Phosphorylation and DNA binding of the regulator DcuR of the fumarate-responsive two-component system DcuSR of Escherichia coli

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The function of the response regulator DcuR of the DcuSR fumarate two-component sensory system of Escherichia coli was analysed in vitro. Isolated DcuR protein was phosphorylated by the sensory histidine kinase, DcuS, and ATP, or by acetyl phosphate. In gel retardation assays with target promoters (frdA, dcbB, dctA), phosphoryl DcuR (DcuR-P) formed a high-affinity complex, with an apparent $K_D$ (app. $K_D$) of 0.2–0.3 μM DcuR-P, and a low-affinity (app. $K_D$ 0.8–2 μM) complex. The high-affinity complex was formed only with promoters transcriptionally-regulated by DcuSR, whereas low-affinity binding was seen also with some DcuSR-independent promoters. The binding site of DcuR-P at the dcbB promoter was determined by DNase I footprinting. One binding site of 42–52 nt (position −359 to −400/−410 nt upstream of the transcriptional start) was identified in the presence of low and high concentrations of DcuR-P. Non-phosphorylated DcuR, or DcuR-D56N mutated in the phosphoryl-accepting Asp56 residue, showed low-affinity binding to target promoters. DcuR-D56N was still able to interact with DcuS. DcuR-D56N increased the phosphorylation of DcuS and competitively inhibited phosphoryl transfer to wild-type DcuR.

INTRODUCTION

In bacteria, two-component sensor–regulators are the preferred system for sensing environmental stimuli and for adapting the cellular response to the prevailing conditions (Aizawa et al., 2000; Parkinson & Kofoid, 1992). Two-component systems are typically composed of a membrane-associated sensor and a cytoplasmic response regulator. The sensory kinase is responsible for receiving environmental stimuli and converting them to a cellular signal, i.e. phosphorylation of a conserved protein histidine residue. The phosphoryl group is then transferred to the response regulator, which is converted to the active state by phosphorylation at a conserved aspartate residue. Phosphorylated response regulators bind to the promoters of target genes and activate or repress the transcription of the corresponding genes.

The DcuSR two-component system of Escherichia coli consists of the sensory kinase DcuS and the regulator DcuR, and controls the expression of genes in response to extracellular C$_4$-dicarboxylates like fumarate and succinate (Jones & Gunsalus, 1987; Zientz et al., 1998; Golby et al., 1999; Davies et al., 1999; Janausch et al., 2002a). The major target genes encode enzymes of aerobic or anaerobic C$_4$-dicarboxylate metabolism, and include the structural gene dcbB for the fumarate/succinate antiporter DcuB, the frdABCD operon for fumarate reductase, and the dctA gene for the succinate uptake carrier DctA. In addition, expression of a number of other genes that are not related to C$_4$-dicarboxylate metabolism is regulated by DcuSR (Oshima et al., 2002).

To date, the sensor of the DcuSR system has mainly been studied, and the structure of the periplasmic domain containing the signal-binding domain has been determined (Janausch et al., 2002b; Pappalardo et al., 2003). The sensor has been isolated and functionally reconstituted in proteoliposomes. After reconstitution, the sensor is functionally intact and responds to the presence of fumarate or succinate (Janausch et al., 2002b). Reconstituted DcuS is able to transfer a phosphoryl group to the response regulator DcuR, which after phosphorylation gains the ability to bind to DNA (Janausch et al., 2002b). However, the details of DNA binding by DcuR are not known, although they would be important for understanding the complete DcuSR system. For this reason, the phosphorylation of DcuR and the binding of DcuR to target DNA were studied. Studies on phosphoryl transfer indicated a specific interaction between DcuR and DcuS during the reaction.
METHODS

Overproduction and preparation of DcuR and DcuR-D56N.
For isolation of DcuR with an N-terminal hexahistidine tag, E. coli BL21DE3(pMW180) (Janausch et al., 2002b) was grown at 30°C under aerobic conditions in 0.8 L LB medium (Miller, 1992), containing 50 μg kanamycin ml⁻¹. When an OD₆₀₀ of 0.7 was reached, the cells were induced with 1 mM IPTG for 2 h. The cells were then harvested, washed, and resuspended in buffer 1 (50 mM potassium phosphate, pH 7-5, 500 mM NaCl, 10 mM imidazole). The cells were broken in a French press and His₆-DcuR was isolated on a Ni-NTA column (Janausch et al., 2002b). The eluted protein was adjusted to 40 % (w/v) glycerol and stored at -20°C.

DcuR-D56N was constructed by site-directed mutagenesis with the QuikChange mutagenesis kit (Stratagene), using plasmid pMW180 (Janausch et al., 2002b) as a template, and primers DcuR-D56N-Mut28-for (GAC CTG ATA TGG CTC AAT ATC TAT ATG) and DcuR-D56N-Mut28-rev (GCA TAT AGA TAT TGA GCA GTA TCA GGT C). In the resulting plasmid pMW267 (encoding DcuR-D56N), the presence of a mutation (D56N) was verified by DNA sequencing. Plasmid pMW267 was used for overproduction and isolation of DcuR-D56N, as described for DcuR.

DNA binding and gel retardation.
Prior to its use in gel retardation assays, His₆-DcuR was phosphorylated by incubation with acetyl phosphate. In this reaction, 10 μg DcuR in 20 μl buffer (50 mM Tris/HC1, pH 7-0, 5 mM MgCl₂, 1 mM dithiothreitol) were incubated for 60 min at 37°C with 50 mM acetyl phosphate and then used immediately for DNA-binding studies. For estimation of the phosphorylation state, various response regulators (DcuR, ArcA, NarL) were subjected to isoelectric focussing. The procedure enabled the separation of phosphorylated and non-phosphorylated regulators (unpublished data). The regulators incubated with acetyl or carboxymethylphosphate were phosphorylated generally to about 50 %, and maximally up to approximately 75% of the total regulator protein. For experiments involving DcuR-P, it was assumed that 50 % of the DcuR protein was present as DcuR-P.

The DNA fragments for gel retardation were obtained by PCR. The intergenic region in front of dcuB was amplified by PCR with the oligonucleotide primers dcuB1EcoRI (GAT AGT GAA GAA TTC CAT GTG) and dcuB2EcoRI (AAG AAA CAA GAA TTC CAG TAA CGG) from E. coli AN387 genomic DNA, and cloned via the EcoRI site into pBluescript KS (Alting-Mees & Short, 1998), resulting in plasmid pMW195 with the complete intergenic region of dcuB. The dcuB promoter fragment (585 bp) for gel retardation was amplified with the same primers from pMW195. The DNA fragment (463 bp) with the frdA promoter region was amplified by PCR with primers frdA1EcoRI (GAC GGA ATT CCG CCA TAA TCG C) and frdA2EcoRI (GCG CCG CAG GAA TTC CAG C) from pN3U1 (Cole, 1982). The DNA fragment (481 bp) with the dctA promoter region was obtained by PCR with primers dctAECori (GTC TGG ATG AAT TCG GCA TGG G) and dctABamHI (CAG AGA GGG GAT CAT AGG GTC TCC) from pMW103 (Zientz et al., 1998). The sdcC promoter region (674 bp) was prepared by PCR from a colony of E. coli AN387 with primers sdcCEcoRI (GAA AGA GGG GAA TTC CTG GGT AC) and sdcECori (GCG GAT GGA TTC AGC CTA TC). In the sequences above, the underlined sequences represent restriction sites.

For gel retardation, the DNA fragments were amplified from the corresponding plasmids, digested with EcoRI or with EcoRI and BamHI, purified with a Qiagen PCR Purification Kit (Qiagen) and labelled with [γ-32P]ATP on both strands. The labelling mixture contained 0-1 pmol DNA fragment, Klenow reaction buffer (MBI Fermentas), 0-25 mM dNTP mix (without labelled nucleotide), 8-1 x 10⁵ Bq [γ-32P]dATP and 5 U Klenow enzyme (exo⁻ fragment). Incubation was carried out for 25 min at 30°C and then for 10 min at 75°C.

Gel retardation assays were performed essentially as described previously (Drapal & Sawers, 1995; Wackwitz et al., 1999). The phosphorylated DcuR protein was incubated with labelled DNA (5 nM) in binding buffer (50 mM Tris/HCl, pH 7-5, 5-5 %, w/v, glycerol, 0-1 mM EDTA, 50 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 5 μg sonicated calf thymus DNA) for 20 min at room temperature. After incubation, the reaction mix was applied to a non-denaturing polyacrylamide gel (5 %) buffered with Tris/borate/EDTA (TBE) buffer (Sambrook et al., 1989).

DNase I footprinting.
Plasmid pMW195, carrying the dcuB promoter, was restricted with HindIII and Cfr42I. The resulting 640 bp fragment, containing the complete dcuB-dcuR intergenic region, was restricted with HindIII, yielding a 421 bp DNA fragment. The dcuB promoter fragments of 640 and 421 bp were labelled on one strand with [γ-32P]dATP for 30 min at 30°C, followed by 10 min at 75°C. The reaction mixture contained 2 pmol DNA fragment, Klenow reaction buffer, 0-25 mM dNTP mix (without labelled nucleotide), 1-63 x 10⁵ Bq [γ-32P]dATP and 20 U Klenow enzyme (exo⁻ fragment). DNase I footprinting was performed essentially as described previously (Drapal & Sawers, 1995). DcuR-P (see Methods, DNA binding and gel retardation) was incubated with labelled DNA (40 nM) for 30 min at room temperature in 50 μl footprinting buffer (50 mM Tris/HCl, pH 7-0, 10 %, w/v, glycerol, 0-1 mM EDTA, 10 mM MgCl₂, 50 mM KCl, 0-1 mM dithiothreitol). The footprinting reaction and electrophoresis were performed as described by Drapal & Sawers (1995). As a size marker, plasmid DNA including the dcuB promoter, sequenced by the chain-termination method (T7-sequencing kit, Pharmacia), was used.

Phosphorylation state of reconstituted DcuS in proteoliposomes.
Detergent-solubilized DcuS was incorporated into liposomes by detergent removal using Bio-Beads, as described previously (Janausch et al., 2002b). An 80 μl aliquot of the proteoliposome suspension with reconstituted DcuS (27 μg DcuS, 530 μg E. coli phospholipids) was mixed with (final concentrations) 10 mM MgCl₂, 1 mM dithiothreitol and 20 mM fumarate, and subjected to three cycles of rapid freezing in liquid nitrogen, with slow thawing at 20°C. After the final thawing, the proteoliposomes were kept for 1 h at 20°C. Then, 2 μl [γ-32P]ATP (110 TBq mmol⁻¹) was added to a final concentration of 0-1 μM ATP, and the reaction mixture incubated for 60 min at 20°C. To test the effect of DcuR and DcuR-D56N on the phosphorylation of DcuS in proteoliposomes, DcuR and DcuR-D56N were included during phosphorylation. At the indicated times, samples (10 μl) were withdrawn, mixed with 10 μl SDS loading buffer, and subjected to SDS-PAGE (Laemmli, 1970). After electrophoresis, the gels were exposed to a phosphorimaging screen (Fuji BAS-MP2040) for identification of radioactive bands in the phosphorimagier (Fuji BAS 1500).

Other methods.
Protein concentration was measured using the method of Bradford (1976). β-Galactosidase activity was determined according to Miller (1992). The method of Laemmli (1970) was used for SDS-PAGE.

RESULTS

Binding of DcuR-P to DcuSR target promoters and formation of two retardation complexes.
Binding of DcuR to target promoters was tested by gel retardation, using purified DcuR. DcuR was purified as a
His$_6$-fusion protein, and phosphorylated by incubation with acetyl phosphate. The promoter DNA fragments composed the complete intergenic region in front of the DcuSR target genes fdrA and dcuB (Zientz et al., 1998; Golby et al., 1999). In the experiment shown in Fig. 1, labelled DNA of the fdrA promoter region was incubated with DcuR-P, and subjected to native DNA PAGE. With increasing concentrations of DcuR-P, free promoter DNA was replaced by two bands of decreased mobility (complex I and complex II). Complex I was replaced by complex II at higher concentrations of DcuR-P. This suggests that complex II binds higher amounts or an oligomeric form of DcuR-P, or that complex II contains DcuR-P bound to an additional (low-affinity) site. Both complexes were found in the presence of a large excess (300-fold) of non-specific competitor DNA and must, therefore, be formed by specific DNA binding. Promoter DNA from the dcb gene, and from dctA, which is transcriptionally activated by DcuSR under aerobic conditions, was retarded by DcuR-P in a similar way, also exhibiting two retarded bands (results not shown). In contrast, the sdhC promoter showed retardation only at high concentrations of DcuR-P ($\geq 1.3$ $\mu$M), and there was only a single retarded species (results not shown). Other genes, like narG, which are not involved in C$_4$-dicarboxylate metabolism, were not retarded by DcuR-P.

**$K_D$ values for binding of DcuR-P and DcuR to promoter DNA**

Table 1 compares app. $K_D$ values for the binding of DcuR to target promoters. The fdrA, dcb, and dctA promoters showed high-affinity binding, and formation of complex I had an app. $K_D$ of 0.2–0.3 $\mu$M for DcuR-P. The app. $K_D$ values for complex II were four- to sevenfold higher (0.8–2 $\mu$M). The DNA fragments that formed high-affinity retardation complexes (fdrA, dcb, dctA) are specifically regulated by DcuR and responsive to the presence of fumarate in vivo (Table 1) (Zientz et al., 1998; Golby et al., 1999). Expression from the sdhC promoter, on the other hand, which showed only low-affinity binding, is not stimulated by the DcuSR system. The significance of this low-affinity binding is not known.

### Requirement of DcuR phosphorylation for high-affinity binding

When non-phosphorylated DcuR was incubated with dcb (Fig. 2) or the fdrA promoter DNA, a retardation complex was formed. In contrast to DcuR-P, only one retardation complex was seen and higher concentrations of DcuR ($\geq 3$ $\mu$M) were required. Therefore, DcuR shows only low affinity for the promoter DNA, and retardation was observed only for some promoters (fdrA, dcb), but not

### Table 1. Apparent (**app.** $K_D$) values for binding of DcuR-P and DcuR to DcuR-regulated promoters

<table>
<thead>
<tr>
<th>Promoter</th>
<th>app. $K_D$ ($\mu$M)</th>
<th>Regulation (fold) by fumarate</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-dcb</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>P-frdA</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>P-dctA</td>
<td>0.3</td>
<td>2</td>
</tr>
<tr>
<td>P-sdhC</td>
<td>na</td>
<td>1.3</td>
</tr>
<tr>
<td>P-narG</td>
<td>nb</td>
<td>nb</td>
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</tbody>
</table>

Fig. 1. Gel retardation of frdA promoter DNA by increasing concentrations of phosphorylated DcuR (DcuR-P). The radioactively labelled fdrA promoter fragment was incubated in the presence of a 300-fold excess of competitor DNA with increasing concentrations of His$_6$-DcuR-P. The protein/DNA mixture was subjected to non-denaturing DNA PAGE. The locations of free promoter DNA and of the retarded DNA/DcuR-P complexes are indicated by arrows.

Fig. 2. Gel retardation of dcb promoter DNA by non-phosphorylated DcuR. Radioactively labelled dcb promoter DNA was incubated with increasing concentrations of non-phosphorylated DcuR, and subjected to non-denaturing DNA PAGE. The locations of free promoter DNA, and of the retarded DNA/DcuR complexes, are indicated by arrows. For other reaction conditions, see legend to Fig. 1.
for all DcuSR-regulated promoters, even when the protein was applied in concentrations as high as 4 μM (Table 1).

Sequence alignments suggest that Asp56 of DcuR represents the conserved phosphorylation site for DcuR-P formation. The Asp56 residue was exchanged for asparagine by site-directed mutagenesis. Strains containing DcuR-D56N instead of DcuR were no longer able to induce dcuB–lacZ in the presence of fumarate. After incubation with acetyl phosphate, purified DcuR-D56N did not bind to the dcuB promoter at concentrations of less than 2 μM DcuR (Fig. 3). Low-affinity binding was observed only at high concentrations of DcuR-D56N (>3 μM) (results not shown). It was concluded that only the high-affinity binding of DcuR-P is functionally relevant in vivo. The binding of non-phosphorylated DcuR, on the other hand, is without physiological significance in vivo, although it appears to be sequence-specific, and it is limited to a few DcuR-regulated promoters.

**Footprint analysis of the DcuR-P binding site at the dcuB promoter**

Footprint analysis of the DcuR-binding site was performed with a 640 bp DNA fragment which included the complete dcuB promoter region. The DNA fragment was incubated with up to 4 μM DcuR-P, then partially digested with DNase I, and the resulting fragments were analysed by denaturing PAGE. In the presence of 0.5 μM DcuR-P (or higher), a region about 400 bp upstream of the dcuB coding sequence was protected from DNase I digestion, as indicated by the lack of fragments. Other regions of the fragment showed no protection, even in the presence of 4 μM DcuR-P. The site protected by DcuR-P was close to the 5′ end of the fragment and poorly resolved (results not shown). For clear identification of the binding site, a 421 bp subfragment of the 5′ end was used for DNase I footprinting in the same manner with DcuR-P (Fig. 4). The footprint showed a region of protection from –359 to –400 or –410 (relative to the transcriptional start site of dcuB). No further sites were protected by DcuR-P from DNase I digestion on this fragment. Therefore, there is only one site in the promoter region which is protected by DcuR-P, even at high concentrations (4 μM). The size of the protected site was the same for all concentrations of DcuR-P used.

**Effect of DcuR and DcuR-D56N on the phosphorylation state of DcuS**

The effects of DcuR and DcuR-D56N on the phosphorylation and dephosphorylation of DcuS-P were tested, using DcuS reconstituted in proteoliposomes (Fig. 5). DcuS is known to be active in autophosphorylation and phosphoryl transfer to DcuR (Janausch et al., 2002b). Incubation of DcuS with [γ-32P]ATP for 30 min caused strong phosphorylation of DcuS (Fig. 5a). It has been shown previously...
that addition of DcuR to autophosphorylated DcuS-P causes rapid phosphoryl transfer to DcuR (Janausch et al., 2002b). Phosphoryl transfer to DcuR-D56N was completely lacking, as expected. When DcuR-D56N and DcuR were present during the phosphorylation of DcuS, they gave rise to different effects on the phosphorylation state of DcuS (Fig. 5a). Taking the extent of DcuS phosphorylation in the presence of DcuR-D56N as 100 %, it was found that DcuS phosphorylation with DcuR present was low (13 %). A greater proportion of the phosphoryl group (20 %) was found as DcuR-P, due to phosphoryl transfer from DcuS-P to DcuR, which is more rapid than autophosphorylation of DcuS. The low amount of overall radioactivity (33 %, compared to the experiment with DcuS plus DcuR-D56N) is explained by the low stability of the phosphoryl group in DcuR-P. In the presence of DcuR-D56N, on the other hand, phosphorylation of DcuS was significantly increased, by 35 %, compared to phosphorylation without DcuR. Thus, DcuR-D56N, which does not function as a phosphoryl acceptor from DcuS-P, stimulated phosphorylation of DcuS.

The interaction of DcuS with DcuR was studied in more detail by DcuR/DcuR-D56N competition experiments with DcuR or DcuR-D56N present at the same time (Fig. 5b). Phosphorylation of DcuS in the presence of DcuR and [γ-32P]ATP reached a constant level after approximately 50 min incubation. When DcuR-D56N was added, the phosphorylation state of DcuS increased and after about 15 min a new steady-state level was achieved which surpassed the previous by about 50 % (Fig. 5b). The changed level can most simply be explained by assuming a decreased rate of DcuS dephosphorylation by competition of DcuR-D56N with wild-type DcuR.

If DcuR-D56N instead of DcuR was present initially, then the level of DcuS phosphorylation was significantly higher, as expected, due to lack of dephosphorylation by DcuR-D56N. Addition of wild-type DcuR in the second phase of the experiment resulted in a decrease in DcuS phosphorylation by about 25 %. The residual phosphorylation of DcuS, therefore, was higher than in the previous experiment (Fig. 5a), when only DcuR was included.

Each of the experiments therefore indicates that DcuR-D56N is still able to interact with DcuS and to compete with DcuR for binding and interaction. Thus, inactivation of the phosphorylation site in DcuR-D56N inhibits only phosphorylation of DcuR and DNA binding, but not interaction with DcuS.

**DISCUSSION**

**Significance of the high-affinity DcuR-P/DNA complex for transcriptional regulation**

Binding of phosphorylated DcuR to target promoters generates two DcuR-P/DNA complexes that differ in app. K_D values. Both complexes were observed for all target promoters, suggesting that complex formation is a property of DcuR, rather than of the frdA, dcuB and dctA promoters. Response regulators are typically found in concentrations of less than 1 μM in bacterial cells. The low-affinity complex is similar to the complex formed with non-phosphorylated DcuR, or with DcuR-D56N, which is not functional in vivo. Therefore, only the high-affinity binding of DcuR-P (app. K_D=0.3 μM), and not the low-affinity binding, seems to be functionally relevant.
Accordingly, only promoters with high-affinity DcuR-P binding (frdA, dcuB, dctA) show DcuSR-dependent regulation in vivo. The site of DNA binding for DcuR and DcuR-P is presumably the same, since binding of DcuR was observed only at promoters containing high-affinity binding sites. In addition, in the footprint experiment, only one binding site was seen, although the DcuR-P preparation contained DcuR.

The formation of two retardation complexes (complexes I and II) could have been due to the presence of two binding sites with high and low affinity. For example, the presence of more than one binding site has been shown for the response regulators ArcA-P and NarL-P at the pfl, aldA, sdhC and narG promoters (Walker & DeMoss, 1994; Drapal & Sawers, 1995; Shen & Gunsalus, 1997; Pellicer et al., 1999). The sdhC promoter contains four independent ArcA-binding sites with differing affinities for ArcA-P (Shen & Gunsalus, 1997).

The formation of two retardation complexes could also be due to binding of DcuR in different oligomeric states. Thus, the ArcA-P binding sites in front of the pfl and sdhC promoters expand with increasing ArcA-P concentrations, since ArcA-P occupies a larger segment on the DNA (Drapal & Sawers, 1995; Lynch & Lin, 1996). The dcuB promoter, on the other hand, contains only one DcuR-binding site, and the size of the protected site remains constant with increasing DcuR concentration. Therefore, the basis of complex II formation in the gel retardation assay is not clear. It cannot be ruled out, however, that complex II is a complex of DNA with non-phosphorylated DcuR. The finding that an equivalent of complex II is formed with all DcuR-specific promoters supports this assumption. In addition, the mobilities and app. $K_D$ values for complex II and the DcuR/DNA complex are similar.

DcuR-P binds at a relatively large site of 42–52 nt at the dcuB promoter (Fig. 6). The protected area is similar in size to sequences of 50–94 bp protected by the response regulator ArcA-P (Tardat & Touati, 1993; Drapal & Sawers, 1995; Lynch & Lin, 1996; Cotter et al., 1997). The protected sequence is AT rich, but there is only a small palindromic sequence (AGTTAA TTAACT) close to the 3' end of the protected sequence. The protected site is centred around −376 bp upstream of the transcriptional start site of dcuB (Golby et al., 1998). The dcuB promoter region contains, in addition, predicted cyclic AMP receptor protein (CRP), FNR and NarL binding sites (Golby et al., 1998), which are all located more than 270 bp downstream of the DcuR site. Identification of DcuR-binding sites at other promoters will be required to derive the DcuR consensus sequence.

**Interaction of DcuR with DcuS**

DcuR has to interact with DcuS during phosphoryl transfer, and there are indications that DcuR/DcuS complexes are formed in this reaction. Thus, DcuS phosphorylation was increased by a factor of 1.5 when DcuR-D56N was present. It has to be assumed that DcuS is more efficiently phosphorylated in the DcuS/DcuR complex than free DcuS. The effect can most easily be explained by assuming that DcuR directly interacts with DcuS to form a complex that affects DcuS autophosphorylation. The interaction is not abolished in the DcuR-D56N mutant, demonstrating that the DcuR-D56N mutant protein has lost only the capability for phosphorylation and high-affinity DNA binding, whereas other functions are still present. The stimulating effect is rather low but significant. Stimulation of sensor kinase autophosphorylation by the presence of response regulators has been observed for other two-component systems, such as FixL/FixJ, EnvZ/OmpR and RegS/RegR (Tuckerman et al., 2001; Mattison & Kenney, 2002; Emmerich et al.,...
1999). In addition, in competition with wild-type DcuR, DcuR-D56N significantly inhibits phosphoryl transfer to DcuR.

After this paper had been accepted for publication, a study on a similar subject was published (Abo-Amer et al., 2004).

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REFERENCES


