13, pUC19	Ap ^r	Yanisch-Peron et al., 1985
M13mp18, M13mp19		Yanisch-Peron et al., 1985
pDctDA ₍₁₋₁₄₂₎	pUC13 with its <i>HincII-EcoRI</i> region replaced by a <i>StuI-EcoRI</i> fragment fusing <i>lacZ</i> codon 12 with <i>R. meliloti</i> <i>dctD</i> codon 143; Ap ^r	Gu et al., 1994
pDctDA _(1-142,405-460)	pDctDA ₍₁₋₁₄₂₎ in which the last 56 residues, I405 to T460, are replaced with SRGSP*	This study
pDctDA _(1-142,405-437)	pDctDA ₍₁₋₁₄₂₎ in which residues 405-437 have been removed by ligating DNA remaining after blunting an <i>NcoI</i> and <i>EcoRV</i> digest.	This study
pRKMAZ:+UAS	pRK290 with <i>EcoRI</i> fragment bearing a Nm ^r gene and a <i>Φ(RmdctA'-EclacZ)(Hyb)</i> fusion gene with UAS	Ledebur and Nixon, 1992

pRKMAZ:-UAS	pRK290 with EcoRI fragment bearing a Nm ^r gene and a $\Phi(RmdctA'$ - $EclacZ$)(Hyb) fusion gene without a UAS	Ledebur and Nixon, 1992
pUCN	pUC13 with <i>R. leguminosarum</i> <i>dctA</i> promoter fragment for isolating <i>Bam</i> HI- <i>Av</i> II fragment containing the UAS; previously called pHLRLBA	Ledebur et al., 1990
pUCD	pUC13 with <i>R. meliloti</i> <i>dctA</i> promoter region base pairs -27 to -222 flanked by <i>Eco</i> R1 and <i>Bam</i> H1 respectively	This study
pBstB.1	pBS+ with the <i>Bst</i> B1- <i>Eco</i> R1 fragment of the <i>R. meliloti</i> <i>dctBD</i> region cloned at <i>Acc</i> I and <i>Eco</i> R1 vector sites.	Lee and Hoover, 1995.
pUCX	pUC13 bearing a fragment of sequence 5'-CTAGTCTAGACTAG-3' inserted into the <i>Sma</i> I site	This study
pDctDA(1-142)/V304...T460	pDctDA ₍₁₋₁₄₂₎ with second indicated residue fused to -SRGSP*, -DSRGSP* or -GASQCHENLSLRQRQSR*	This study
pLA114	pUC19Cm ^r containing <i>Bam</i> HI- <i>Hin</i> DIII fragment of the <i>R. leguminosarum</i> <i>dct</i> locus	Jiang et al., 1989
pBG1	pUC13 with <i>Bam</i> HI- <i>Hin</i> DIII fragment of pLA114	Jiang et al., 1989
pNifA3	<i>Nr</i> II fragment encoding the C-terminus of <i>R. meliloti</i> NifA cloned in pUC13	This study
pBS13	pUC13 bearing the <i>Bam</i> HI- <i>Sa</i> II fragment of pLA114	This study
pRK290	IncP Tc ^r	Ditta et al., 1980
pRK2013	<i>rep</i> (ColE1) Mob ⁺ Tra ⁺ Km ^r	Ditta et al., 1980
pBGΩ	pRK290 with Ω-fragment replacing <i>tet</i> gene, replicates in <i>E. coli</i> and <i>R. meliloti</i> , mobilizable into <i>R. meliloti</i> , Sp ^r , Gm ^r	Jiang et al., 1989
pBGΩ-pBG1	pBGΩ joined at <i>Hin</i> DIII site to pBG1	Jiang et al., 1989
pBGΩ- pDctDA ₍₁₋₁₄₂₎ ...	pBGΩ made biphasic with pDctDA ₍₁₋₁₄₂₎ (etc) by joining at <i>Eco</i> RI sites, Sp ^r , Gm ^r	This study
M13-E96...	M13mp19 containing <i>Sa</i> II fragments encoding the fusion of <i>R. leguminosarum</i> DctD and <i>R. meliloti</i> NifA residues as indicated in Figure 6	This study
pE96...	pBS13 bearing the <i>Sa</i> II fragments encoding the named DctD-NifA fusions as described in Figure 6	This study
pRMHUAS	base pairs -68 to -166 of the <i>R. meliloti</i> <i>nifH</i> promoter region (<i>Mlu</i> I(blunted)- <i>Bgl</i> II fragment) cloned in the <i>Hin</i> DII and <i>Bam</i> HI sites of pUC19	This study

pMal-c2	Maltose-binding protein fusion vector	New England Biolabs
pMal-E96...	pMal-c2 containing PCR-amplified fragments fusing MBP to residue Q140 of DctD-NifA fusions as described in Figure 6	This study

FIGURE LEGENDS

Figure 1. *In vitro* binding activity of whole cell extracts containing DctDA₍₁₋₁₄₂₎ and DctDA_(1-142,405-460). (A) Samples of *E. coli* JM109 cells containing pDctDA₍₁₋₁₄₂₎ or pDctDA_(1-142,405-460) that were grown in LB medium (O.D.₆₀₀ = 0.8) were applied to an SDS-PAGE gel. Bands representing the respective proteins are labeled 1 and 2. Lysates made from (B) *E. coli* JM109 cultures carrying pDctDA₍₁₋₁₄₂₎, pDctDA_(1-142,405-460), or no plasmid, or (C) *R. meliloti* Rm6000 cultures carrying pBGΩ- pDctDA₍₁₋₁₄₂₎, pBGΩ- pDctDA_(1-142,405-460), or no plasmid were incubated with approximately 1 ng of ³²P-radiolabelled DNA fragment containing the *R. leguminosarum* *dctA* upstream region with 2 μg of poly dI-dC as nonspecific competitor and subject to gel-shift analysis. A 100-fold excess of unlabelled, specific DNA was included in the indicated binding reactions. Arrows identify the DctDA₍₁₋₁₄₂₎ / DNA and DctD / DNA complexes, that latter being provided by extracts of the wild type *R. meliloti* strain Rm1021 instead of the *dctD* insertion strain Rm6000.

Figure 2. Sequence alignment of *R. meliloti* DctD, *R. leguminosarum* DctD, and the C-terminal portion of *R. meliloti* NifA. Indicated are the boundaries between (A) the N-terminal and central domains (residues P144 to P150), and (B) the central and C-terminal domains of DctD (*Rm* residues G381 to A384); (C) the residues deleted from the C-terminal domain in *Rm* DctDA_(1-142,405-437); and the putative HtH DNA-binding motifs of the DctD and NifA proteins.

Figure 3. Non-specific DNA binding activity of purified DctDA_(1-142,405-437). (A) DNaseI digestion of DNA containing the *R. meliloti* *dctA* UAS was interfered with by 30 nM DctDA₍₁₋₁₄₂₎ (Control, showing specific binding), or increasing amounts of buffer or buffer containing 200 pM to 5 μM purified DctDA_(1-142,405-437). (B) Gel-shift of 3 nM to 10 μM DctDA_(1-142,405-437) plus DNA containing or lacking the *R. meliloti* *dctA* UAS. (C) Quantitation of results: interference with DNaseI digestion (triangles; open for buffer, solid for DctDA_(1-142,405-437)); gel-mobility shift (circles; open for +UAS, solid for -UAS).

Figure 4. *In vitro* transcriptional activation by a mutant form of DctD that lacks the HTH motif. DctDA₍₁₋₁₄₂₎ (circles) and DctDA_(1-142,405-437) (triangles) were compared in a coupled transcription / translation assay. The ability to activate transcription from an *R. meliloti* $\Phi(dctA'$ -*lacZ*) reporter gene containing (solid symbols) or lacking (open symbols) the *dctA* UAS was determined. Reported results are the average of two independent experiments, and a relative activity of 1.0 is equal to 54 nmoles of ONPG hydrolyzed per minute for the entire 50 μl reaction mixture.

Figure 5. Delineation of the boundary between C-terminal and central domains of DctD. A battery of 3'-deletions of the coding region of *dctDA*₍₁₋₁₄₂₎ was generated with *Bal31* nuclease, and joined with DNA encoding the indicated C-terminal residues. The doubly deleted mutants were expressed in *E. coli* to determine their ability to activate transcription of +/- UAS $\Phi(dctA'$ -*lacZ*) reporter genes. Background β-galactosidase activity, supported by the reporter gene alone, was less than 10 units.

Figure 6. Activity of chimeric DctD-NifA proteins on *dctA* and *nifH* target promoters. The thirteen *R. leguminosarum* DctD-*R. meliloti* NifA mutant proteins are displayed at left, along with the position of fusion. Black balls represent HTH DNA-binding motifs. Activities of the chimeric proteins were assessed in glucose and succinate minimal media in both Rm6000 and Rm4000. nd: not done; ng: cells failed to grow in that medium.

Figure 7. Carbon source shift experiments with active chimeras E96 and D20. *Rm4000* cells containing pBGΩ-pD20 (diamonds), pBGΩ-pE96 (squares), or the vector pBGΩ (circles) were grown in minimal-glucose media for more than 24 hours until mid-log density was obtained. Cells were harvested by centrifugation, washed once in minimal medium lacking a carbon source, and resuspended in minimal medium containing glucose (solid symbols) or succinate (open symbols) as the sole carbon source. β-galactosidase activity resulting from the $\Phi(nifH'$ - $lacZ)$ (Hyb) reporter gene was determined at the indicated times.

Figure 8. A) Coomassie Blue-stained SDS Polyacrylamide (7.5%) gel analysis of MBP-DctD-NifA fusion protein samples used in quantitative gel mobility shift assays. 5 μl of the indicated purified protein samples were resuspended in SDS-loading buffer. Lane 1: whole cell lysate of the *E. coli* TB1 host carrying pMAL-E96, with no IPTG added; lane 9: purified *R. meliloti* DctD. B) Competition gel-shift for MBP-DctD-NifA chimera C17. Aliquots of 0.02 to 10 μl of purified MBP-DctD-NifA protein C17 were added to an equimolar mix of labeled DNA containing the *R. meliloti nifH* (120 bp) or the *R. leguminosarum dctA* (200 bp) upstream regions in binding reactions and then electrophoresed through native polyacrylamide gels.

Figure 9. *In vitro*, UAS-Specific DNA Binding by the MBP-DctD-NifA fusion proteins. Competition gel shift assays as described in the legend to Figure 8 were carried out for the indicated MBP-DctD-NifA chimeric proteins and DNA fragments containing the UAS of *dctA* (closed symbols) or *nifH* (open symbols). Results were quantified by phosphorimager analysis, and plotted as the fraction of free DNA versus the amount of protein added.

Gu et al., Figure 2

Rm DctD	1	MSAAPSVFLIDDDRDLRKAMQQTLELAGFTVSSFASATEALAGLSADFAG	50
Rl DctD	1	MDTLMPVALIDDDKDLRRATAQTLELAGFSVSAYDGAKAALADLPADFAG	50
Rm DctD	51	IVISDIRMPGMDGLALFRKILALDPDLPMILVTGHGDIPMAVQAIQDGAY	100
Rl DctD	51	PVVTDIRMPEIDGLQLFATLQGMVDLPVILMTGHGDIPMAVQAIQDGAY	100
Rm DctD	101	DFIAKPFAADRLVQSARRAEKRRRLVMENRSLRRAEAAASEGLPLIGQTP	150
Rl DctD	101	DFIAKPFAADRLVQSVRRASEKRRRLVLENRMLRKAEDAQENLPLIGQTP	150
Rm DctD	151	VMERLRQTLKHIADTDVDVLVAGETGSGKEVVATLLHQWSRRRTGNFVAL	200
Rl DctD	151	VMENLRNILRHIADTDVDVLVAGETGSGKEVVAQILHQWSHRRKGNFVAL	200
Rm DctD	201	NCGALPETVIESELFGEHPGAFTGAVKKRIGRIEHASGGTLFLDEIEAMP	250
Rl DctD	201	NCGALPETVIESELFGERGAFTGAQKRRTGRIEHASGGTLFLDEIESMP	250
Rm DctD	251	PATQVKMLRVLEAREITPLGTNLTRPVDIRVVAAAKVDLGDPAAARGDFRE	300
Rl DctD	251	AATQVKMLRVLEMREITPLGTNEVRPVNLRVVAAAKIDLGDPAVRGDFRE	300
Rm DctD	301	DLYYRLNVVTLSSIPPLRERRDDIPLLFSSHFLARASERFGREVPISAAMR	350
Rl DctD	301	DLYYRLNVVTISIPPLRERRDDIPLLFSHFAARAAERFRDVPPLSPDVR	350
Rm DctD	351	AYLATHSWPGNVRELSHFAERVALGVEGNLGVPAAPASSGATLPERLE -	399
Rl DctD	351	RHLASHTWPGNVRELSHYAERVVLGVEG.GGAAAVPPQPTGATLPERLE -	398
Rm Nifa	405	DHLSKCKFPGNVRELENCVVRTATLARSKITITSSDFACQTDQCFSSRLWK	454
Rm DctD		-----	399
Rl DctD		-----	398
Rm Nifa	455	GVHCSHGHIIDAPAGTTPLL GAPANDVPPKEPGSAGVASN	495
Rm DctD	400	RYEADILKQALTAHCGDVKETLQALGIPRKTFYDKLQRHGINRADYVERAG	450
Rl DctD	399	RYEAEIIRDITLSANDGDVVRTIEALGIPRKTFYDKLQRHGINRGGYSSRK*	448
Rm Nifa	496	LIERDRLISALEEAGWNQAKAARILEKTPRQVGYALRRHGVDVRKL*	541
		helix -T- helix	
Rm DctD	451	PGRPNAISK T*	460