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Identification of Amino Acid Residues in the Carboxyl Terminus Required for Malonate-Responsive Transcriptional Regulation of MatR in *Rhizobium leguminosarum* bv. *trifolii*

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MatR in *Rhizobium trifolii* is a malonate-responsive transcription factor that regulates the expression of genes, *mat*ABC, enabling decarboxylation of malonyl-CoA into acetyl-CoA, synthesis of malonyl-CoA from malonate and CoA, and malonate transport. According to an analysis of the amino acid sequence homology, MatR belongs to the GntR family. The proteins of this family have two-domain folds, the N-terminal helix-turn-helix DNA-binding domain and the C-terminal ligand-binding domain. In order to find the malonate binding site and amino acid residues that interact with RNA polymerase, a site-directed mutagenesis was performed. Analysis of the mutant MatR suggests that Arg-160 might be involved in malonate binding, whereas Arg-102 and Arg-174 are critical for the repression activity by interacting with RNA polymerase.

Keywords: EMSA, MatR, *Rhizobium leguminosarum* bv. *trifolii*, Site-directed mutagenesis, Transcription factor.

Introduction

The transcriptional regulator, MatR, was identified and characterized in *Rhizobium leguminosarum* bv. *trifolii,* which is symbiotic with clover. By interacting with the upstream operator region of *matABC*, MatR down-regulated the transcription of the *matABC* operon that encodes enzymes for malonate catabolism (Lee *at al.*, 2000). The binding sites of the MatR protein, identified by an electrophoretic mobility shift and DNase I protection assays, were very similar to the sequences of the *B. subtilis gnt* operator site (ATACTTGTA/TACAAGTAT) (Fujita *et al.*, 1986, 1989). Furthermore, the deduced amino acid sequence of MatR showed similarity with that of GntR from *B. subtilis* in the N-terminal region that encodes a helix-turn-helix DNA-binding domain, but not with that of the C-terminal region, specifying a gluconate-binding region. The *gnt* operon of *B. subtilis* is involved in gluconate catabolism. The GntR protein represses *gnt* promoter-activated mRNA synthesis by binding to the *gnt* operator, and this binding is suppressed by gluconate. In spite of the amino acid sequence similarities of the MatR and GntR proteins, MatR bound its operator sites with increasing affinity in the presence of malonate, a positive transcriptional inducer for the *mat* operon.

Recently, another GntR-like transcriptional regulator, MdcY, was reported to down-regulate the transcription of the *mdc* operon with malonate as a positive inducer in *Acinetobacter calcoaceticus* (Koo *at al.*, 2000). The *mdc* operon encodes malonate decarboxylase, which converts malonate to acetate. The MdcY protein is homologous to MatR in the amino acid sequence by 65% and has a similar operator site (ATTGTA/TACAAT). However, MdcY binding to its operator was unaffected by malonate. This indicates that MdcY and MatR carry out the transcriptional repression by a mechanism different than GntR.

In the present study, we used site-directed mutagenesis to examine the presumed C-terminal malonate-binding domain of MatR. A sequence comparison with MdcY protein detected a number of Arg-residues that are conserved throughout the C-terminal domain. Here, Arg-68, Arg-102, Arg-160, and Arg-174 of MatR were selected for site-directed mutagenesis. Each of the mutant proteins were purified and characterized using a DNA-protein gel shift and reporter gene assays. The results reported here suggest that Arg-160 might be involved in malonate binding, whereas Arg-102 and Arg-174 are critical for the repression activity by interacting with RNA polymerase.

Materials and Methods

Strains and plasmids The *Escherichia coli* strain BL21 (DE3) was used to over-express the recombinant protein. For the reporter

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gene assay, *E. coli* NovaBlue (DE3) from Novagen (Madison, USA) was used as a host strain. The pET22b vector for over-expression was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Antibiotics were used at the following concentrations (in $\mu g \cdot ml^{-1}$): for *E. coli*, ampicillin at 100, chloramphenicol at 25. All manipulations with recombinant DNA were carried out according to standard procedures.

Enzymes and chemicals Restriction enzymes and the *Klenow* fragment were purchased from Boehringer Mannheim (Mannheim, Germany). Cloned *Pfu* polymerase was purchased from Stratagene (La Jolla, USA). The ^{32}P-labeled nucleotides were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The synthetic oligomers were purchased from the Bioneer Co., Korea.

Site-directed mutagenesis Appropriate pairs of mutagenic primers (Table 1) were synthesized and used to generate the mutated MatR by PCR, according to the method of QuickChange site-directed mutagenesis from Stratagene (La Jolla, USA). Here, the pMATR was used as the template for the *Pfu* DNA polymerase (Stratagene) (Lee *at al.*, 2000). After PCR, the wild-type parental plasmid that remained in the PCR product was selectively digested by the *Dpn*I restriction enzyme. The resultant mixture was used to transform the chemically competent *E. coli* BL21 (DE3). The desired mutants were sequenced to identify each mutation and confirm their fidelity.

Expression and purification of wild type and mutant MatR The expression and purification of wild type and mutant enzymes were carried out using His-bind resin, according to the manufacturer's instructions (Novagen, WI, USA).

Electrophoresis mobility shift assay (EMSA) To investigate the binding of wild type and mutant MatR to its operator site, a 295-bp DNA fragment that contained an upstream region of *matA* gene was used as a probe. It was end-labeled with [α-^{32}P]dATP using the *Klenow* fragment, and used for EMSA by the method described previously (Kwon *et al.*, 1999).

Luciferase assay Plasmid pGL3-Basic (Promega, Madison, USA) containing the firefly luciferase gene, was used as the reporter system. The plasmid, pMAT-L, was co-transformed with the pMATR wild type and mutant plasmids into *E. coli* NovaBlue (DE3) (Novagen, Madison, USA) (Lee *at al.*, 2000). The bacteria for the luciferase assay were grown at 37°C in M9-glucose minimal media. At $OD_{600} = 0.5$, malonate was added to the culture medium for the final concentration of 4 mM. The culture was then incubated at 20°C. After a 20-h incubation, samples were taken and assayed directly for luminescence in the Turner Designs Model TD-20/20 Luminometer, according to the manufacturer's instructions (Promega, Madison, USA).

Fig. 1. Sequence alignment of the amino acid sequences of *R. leguminosarum* bv. *trifolii* MatR with *A. calcoaceticus* MdcY. The predicted secondary α-helix structure of MatR is indicated by gray bars.

Results

Selection of putative ligand-binding residues The amino acid sequence of MatR identified this protein as a member of the GntR transcription factor family (Lee *et al.*, 2000). The sequence homology of the N-terminal helix-turn-helix DNA binding motif is representative of the GntR family (Haydon and Guest, 1991). However, the C-terminal region, specifying a putative ligand-binding region, shows little sequence homology among the GntR family members. Interestingly, there is 49% identity and 65% homology between amino acid sequences of MatR and MdcY, both of which belong to the GntR family, and are related to the malonate metabolism (Fig.

Table 1. Site-directed matR gene mutations and corresponding mutagenic oligonucleotides

MatR gene allele	Mutagenic primer set[a]
R68G	CGAAATCCGACCGGGCAGAGGCGCTATTGTCA
	ATAGCGCCTCTGCCCGGTCGGATTTCGATGAG
R102G	GCTCGCCGCGCGGGGGCTGGACAAGACCTCTC
	GTCTTGTCCAGCCCCCGCGCGGCGAGCGACCC
R160G	GCAATTGCATCGGGGCCTGCGGCCAGATCGCC
	TCTGGCCGCAGGCCCCGATGCAATTGCAGACA
R174G	GCGCGTGCGCAACGGCCTGTCGACGTCCTTCT
	GACGTCGACAGGCCGTTGCGCACGCGCAGTTG

[a]Nucleotide sequences that correspond to the mutated amino acid residues are underlined.

1). Throughout the whole amino acid sequence, 12 Arg-residues are conserved in MatR and MdcY protein. Considering this, the arginine residue was expected to be a candidate for the malonate-binding amino acid residue of MatR. Therefore, Arg-68, Arg-102, Arg-160, and Arg-174 were selected for mutagenesis.

Construction and purification of mutant enzymes All of the mutants were constructed using the PCR method and expressed in the same cell line that was used to express the wild-type gene: *E. coli* BL21 (DE3). All of the mutants were purified from His-bind resin under the same conditions as the purification of the wild-type MatR (Fig. 2). From 200 ml of the culture that was induced with 1 mM IPTG, the yields obtained of the purified enzymes were: 0.6 mg of wild type, 0.8 mg of R68G, 0. 5mg of R102G, 0. 2mg of R160G, and 0.5 mg of R174G MatR protein.

Binding properties of wild-type and mutant MatR Binding of the wild type and mutant MatR protein to the operator site of the *mat* operon was analyzed by EMSA using the ^{32}P-labeled DNA fragment that contained the region between –129 and +26. As shown in Fig. 3, R102G, R160G, and R174G MatR protein could form the DNA-protein complex. In the presence of malonate, R102G, R174G, but not R160G mutant MatR seemed to form a more retarded DNA-protein complex. This suggests Arg-160 as a malonate binding residue. However, R68G did not form the DNA-protein complex, which indicates that the mutagenesis of Arg-68 led to the conformational change in the DNA-binding helix-turn-helix domain.

Transcriptional regulation of wild type and mutant MatR on the *mat* promoter A derivative of pBBR1MCS, pMAT-L, was used to investigate which residue of the MatR protein is involved in the transcriptional repression and malonate binding. *E. coli,* carrying pMAT-L and pET22b, was used as a control (LU 828-1130). *E. coli,* which expressed wild-type MatR, showed a 80-fold decrease of luciferase activity (LU 16) in the absence of malonate. It showed an increase in luciferase activity (LU 80) in the presence of malonate. *E. coli,* which expressed R68G, showed similar luciferase activity of the wild-type MatR (LU 1146-1360). This is in accordance with the result of EMSA. *E. coli,* which expressed R102G, showed a super-activation phenotype (LU 10470-11885). It seems that this mutant has some character that led to the effective access of RNA polymerase to the promoter. *E. coli,* which expressed R160G, showed a decrease in transcriptional repression (LU 406-375) in the presence or absence of malonate. Also, EMSA using this mutant protein had little difference in the presence or absence of malonate. *E. coli,* which expressed R174G, had the similar luciferase activity (LU 964-1128) compared with the control, but EMSA with R174G mutant showed a DNA-binding character.

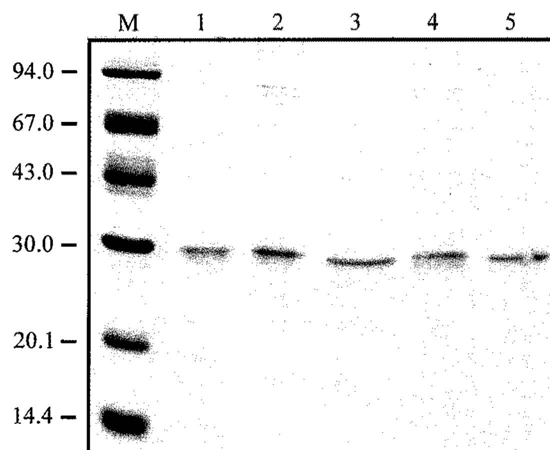

Fig. 2. SDS-PAGE of the wild-type and mutant MatR that are purified from the extracts of each *E. coli* transformant. Lane 1; wild-type, lane 2; R68G, lane 3; R102G, lane 4; R160G, lane 5; R174G.

Discussion

According to the sequence analysis, MatR from *Rhizobium leguminosarum* bv. *trifolii* belongs to the GntR family, together with MdcY from *Acinetobacter calcoaceticus* (Koo at al., 2000). However, structures of many of the GntR family proteins cannot be directly superimposed because of their overall shape, which is probably related to their diverse functions. A high sequence homology of MatR and MdcY implies that conserved regions of the two malonate-responsive proteins contain a novel motif, whose function is to interact with malonate.

In EMSA and luciferase assays, using mutant MatR proteins, R160G showed little difference in the DNA-binding character and luciferase activity in the presence or absence of malonate. This implies that Arg-160 may contribute to malonate binding of MatR. R174G and R102G could bind the operator site in spite of the reduced repressor activity. In the previous report, MatR was suggested to carry out the repressor activity by inhibiting the promoter clearance of RNA polymerase (Lee *et al.*, 2000). R174G and R102G mutant proteins seem to be unable to bind RNA polymerase tightly enough to inhibit the promoter clearance, though these proteins still bound the operator site. Therefore, Arg-174 and Arg-102 might be related to the interaction with RNA polymerase. Also, Arg-68, which belongs to the N-terminal DNA-binding domain, was mutated. It could not bind the DNA and showed no repressor activity.

Recently, the crystal structure of FadR, a fatty acid-responsive GntR-like transcription factor from *E. coli,* was determined (van Aalten *et al.*, 2000). The structure reveals a two-domain fold, DNA-binding and acyl-CoA binding sites that are located in the N-terminal and C-terminal domains, respectively. The N-terminal domain contains a winged helix-

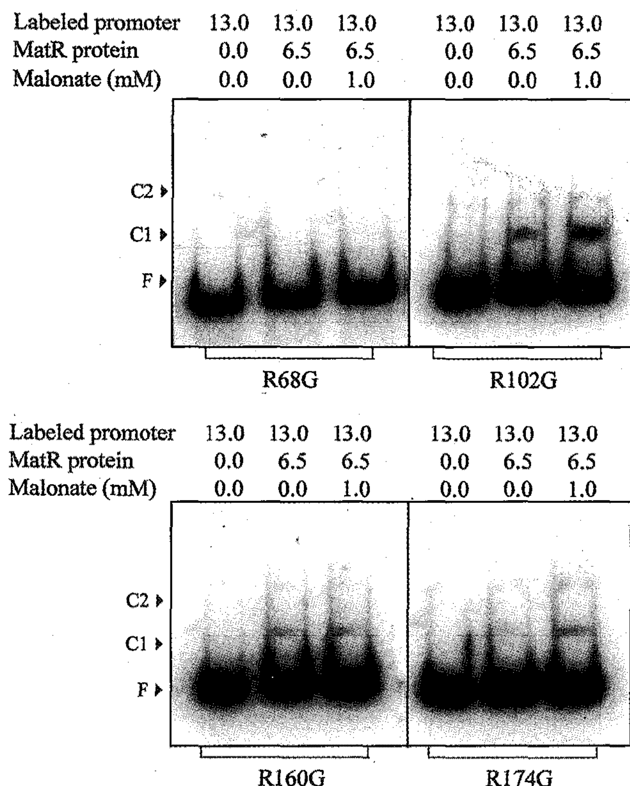

Fig. 3. Electrophoretic mobility shift assays of wild-type and mutant MatR binding to operator site. Radiolabeled 295-bp mat operator/promoter was incubated with wild-type and mutant MatR in the presence or absence of malonate. The concentration of the labeled probe and the MatR are in fM, indicated at the top. F, C1 and C2 indicate bands corresponding to free, DNA-MatR complex 1 and DNA-MatR complex 2, respectively. Malonate was used in 1 mM concentration.

turn-helix prokaryotic DNA-binding fold. The C-terminal domain has a novel fold, consisting of a seven-helical bundle with a crossover topology. Careful analysis of the structure, together with the mutational and biochemical data, revealed a putative hydrophobic acyl-CoA-binding site that is buried in the core of the seven-helical bundle. In the detailed FadR structure, the acyl-CoA binding motif is located in the cavity that is formed with the following residues: Leu-102, Arg-105, Ala-142, Asp-145, Phe-149, Tyr-172, Ile-175, Gly-176, Tyr-179, Phe-180, Tyr-215, Ser-219, Gly-229, and Trp-223.

According to the secondary structure prediction, MatR has seven-helixes in the C-terminal domain, implying a similar helical topology of FadR. Each of the Arg residues was located on helical domains. They corresponded to the acyl-CoA binding motif of FadR. The presumed malonate binding motif of MatR, which is different from the hydrophobic acyl-CoA binding motif of FadR, seems to contain more charged residues. This is probably due to the property of malonate. For a detailed view of the malonate binding motif, further study using genetic or biochemical experiments with structure studies might be required. However, this study provides

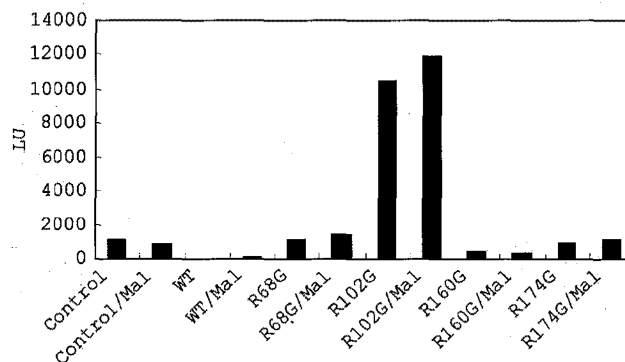

Fig. 4. Expression of pMAT-L, pMAT-L/wild-type MatR, and pMAT-L/mutant MatR in the presence or absence of malonate. Transcriptional responses in luxes(LU) per OD600 unit (A600) of *E. coli* NovaBlue (DE3) that contained pMAT-L, pMAT-L/wild-type MatR, and pMAT-L/mutant MatR were measured in the presence or absence of malonate. Data are averaged from at least three independent aerobic cultures that were grown in a YM medium with or without 4 mM malonate.

evidence that the GntR family members have some similar structural scaffold, not only for N-terminal helix-turn-helix DNA-binding domains, but also for the C-terminal effector molecule-binding domains.

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