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ChpR Is a Chlorpyrifos-Responsive Transcription Regulator in *Sinorhizobium meliloti*

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Key Words
Pesticide · Biosensor · Transcription activator · Insecticide responsive promoter

Abstract
The broad-spectrum organophosphate insecticide chlorpyrifos (CPF)-inducible locus, *chpAB,* was identified on the endogenous plasmid pSymB in *Sinorhizobium meliloti*. The *S. meliloti chpA* promoter was highly induced by CPF and was induced at much lower levels by diazinon and ethion. Transcription of *chpA* was dependent on *chpR,* a CadC family transcriptional regulator located upstream of, and divergently transcribed from, *chpAB*. ChpR was able to mediate the CPF-inducible expression of the *S. meliloti chpA* promoter in *Escherichia coli* through direct interaction with the *chpAB* promoter. The *chpR-chpA* intergenic regions of several bacterial *chpRAB* operons were aligned and a putative ChpR-binding sequence was proposed. Both the ChpR transcription factor and *chpA* promoter constitute a good candidate system for genetic-based biosensor development.

Chlorpyrifos (CPF, *O*,*O*-diethyl-*O*-3,5,6-trichloro-2-pyridyl phosphorothioate) is a broad-spectrum organophosphate insecticide. Its wide use raises environmental concerns because it is particularly toxic to birds as well as aquatic and marine fish and invertebrates [Barron and Woodburn, 1995]. Analytical methods for the detection of CPF residues are generally based on chromatographic separation and are frequently time consuming and expensive. High-performance liquid chromatography with mass spectrometry [Curwin et al., 2009], gas chromatography with electron capture detection, nitrogen-phosphorus detection [Jaggi et al., 2001], micellar electrokinetic chromatography [Klotz et al., 2001], and high-performance liquid chromatography with fluorescence detection [Fillion et al., 2000] are all methods that have been proposed for use in the detection of CPF.

A potentially cheaper, yet specific and sensitive, technique for the detection of pesticides is the use of biosensors based on the genetic systems of microorganisms that have been engineered to recognize and produce a measurable signal in response to exposure to specific chemicals. Upon exposure to a pesticide, the regulator/promoter sequence triggers the expression of the reporter gene and leads to a detectable signal. There are relatively few

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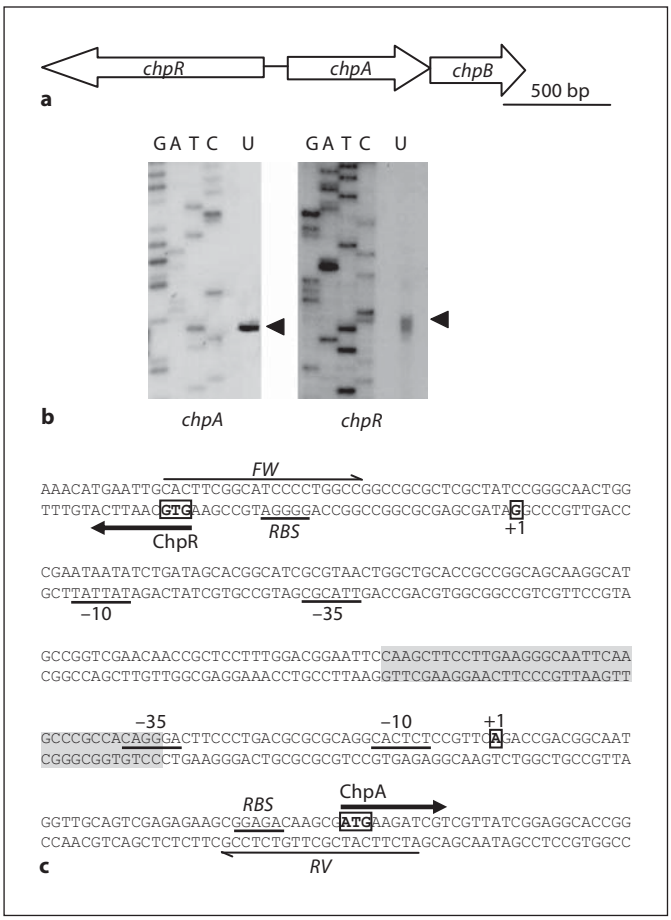

Fig. 1. Organization of genes involved in CPF response and their promoter analyses. **a** Map of *chpRAB* operon. Arrows indicate direction of transcription. **b** Localization of the *chpA* and *chpR* transcription start sites. The first four lanes show sequencing ladders (labeled GATC). The extension products (labeled U) are indicated by arrowheads. **c** Sequence of the *chpR-chpA* intergenic region. The transcription start sites of *chpR* and *chpA* are denoted by +1. Promoter sequences (–10 and –35) and ribosome-binding sites (RBS) are underlined. The translation start sites are in bold and box. The putative ChpR-binding site is shaded in gray. Primer FW and RV positions are indicated.

reports on genetically engineered whole-cell sensing systems to detect pesticides, i.e. use of the *Ralstonia eutropha tfdDII* promoter to detect 2,4-dichlorophenoxyacetic acid and 2,4-dichlorophenol [Hay et al., 2000] and an uncharacterized promoter from *Synechocystis* that is responsive to herbicides [Shao et al., 2002]. One main requirement of this approach is the isolation of a promoter that responds specifically to the molecule to be monitored.

The ultimate aim of this work is to construct a genetic-based bacterial biosensor composed of a reporter protein fused to a CPF-inducible promoter that is under the control of a CPF-responsive transcription factor [Belkin, 2003]. The first step toward this goal was to identify bacterial gene promoters that show increased expression in the presence of CPF, as well as identify the regulator(s) that control them. In this study, *Sinorhizobium meliloti* was selected as a likely source of CPF-inducible genes since it is an agriculturally important soil microbe that is found in environments likely to be exposed to pesticides, especially CPF.

To our knowledge, there is no information available concerning the development of biosensor systems specific to CPF. Here we report the identification of a CPF-inducible promoter controlling the transcription of an *S. meliloti* operon encoding ORFs, SMb20700 and SMb20701. CPF-inducible expression of this promoter is mediated by a novel organophosphate-responsive transcription activator encoded by the closely linked ORF SMb20702.

Results and Discussion

Cloning of the CPF-Inducible Operon

A randomly integrated library of Tn5 mutants in *S. meliloti* Rm1021 was constructed using pUTTn5lacZ1-Gm that carries Tn5 containing a promoterless *lacZ* and a gentamicin (Gm) resistance cassette [de Lorenzo et al., 1990]. Integration of Tn5lacZ1Gm into DNA can result in the fusion of the promoterless *lacZ* to various cellular promoters. Strains in which Tn5lacZ1 had inserted into CPF-inducible loci were identified on Luria-Bertani medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LB/MC medium) containing Gm, X-gal and CPF. One dark blue colony, designated chp (CPF inducible), that expressed *lacZ* only in the presence of CPF was selected for further study.

Subcloning and sequencing of the Tn5-tagged locus revealed that the site of the Tn5 insertion was within the ORF, SMb20700 that is located on pSymB, a large (1,683 kb) endogenous megaplasmid of *S. meliloti* Rm1021 [Finan et al., 2001]. SMb20700, denoted *chpB*, is predicted to encode a protein of unknown function that is 137 amino acids in length with a molecular mass of 14,221 Da. Blast searches against the protein database indicate that ChpB is a member of the cupin superfamily of proteins [Dunwell et al., 2004]. Two additional ORFs upstream of *chpB* were also identified (fig. 1a). ORF SMb20701, designated

chpA, is oriented in the same direction and is separated from *chpB* by 78 bps. *chpA* is predicted to encode a 250-amino-acid protein of molecular mass 26,155 Da. The deduced amino acid sequence of ChpA is similar to nucleotide diphosphate sugar epimerases and an NmrA family protein [Stammers et al., 2001]; however, its true function is unknown. 180 bps upstream and divergently transcribed from *chpA* is an ORF (SMb20702), designated *chpR*, which is predicted to encode a 554-amino-acid protein of 60,005 Da that shares 76% identity with a CadC family transcriptional regulator of *Ralstonia eutropha* H16 (YP_840943) [Pohlmann et al., 2006]. The ChpR N-terminal domain spanning residues 25–104 is similar to the CadC DNA-binding winged helix-turn-helix domain. The C-terminal half of the ChpR contains a tetratricopeptide repeat domain, which is known to be involved in a variety of functions, such as protein-protein interaction, chaperone activity, transcription and protein transport [Das et al., 1998]. Currently, no information is available concerning CadC type transcription factors involved in insecticide-inducible responses.

CPF-Inducible Transcription of chpA *and* chpB

Northern blots of total RNA extracted from *S. meliloti* wild-type and *chpR* insertion-mutant strains, grown in the presence and absence of 100 μM CPF, were hybridized with ^{32}P-radiolabeled probes specific for *chpA* and *chpB* as described previously [Loprasert et al., 2007]. Hybridization with either *chpA*- or *chpB*-specific probes detected a 1.4-kb mRNA that was present only in cultures grown in media containing 100 μM CPF. This indicates that *chpA* and *chpB* are cotranscribed since the 1.4-kb message detected by Northern blot analysis is close to the minimum message size of 1,291 nucleotides that is necessary to encode both genes (fig. 2a).

In order to more clearly define the promoter regions of *chpA* and *chpR*, primer extension mapping was performed using total RNA extracted from *S. meliloti* grown in the presence of 100 μM CPF. The transcription of *chpA* was found to begin at an A residue 44 upstream from the *chpA* translation start site, while *chpR* is transcribed from a G residue 33 bps upstream of the *chpR* translation start (fig. 1b, c). The regions upstream of each transcription start site contained sequences with some similarity to *Escherichia coli* consensus –10 (TATAAT) and –35 (TTGACA) promoter elements (fig. 1c). Similarity to the *S. meliloti* –35 and –10 consensus promoter, CTTGAC-N17-CTATAT identified by MacLellan et al. [2006] is limited to the putative –10 region of the *chpR* promoter. The –35 elements of both the *chpR* and *chpA* promoters

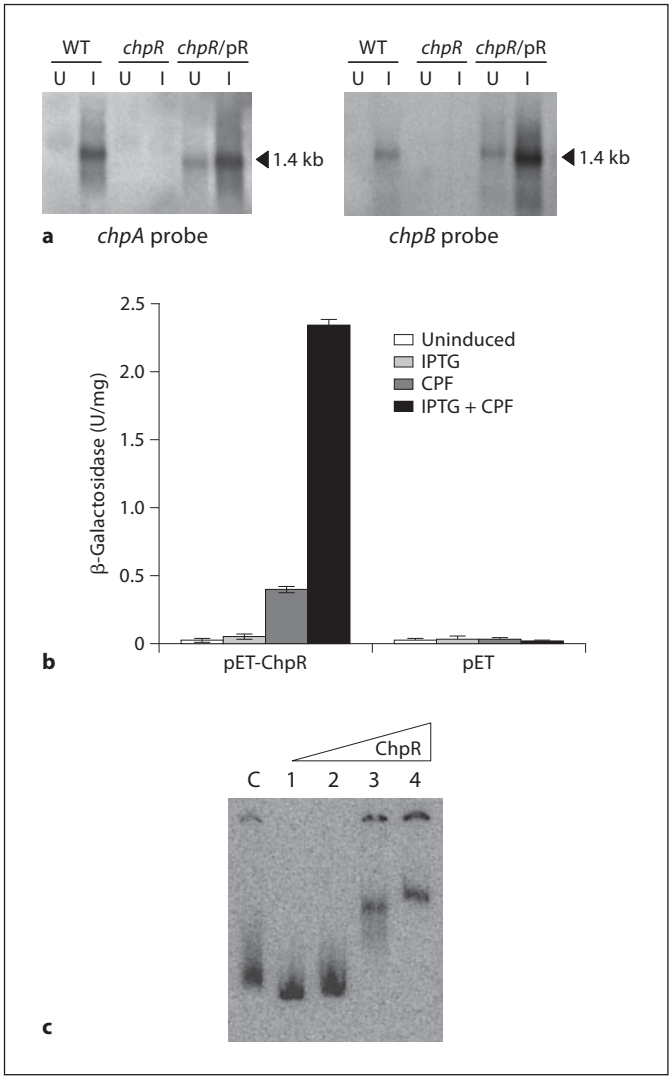

Fig. 2. a An autoradiogram of a Northern blot of total RNA extracted from *S. meliloti* wild type (WT), the *S. meliloti chpR* insertion mutant *(chpR)* and the mutant strain *chpR* complemented with a functional *chpR (chpR*/pR*),* probed with a ^{32}P-labeled gene internal fragments of *chpA* and *chpB.* Cells were grown in medium containing 100 μM CPF (labeled I) or no inducer (labeled U). Arrowheads indicate *chpAB* transcripts. **b** ChpR-dependent activation of *S. meliloti chpA* expression in response to CPF in *E. coli.* Results of β-galactosidase assays of *E. coli* DE3 containing pMP-*chpA*p and pET-ChpR or pET vector during growth in the presence of 100 μM IPTG or 100 μM CPF. Exponentially grown cultures were either uninduced or induced for 1 h with IPTG, CPF and both prior to the assay. Error bars show standard deviations. **c** Binding of ChpR to the *chpA* promoter region. An autoradiogram of a gel mobility shift assay performed using crude extract of an *E. coli* expression strain containing ChpR and a ^{32}P-labeled *chpA* promoter fragment. Lane C contains a gel shift reaction mixture with 53 μg of crude extract of *E. coli* harboring the pDuet expression vector with no insert. Lanes 1–4 contain reaction mixtures to which ChpR expression strain crude extract was added to a final concentration of 7, 20, 33, and 66 μg of protein/reaction.

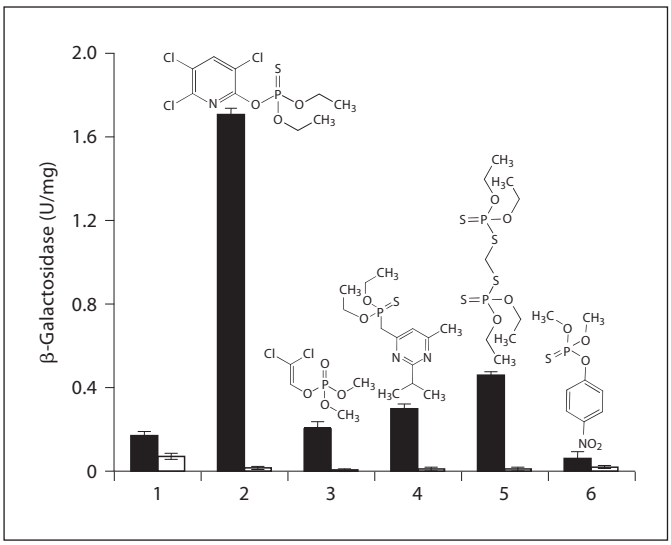

Fig. 3. Specificity of ChpR-mediated transcription activation in response to organophosphate pesticides. Results of β-galactosidase assays in *S. meliloti* wild type (black bars) and *chpR* mutant (white bars) carrying pMP-chpAp during growth in the absence (column 1) or presence of 100 μM CPF (column 2), dichlorvos (column 3), diazinon (column 4), ethion (column 5), and methyl parathion (column 6). Error bars show standard deviations. Structures of the pesticides are also shown.

are not similar to the consensus and do not contain the 100% conserved TTG sequence element.

ChpR Is a Positive Regulator of chpAB *Operon Transcription*

Given their close proximity, it seemed likely that the putative CadC-type regulator, ChpR, was involved in the regulation of the *chpAB* operon. The *chpR* insertional inactivation mutant strain, *S. meliloti chpR*, was constructed using pKNOCKGm [Alexeyev, 1999] to test this. Inactivation of *chpR* resulted in the disappearance of the 1.4-kb *chpA-chpA1* transcript in cultures exposed to CPF (fig. 2a). CPF-dependent expression of the *chp* operon was restored when plasmid pR, consisting of pBBR1MCS [Kovach et al., 1995] carrying full-length *chpR*, was introduced into *S. meliloti chpR* (fig. 2a).

The analysis of *chpA* regulation was extended using the *chpA* promoter-*lacZ* transcriptional fusion plasmid, pMP-chpAp, which contains 480 bps of sequence upstream of *chpA*. *lacZ* activity was assayed in *S. meliloti* carrying the *chpA* promoter-*lacZ* transcriptional fusion plasmid, pMP-chpAp, during growth in the presence and absence of CPF. The results confirmed those of the Northern hybridization showing that *chpA* expression was increased about 10-fold in the presence of 100 μM CPF, while no increase was observed in the *S. meliloti chpR* mutant (data not shown). Thus, ChpR is a positive regulator that mediates the CPF-dependent induction of *chp* operon transcription.

Further analyses were performed to determine whether ChpR-mediated transcription activation occurs in response to other organophosphate pesticides. Induction of *lacZ* was assayed in *S. meliloti* wild type and *chpR* mutants carrying pMP-chpAp grown in the presence of 100 μM of the organophosphate pesticides: CPF, dichlorvos, diazinon, ethion, and methylparathion (fig. 3). Growth in the presence of CPF resulted in the highest *chpA* promoter activity that was over 10-fold higher than that of *S. meliloti*::pMP-chpAp grown in the absence of pesticide (fig. 3, compare columns 1 and 2). No significant induction was seen in *S. meliloti*::pMP-chpAp grown in the presence of either dichlorovos or methyl parathion (fig. 3, compare column 1 with columns 3 and 6). However, growth in the presence of diazion and ethion did result in a lower level of induction of about 2-fold and 4-fold, respectively. No significant *lacZ* expression was observed in the *S. meliloti chpR* mutants and wild type carrying the *lacZ* fusion parent vector, pMP220 (data not shown).

In order to demonstrate the direct involvement of ChpR in *chp* operon transcription regulation, it was determined whether expression of *chpR* from a plasmid could activate the *S. meliloti chpA* promoter in *E. coli*, which contains no close homologs of *chpR*, *chpA* or *chpB* [Loprasert, personal obs.]. The *chpA* promoter-*lacZ* fusion plasmid pMP-chpAp was cotransformed into *E. coli* strain DE3, which contains an isopropyl-beta-D-thiogalactopyranoside (IPTG)-inducible T7-RNA polymerase gene, along with plasmid pET-ChpR. pET-ChpR carries *chpR*, C-terminally fused to six His codons under the control of a T7 RNA polymerase-dependent promoter in the expression vector pETBlue2 (Novagen). High-level induction of the *chpA* promoter by CPF required ChpR. This was evidenced by the observation that in *E. coli* DE3, *lacZ* expression from the *chpA* promoter-*lacZ* fusion, pMP-chpAp, required both IPTG-induced expression of ChpR from pET-ChpR and CPF (1 h induction) (fig. 2b). A small amount of *chpA* promoter activity was also observed during growth in the presence of CPF alone that was likely due to leaky expression of *chpR* from pET-ChpR. CPF did not stimulate *chpA* promoter activity in *E. coli* DE3 containing pMP-chpAp and pETBlue2 during growth in the presence of IPTG (fig. 2b). The ability of

ChpR to mediate CPF-dependent activation of *chpA* transcription in *E. coli*, an organism that does not possess close homologs to either a *chpR* or *chpA*, suggests that ChpR is sufficient for sensing the CPF signal.

The ChpR-mediated regulation of *chpA* in *E. coli* also suggested that CPF-dependent induction of the *chp* operon was the direct result of ChpR binding to the promoter. Gel mobility shift assays were performed to confirm this. Since overproduction of His-tagged ChpR in *E. coli* using pET-ChpR resulted in inactive protein due to the formation of ChpR inclusion bodies in the crude lysate, we created a native non-His-tagged ChpR expression construct in pDuet (Novagen) denoted pDuet-ChpR. ChpR was overproduced in IPTG-induced *E. coli* DE3 harboring pDuet-ChpR. Soluble extracts of these cultures contained DNA-binding activity specific for a ^{32}P-labeled 263-bp PCR fragment spanning the promoter region of *chpA* (fig. 2c). Binding activity was not present in extracts of the vector control strain containing pDuet (fig. 2c).

The results clearly indicate that ChpR interacts directly with the *chpA* promoter and is required for induction of the *chpA* promoter in response to CPF exposure. However, the exact nature of the inducing signal remains unknown. It is tempting to speculate that ChpR senses CPF directly. The fact that the organophosphates diazinon and ethion also induce transcription could argue against direct sensing due to their gross differences in structure; however, the structure of the *O*,*O*-diethyl phosphorothioate group is similar. It is also possible that the signal is a CPF breakdown product or an indirect result of stress induced by the exposure. In this regard, no induction of *chp* operon transcription was observed in response the oxidative stress inducers: H_2O_2, menadione, *tert*-butyl hydroperoxide or cumene hydroperoxide (data not shown).

Regulation of a Pseudomonas fluorescens chpA *Homolog by* S. meliloti *ChpR*

Interestingly, inspection of the *P. fluorescens* Pf0–1 genome sequence revealed the presence of a similar *chpRAB* operon (Pf01_2276, 2275, and 2274), which shares the same gene organization and a high degree of amino acid identity (75, 78 and 71%, for ChpR, ChpA and ChpB, respectively). To examine whether *S. meliloti* ChpR can control *P. fluorescens chpA* expression, we cloned a 690-bp *P. fluorescens chpA* promoter fragment into the fusion vector pMP220, which contains a promoterless *lacZ* [Spaink, 1987], yielding pPF-chpAp. *lacZ* determinations in *E. coli* carrying both pPF-chpAp and pET-ChpR confirmed that *S. meliloti* ChpR can regulate the *P. fluorescens chpAB* operon since *lacZ* expression occurred only when ChpR expression was induced by IPTG and was maximal in the presence of CPF (data not shown). Similar experiments were also performed in *S. meliloti* wild type and ChpR-mutant strains carrying pPF-chpAp, showing that CPF-inducible expression of *P. fluorescens chpAB* in *S. meliloti* required ChpR (data not shown).

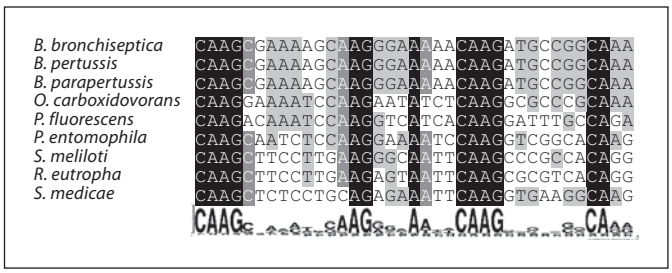

Fig. 4. Comparison of ChpR-binding site sequences. A comparison of the putative *S. meliloti* ChpR-binding sequence in the *chpA* promoter with sequences present in the *chpA* promoters of *B. bronchiseptica, B. pertussis, B. parapertussis, O. carboxidovorans, P. fluorescens* Pf0–1, *P. entomophila, S. medicae, and R. eutropha* H16. A consensus sequence is shown at the bottom. The height of each letter is proportional to the frequency of the residue at that position [Crooks et al., 2004].

The Proposed ChpR-Binding Sequence

The finding that *S. meliloti* ChpR can activate expression of the *P. fluorescens chpA* promoter prompted us to examine these and other *chp*-operon promoters to identify a putative ChpR-binding sequence. Interestingly, conserved direct repeat sequences were found within the intergenic regions between putative *chpR* and *chpA* in alpha-, beta- and gammaproteobacteria: *S. meliloti* Rm1021, *Sinorhizobium medicae, P. fluorescens* Pf0–1, *Pseudomonas entomophila, Bordetella parapertussis, Bordetella bronchiseptica, Bordetella pertussis, Oligotropha carboxidovorans,* and *Ralstonia eutropha* H16. All these organisms possess a ChpR homolog together with ChpAB except *O. carboxidovorans,* which does not have ChpB. The putative ChpR-binding sites of these organisms were aligned and the ChpR consensus binding sequence CAAG – N_7 – ($^{C}/_{G}$)AAG – N_7 – CAAG – N_7 – CA($^{A}/_{G}$) ($^{A}/_{G}$) was identified that contains 4 imperfect CAAG repeats separated by 7 nucleotides (fig. 4). This element is immediately upstream of and partially overlaps the putative –35 promoter region of the *S. meliloti chpAB* operon (fig. 1c).

Table 1. Plasmids and primers used in this study

Plasmid or primer	Properties or product	Source or reference
Plasmids		
pUTTn5lacZ	*Tn* integrated library construction	de Lorenzo et al. [1990]
pKNOCKGm	*chpR* insertion-mutant construction	Alexeyev [1999]
pMP220	Promoter probe vector	Spaink [1987]
pETBlue2	His-tagged protein construction	Novagen
pRSFDuet-1	Expression vector	Novagen
pBBR1MCS	Broad host range vector	Kovach et al.[1995]
Primers		
LA	*lacZ*-specific primer, GCGCGTCGCCGCTTTCATC	this study
CHA	*chpA* primer extension ATCTTCATCGCTTGTCTCCG	this study
CHR	*chpR* primer extension CACTTCGGCATCCCCTGGCC	this study
KP and PS	*chpA* promoter-*lacZ* fusion construction CCGCCGGTACCGCCGATGGC and CGGCAGCTGCAGCAGCCAGC	this study
NC and XH	His-tagged ChpR plasmid construction ATGCCGCCATGGAATTCATG and CGATCACTCGAGCAATCC	this study
FW and RV	*chpA* promoter for gel shift experiment CACTTCGGCATCCCCTGGCC and ATCTTCATCGCTTGTCTCCG	this study
PF1 and PF2	*P. fluorescens chpA* promoter fusion GCCAGGTGAATTCCCGGCGC and GCAGGCGGTACCGAATGCCC	this study

This work reports the isolation of the CPF-inducible *chpAB* operon in *S. meliloti* and the identification and partial characterization of ChpR, a new member of the CadC family of transcriptional activators that is a direct activator of *chpA* in response to CPF exposure. The fact that ChpR can mediate CPF-induced expression of the *S. meliloti chp* operon in *E. coli*, an unrelated bacterium, suggests that this system is a good candidate for further biosensor development. Further efforts are also centered on elucidating the function of *chpAB* in *S. meliloti*.

Experimental Procedures

Media and Growth Conditions. S. meliloti Rm1021was grown aerobically at 28ºC in LB/MC medium [Glazebrook and Walker, 1991]. All *E. coli* strains were grown aerobically in Luria-Bertani broth at 37°C. Gm (15 μg/ml), kanamycin (400 μg/ml for *S. meliloti*, 15 μg/ml for E. coli), ampicillin (100 μg/ml) and CPF (Sigma) (100 μM) were added to media when required.

Nucleic Acid Extraction and Transformation. Genomic DNA extraction from *S. meliloti* was performed according to the method of Mongkolsuk et al [1996]. Total RNA was isolated by the hot-phenol method [Mongkolsuk et al., 1996]. *S. meliloti* was genetically transformed by electroporation [Mongkolsuk et al., 1996] or conjugation [Glazebrook and Walker, 1991].

Transposon Integrated Library Construction. CPF-inducible genes were identified by generating a randomly integrated library of Tn5 mutants in *S. meliloti* Rm1021 using pUTTn5lacZ1Gm, which carries Tn5 containing a promoterless *lacZ* and a Gm resistance cassette substituted for the Km resistance cassette of its parent vector, pUTTn5lacZ [de Lorenzo et al., 1990].

Cloning of the Tn5 Flanking Fragment. S. meliloti strain *chp* chromosomal DNA was digested with *Sal*I and ligated with *Xho*I-digested pBluescript KS (Strategene). Sequencing of the Gm-resistant clone, pKS-chpB, was performed with the *lacZ*-specific primer (table 1).

Determination of Transcription Start Sites. Primer extension experiment using total RNA extracted from *S. meliloti* and Superscript III MMLV reverse transcriptase was carried out following a previously described protocol [Loprasert et al., 2000, 2007]. Primers used for *chpA* and *chpR* are: CHA and CHR (table 1).

chpR *Insertion-Mutant Construction.* An insertion mutant of *chpR* was constructed using the insertion vector pKNOCKGm [Alexeyev, 1999] carrying an 800-bp gene internal *Pst*I-*Sac*I fragment of *chpR* to generate the strain *R. meliloti chpR*. The mutant was confirmed as carrying a single insertion of pKNOCKGm with *chpR* by Southern analysis.

chpA *Promoter*-lac*Z Transcriptional Fusion Construction.* The plasmid pMP-chpAp contains an 800-bp *Kpn*I/*Pst*I PCR-amplified *chpA* promoter fragment transcriptionally fused to a pro-

moterless *lacZ* in pMP220 [Spaink, 1987] using primers KP and PS (table 1).

His-Tagged chpR *and Native* chpR *Plasmid Construction.* Plasmid pET-ChpR (a derivative of pETBlue2, Novagen) which carries *chpR*, C-terminally fused to 6 His codons was created. A 1.6-kb *Nco*I/*Xho*I fragment containing the *chpR* gene was amplified by PCR using primer NC and primer XH (table 1). Plasmid pDuet-ChpR was made by insertion of a 1.6-kb *Nco*I/*Hin*dIII PCR-amplified fragment into *Nco*I/*Hin*dIII digested pRSFDuet-1 (Novagen). The 1.6-kb chpR coding fragment was generated by PCR amplification using plasmid pBBR1MCS [Kovach et al., 1995] carrying *chpR* (pR) as the template and primers NC and M13 forward.

Gel Mobility Shift Assays. The experiments were performed as previously described [Loprasert et al., 2007]. Briefly, a 263-bp PCR fragment (primer FW and RV, table 1) spanning the promoter region of *chpA* was end-labeled using [γ-^{32}P]ATP and T_4 kinase. Gel shift reactions were performed by adding labeled probe to binding buffer containing 100 mM Tris-HCl pH 7.4, 4 mM DTT, 5 mM $MgCl_2$, 5% glycerol, 2 μg salmon sperm DNA, 100 ng bovine serum albumin.

P. fluorescens chpA *Promoter Fusion Plasmid Construction.* We amplified a 690-bp *P. fluorescens chpA* promoter fragment by PCR using primers PF1 and PF2 (table 1). The fragment was cloned into the fusion vector pMP220, which contains a promoterless *lacZ* [Spaink, 1987], yielding pPF-chpAp.

Acknowledgments

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