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Functional roles for the GerE-family carboxyl-terminal domains of nitrate response regulators NarL and NarP of *Escherichia coli* K-12

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NarL and NarP are paralogous response regulators that control anaerobic gene expression in response to the favoured electron acceptors nitrate and nitrite. Their DNA-binding carboxyl termini are in the widespread GerE–LuxR–FixJ subfamily of tetrahelical helix–turn–helix domains. Previous biochemical and crystallographic studies with NarL suggest that dimerization and DNA binding by the carboxyl-terminal domain (CTD) is inhibited by the unphosphorylated amino-terminal receiver domain. We report here that NarL-CTD and NarP-CTD, liberated from their receiver domains, activated transcription *in vivo* from the class II *napF* and *yeaR* operon control regions, but failed to activate from the class I *narG* and *fdnG* operon control regions. Alanine substitutions were made to examine requirements for residues in the NarL DNA recognition helix. Substitutions for Val-189 and Arg-192 blocked DNA binding as assayed both *in vivo* and *in vitro*, whereas substitution for Arg-188 had a strong effect only *in vivo*. Similar results were obtained with the corresponding residues in NarP. Finally, Ala substitutions identified residues within the NarL CTD as important for transcription activation. Overall, results are congruent with those obtained for other GerE-family members, including GerE, TraR, LuxR and FixJ.

INTRODUCTION

The helix–turn–helix (HTH) is a widespread DNA-binding domain. One variation, the tetrahelical HTH superfamily (Aravind *et al.*, 2005), includes one family defined initially through sequence similarity to the *Vibrio fischeri* LuxR quorum sensor, response regulators such as *Sinorhizobium meliloti* FixJ, and the single-domain GerE regulator from *Bacillus subtilis* (Henikoff *et al.*, 1990). This family is annotated in domain databases as GerE (pfam00196), HTH_LuxR (smrt00421) and LuxR_C_like (cd06170) (Marchler-Bauer *et al.*, 2009). Approximately one quarter of all DNA-binding response regulators have the GerE-family carboxyl-terminal domain (CTD) (Galperin, 2006), including *Escherichia coli* NarL, which mediates nitrate-responsive transcriptional regulation (Stewart & Rabin, 1995).

NarL has been analysed by X-ray crystallography of both the unphosphorylated monomeric protein (Baikalov *et al.*, 1996) and the isolated dimeric CTD in complex with DNA (Maris *et al.*, 2005, 2002). The CTD comprises scaffold and DNA-recognition helices (α8 and α9, respectively), which form the HTH per se, as well as support and dimerization helices (α7 and α10, respectively; Fig. 1). Superimposable structures have been determined for other GerE-family proteins, including GerE (Ducros *et al.*, 2001), FixJ (Kurashima-Ito *et al.*, 2005) and *Agrobacterium tumefaciens* TraR (Vannini *et al.*, 2002; Zhang *et al.*, 2002), a LuxR homologue (Nasser & Reverchon, 2007; Pappas *et al.*, 2004). In full-length NarL, the recognition helix is blocked by the receiver, whereas the dimerization helix is packed against the interdomain linker. Receiver phosphorylation results in domain rearrangement to relieve this inhibition of DNA binding (Eldridge *et al.*, 2002).

The NarL CTD binds as an antiparallel dimer to inverted repeat DNA sequences (Maris *et al.*, 2005, 2002) termed 7–2–7 heptamer pairs. Heptamers have a consensus of 5′-TACYYMT-3′, where Y=C or T and M=A or C (Darwin *et al.*, 1997). The two nucleotides separating the heptamers are usually A or T to accommodate dimerization over the minor groove (Maris *et al.*, 2005). In cocrystals, protein–DNA interaction occurs through primary base-pair recognition by residues Lys-188, Val-189 and Lys-192, as well as secondary DNA backbone interactions (Maris *et al.*, 2005, 2002). TraR interacts similarly with its DNA targets (White & Winans, 2007).

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Abbreviations: CTD, carboxyl-terminal domain; EMSA, electrophoretic mobility shift assay; HTH, helix–turn–helix; PC, positive control.

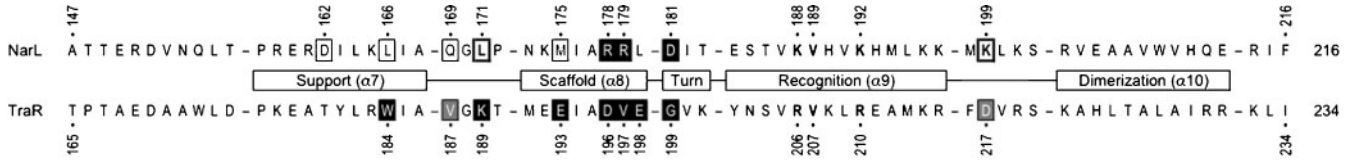

Fig. 1. NarL and TraR CTD sequences. The four α-helices include the central HTH element. Results for TraR are from Qin *et al.* (2009) and White & Winans (2005). Boxed residues indicate phenotypes for Ala substitutions: black, PC; white, functional; bold type and outline, deficient. Grey-shaded boxes indicate positions where substitution with residues other than Ala results in the PC phenotype. Residues in bold type are implicated in direct recognition of DNA (Maris *et al.*, 2005; White & Winans, 2007). The TraR-CTD sequence shown is from plasmid pTiR10 (White & Winans, 2005); the TraR-CTD sequence from plasmid pTiC58 differs at three positions (Val-168, Met-189 and Val-194) (Qin *et al.*, 2009).

NarL and its paralogue NarP regulate transcription from target operon control regions with various binding-site architectures (Stewart & Rabin, 1995) (Fig. 2). Most of these are activated by Fnr, which functions during anaerobiosis (Browning *et al.*, 2002; Kiley & Beinert, 1998). Control regions are classified according to the location of the activator binding site (Barnard *et al.*, 2004). Nar class II control regions, exemplified by the *napF* and *yeaR* operons (Lin *et al.*, 2007; Squire *et al.*, 2009; Stewart & Bledsoe, 2005), have a single Nar-binding site immediately adjacent to the promoter. Nar class I control regions, exemplified by the *narG* and *fdnG* operons, have two Nar-binding sites upstream of the promoter (Stewart & Rabin, 1995). For the *nirB* and *nrfA* control regions, the single upstream Nar-binding site functions in remodelling an inhibitory nucleoprotein complex (Barnard *et al.*, 2004). Finally, NarL and NarP repress transcription from several control regions, exemplified by synthetic constructs in which a Nar 7–2–7 binding site has replaced the *lacZ* operon primary operator O1 (Stewart & Bledsoe, 2003).

GerE functions as a direct transcriptional activator (Zheng *et al.*, 1992), demonstrating that the GerE-family domain can make activating contacts with RNA polymerase. Likewise, the isolated LuxR (Choi & Greenberg, 1991) and FixJ (Kahn & Ditta, 1991) CTDs function as class II activators at some promoters. Residues required for activation have been identified in GerE (Crater & Moran, 2002), LuxR (Egland & Greenberg, 2001), TraR (Costa *et al.*, 2009; Qin *et al.*, 2009; White & Winans, 2005) and FixJ (Ton-Hoang *et al.*, 2001).

Here we report results from experiments designed to examine DNA binding and transcriptional activation by

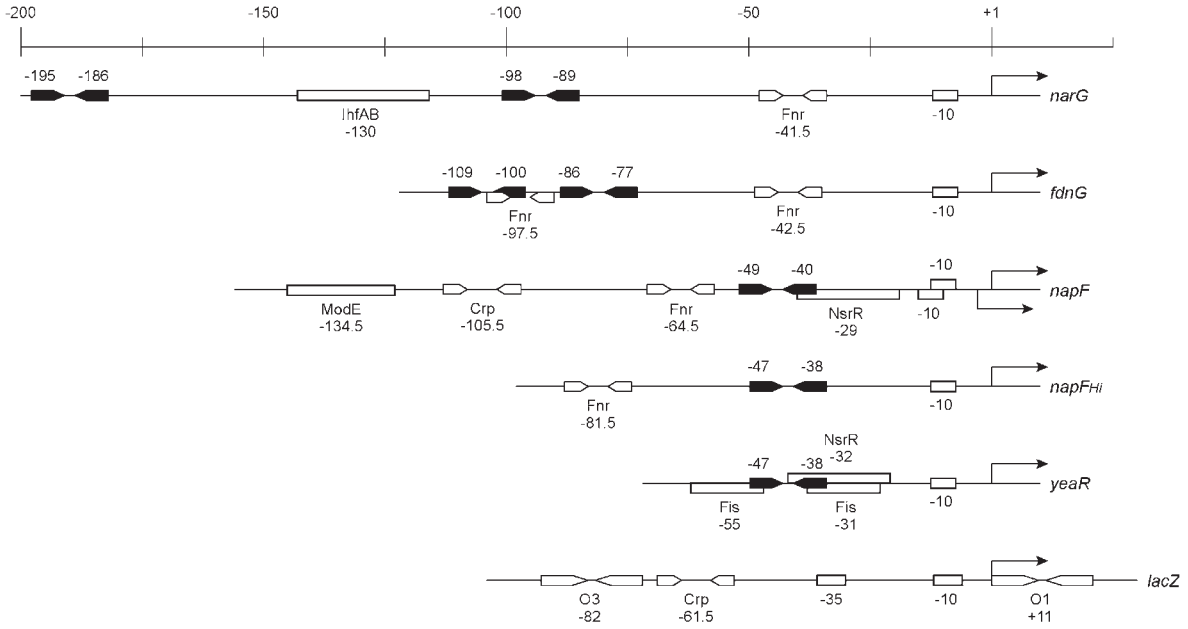

Fig. 2. Control regions used in this study. The scale is in nucleotides. Nar 7–2–7 heptamer pairs are depicted as black inverted arrows, whereas sites for other proteins are depicted as white boxes or inverted arrows. Numbers show positions of binding-site centres relative to the transcription initiation point.

NarL and NarP. The findings provide context for understanding NarL structure in relation to its functions.

METHODS

Mutants and their analysis

Strain construction. Strains and plasmids are listed in Table 1. Mutant alleles were transferred between strains by bacteriophage P1-mediated generalized transduction (Miller, 1972). For some strains, *att*80-integrated alleles were transferred by selection for the adjacent *trp*$^+$ marker. Standard methods were used for restriction endonuclease digestion, ligation, transformation and PCR amplification (Maloy *et al.*, 1996). Oligonucleotide-directed site-specific mutagenesis followed the QuikChange protocol (Stratagene Cloning Systems), as described previously (Lin *et al.*, 2007).

Null alleles. The Δ*narL261* allele was constructed through λRed-mediated recombineering (Datsenko & Wanner, 2000), using PCR primers LLC1287 and LLC1288 (5′-ATGGCACCAGATATCACCG-TGGTTGGCGAAGCGAGTgtgtaggctggagctgcttc-3′ and 5′-CATTTT-CTTCAGCATGTGCTTGACGTGCACTTTTACattccggggatccgtcgacc-3′, respectively) with plasmid pKD13 as template (sequence complementary to pKD13 is shown in lower case). This results in deletion of codons Asn-40 to Thr-186 (216 codons in total).

The Δ*narP262* allele was constructed by using primers LLC1281 and LLC1282 (5′-ATGCCTGAAGCAACACCTTTTCAGGTGATGATTG-TGgtgtaggctggagctgcttc-3′ and 5′-TTATTGTGCCCCGCGTTGTTG-CAGGAACAGAATGGTattccggggatccgtcgacc-3′, respectively). This results in deletion of codons Asp-13 to Ala-204 (215 codons in total).

The Δ*narQ264* allele was constructed by using primers LLC1279 and LLC1280 (5′-GTGATTGTTAAACGACCCGTCTCGGCCAGTCTGG-CCgtgtaggctggagctgcttc-3′ and 5′-TTACATTAACTGACTTTCCTCAC-CCTCCGCAGAGCGattccggggatccgtcgacc-3′, respectively). This results in deletion of codons Arg-13 to Phe-555 (566 codons in total). For all three of these alleles, the residual scar sequence following excision of the *aphA* gene includes an in-frame nonsense codon.

The Δ*narX263* allele, designed to mimic the previously characterized Δ*narX242* allele (Egan & Stewart, 1990), was constructed by using primers LLC1285 and LLC1286 (5′-ATGGCGATGCTTGGAACTG-CGTTGAACAATATGTCTattccggggatccgtcgacc-3′ and 5′-GCGGAA-TGTGGTGAGCAATTCACGCAACTGCGCCCgtgtaggctggagctgcttc-3′, respectively). This results in deletion of codons Ala-221 to Ser-441 (598 codons in total). The residual scar sequence does not contain any in-frame nonsense codons. Thus, this deletion does not have polar effects on expression of the overlapping *narL* gene (Egan & Stewart, 1990).

***narL*† and *narP*† alleles.** The *narL* gene is autoregulated, whereas the *narP* gene is not. Thus, to ensure equivalent expression levels, the *narP* upstream transcription control region and Shine–Dalgarno sequence were fused to the *narL* initiation codon. This was accomplished by introducing *Nde*I restriction endonuclease sites overlapping the initiation codons for *narL* (CCC ATG changed to CAT ATG) and *narP* (ACT ATG changed to CAT ATG), and then subcloning the *narL* gene from *Nde*I to a downstream *Bam*HI site into the *narP* plasmid, replacing the corresponding *narP* sequence.

These *Nde*I-modified alleles were engineered to contain two additional restriction endonuclease sites. *Ngo*MIV sites were introduced near the end of the receiver domain-coding regions (helix α5; *narL* codons 125–126, GCT GGC changed to GCC GGC; *narP* codons 124–125, GCG AAA changed to GCC GGC), and *Xho*I sites were introduced near the beginning of the CTD-coding regions (helix α7; *narL* codons 164–165, CTC AAG changed to CTC GAG; *narP* codons 162–163, CTG CAC changed to CTC GAG). These sites, which result in missense substitutions (NarL, Lys-165 to Glu; NarP, Lys-125 to Gly and His-163 to Glu), were designed for a separate study to analyse properties of NarL–NarP chimerae. Control experiments established that these missense substitutions did not influence regulatory phenotypes.

These modified genes are denoted as *narL*† and *narP*† to distinguish them from the wild-type. The *narL*† and *narP*† genes were recloned, from the *Eco*RI site 490 nt upstream of the *narP* initiation codon to the downstream *Bam*HI site, into the moderate-copy-number plasmid pSU19. The cloned inserts are in opposite orientation to that of the vector *lacZ* promoter.

Monocopy *narL*† and *narP*† alleles. Conditional-replication, integration and modular (CRIM) plasmids of Haldimann & Wanner (2001) were used to place modified *narL* and *narP* alleles at the chromosomal prophage attachment site for Φ80 (centisome 28; plasmid pAH153). Chromosomal integration and PCR analysis to confirm the resulting strains were performed essentially as described by Haldimann & Wanner (2001).

***narL-CTD* and *narP-CTD* alleles.** *Sph*I sites were introduced in the interdomain linker coding regions (*narL* codons 147–148, GCC ACT changed to GCA TGC; *narP* codons 146–147, GCG GAA changed to GCA TGC). Fragments (*Sph*I–*Hin*dIII) were cloned into plasmid LITMUS 39, and then recloned (*Sal*I–*Hin*dIII) into plasmid pSU18. The resulting plasmids were designed to express NarL-CTD and NarP-CTD with vector-derived amino-terminal extensions of 20 residues (MTMITNSSSVPGDPLESTAC), corresponding to the LacZ amino terminus and polylinker.

Culture media and conditions. Defined, complex and indicator media for genetic manipulations were used as described previously (Maloy *et al.*, 1996). Defined medium to grow cultures for enzyme assays was buffered with MOPS, as described previously (Stewart & Parales, 1988). Medium for overnight cultures arrested in the mid-exponential phase contained glucose (6 mM) or glucose plus $NaNO_3$ (4 and 10 mM, respectively) as indicated (Stewart & Bledsoe, 2003).

Plasmid-bearing strains were cultured in tryptone yeast extract glucose (TYEG) medium, which contains 0.8 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, Vogel–Bonner phosphate-buffered salts (Maloy *et al.*, 1996) and 10 mM glucose. $NaNO_3$ (40 mM) was added as indicated.

Cultures were grown at 37 °C to the mid-exponential phase, about 35–40 Klett units. Culture densities were monitored with a Klett–Summerson photoelectric colorimeter (Klett Manufacturing) equipped with a number 66 (red) filter. Anaerobic cultures were grown in screw-capped tubes, as described previously (Stewart & Parales, 1988).

Transcription reporters. Control region output was measured from *lacZ* fusions (Table 1) integrated at the chromosomal prophage attachment site for λ (centisome 17). The Φ(*narG–lacZ*) and Φ(*fdnG–lacZ*) reporters are activated by NarL but only weakly by NarP (Stewart & Rabin, 1995). Activities (Miller units) after growth in the absence and presence of nitrate were 37 and 2610 (*narG*), and 22 and 490 (*fdnG*).

The Φ($napF_{Ec}$–*lacZ*) and Φ($napF_{Hi}$–*lacZ*) reporters, from *E. coli* and *Haemophilus influenzae*, respectively, are activated by NarP. The Φ($napF_{Hi}$–*lacZ*) reporter is also activated by NarL (Stewart & Bledsoe, 2005), as is the P2$^-$ promoter mutant version of the Φ($napF_{Ec}$–*lacZ*) reporter (Stewart *et al.*, 2003). The Φ(*yeaR–lacZ*) reporter is an Fnr-independent Nar class II control region; the version used here lacks the binding site for the nitric oxide-responsive NsrR repressor (Lin *et al.*, 2007). Activities (Miller units) after growth in the absence and

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
***E. coli* K-12 strains**		
BW25113	*hsdR lacI*^{Q} Δ(*lacZ*) Δ(*araBAD*) Δ(*rhaBAD*)	Datsenko & Wanner (2000)
VJS632	F^{-} λ^{-} prototroph	Stewart & Parales (1988)
VJS676	As VJS632 but Δ(*argF–lacIZYA*)*U169*	Stewart & Parales (1988)
VJS8364	As VJS632 but Δ*lacZ*	Lin *et al.* (2007)
Derivatives of VJS676		
VJS2197	λΦ(*narG–lacZ*)	Rabin & Stewart (1992)
VJS4147	λΦ(*fdnG–lacZ*) [Δ313; Fnr 2^{-}]	Li & Stewart (1992)
VJS6317	λ[O1-*nirB lacZ*$^{+}$ *Y*$^{+}$ *A*$^{+}$] *narX*$^{+}$ *narL215*::Tn*10 narQ*$^{+}$ *narP253*::Tn*10*d(Cm)	Stewart & Bledsoe (2003)
VJS6906	λΦ(*napF$_{Hi}$–lacZ*) [Δ260]	Stewart & Bledsoe (2005)
VJS6990	λΦ(*napF–lacZ*) [Δ146; P2^{-}]	Stewart *et al.* (2003)
VJS7449	λ^{-}Δ(*attλ-lom*)::*bla* [O1-*napF lacZ*$^{+}$]	Stewart & Bledsoe (2003)
VJS7489	λ^{-}Δ(*attλ-lom*)::*bla* [O1-*nrfA lacZ*$^{+}$]	Stewart & Bledsoe (2003)
VJS7623	λ^{-}Δ(*attλ-lom*)::*bla* [O1-*nirB* −74/−74 *lacZ*$^{+}$]	This work
VJS9284	λΦ(*napF$_{Hi}$–lacZ*) [Δ260] *narX*$^{+}$ Δ*narL261 narQ*$^{+}$ Δ*narP262*	This work
VJS9719	λ^{-}Δ(*attλ-lom*)::*bla* [O1-*napF lacZ*$^{+}$] *narX*$^{+}$ Δ*narL261 narQ*$^{+}$ Δ*narP262*	This work
VJS10030	λ[O1-*nirB lacZ*$^{+}$ *Y*$^{+}$ *A*$^{+}$] *narX*$^{+}$ *narL215*::Tn*10* Δ*narQ264*::*aph narP253*::Tn*10*d(Cm)	This work
VJS10054	λ^{-}Δ(*attλ-lom*)::*bla* [O1-*nirB* −74/−74 *lacZ*$^{+}$] *narX*$^{+}$ Δ*narL261*::*aph* Δ*narQ264* Δ*narP262 trpB*::Tn*10*	This work
VJS10247	λΦ(*napF$_{Hi}$–lacZ*) [Δ260] *narX*$^{+}$ Δ*narL261* Δ*narQ264* Δ*narP262*	This work
VJS10248	λΦ(*fdnG–lacZ*) [Δ313; Fnr2^{-}] *narX*$^{+}$ Δ*narL261* Δ*narQ264* Δ*narP262*	This work
VJS10258	λΦ(*narG–lacZ*) *narX*$^{+}$ Δ*narL261*::*aph* Δ*narQ264* Δ*narP262*	This work
VJS10461	λ^{-}Δ(*attλ-lom*)::*bla* [O1-*nrfA lacZ*$^{+}$] *narX*$^{+}$ Δ*narL261*::*aph* Δ*narQ264* Δ*narP262 trpB*::Tn*10*	This work
VJS10649	λΦ(*napF–lacZ*) [Δ146; P2^{-}] *narX*$^{+}$ Δ*narL261*::*aph* Δ*narQ264* Δ*narP262*	This work
VJS10665	λ^{-}Δ(*attλ-lom*)::*bla* [O1-*napF lacZ*$^{+}$] *narX*$^{+}$ Δ*narL261*::*aph* Δ*narQ264* Δ*narP262*	This work
Derivatives of VJS8364		
VJS9565	λ^{-}Δ(*attλ-lom*)::*bla* {Φ(*yeaR–lacZ*) [Δ175]} (NsrR^{-})	Lin *et al.* (2007)
VJS10983	λ^{-}Δ(*attλ-lom*)::*bla* {Φ(*yeaR–lacZ*) [Δ175]} (NsrR^{-}) *narX*$^{+}$ Δ*narL261 narQ*$^{+}$ Δ*narP262*	This work
VJS11159	λ^{-}Δ(*attλ-lom*)::*bla* {Φ(*yeaR–lacZ*) [Δ175]} (NsrR^{-}) *narX*$^{+}$ Δ*narL261 narQ*$^{+}$ Δ*narP262 trpB*::Tn*10*	This work
Plasmids		
LITMUS 39	Ap^{r}; *ori* pMB9	New England Biolabs
pACYC184	Cm^{r}, Tc^{r}; *ori* P15A	Chang & Cohen (1978)
pAH69	Ap^{r}; *int* HK022; *ori* pSC101(Ts)	Haldimann & Wanner (2001)
pAH123	Ap^{r}; *int* Φ80; *ori* pSC101(Ts)	Haldimann & Wanner (2001)
pAH144	Sm^{r}; *att* HK022; *ori* R6Kγ	Haldimann & Wanner (2001)
pAH153	Gm^{r}; *att* Φ80; *ori* R6Kγ	Haldimann & Wanner (2001)
pCP20	Ap^{r}, Cm^{r}; Flp^{+}; *ori* pSC101(Ts)	Datsenko & Wanner (2000)
pKD13	Ap^{r}, Km^{r}; *ori* R6Kγ	Datsenko & Wanner (2000)
pKD46	Ap^{r}; Red^{+}; *ori* pSC101(Ts)	Datsenko & Wanner (2000)
pQE30	Ap^{r}; *ori* pMB9	Qiagen
pQE32	Ap^{r}; *ori* pMB9	Qiagen
pSU18	Cm^{r}; *ori* P15A; pUC18 polylinker	Bartolomé *et al.* (1991)
pSU19	Cm^{r}; *ori* P15A; pUC19 polylinker	Bartolomé *et al.* (1991)
pVJS2288	Ap^{r}; His$_{6}$-*narP* in pQE32	Noriega *et al.* (2010)
pVJS2294	Ap^{r}; His$_{6}$-*narL* in pQE30	Noriega *et al.* (2010)
pVJS4095	Cm^{r}; *narL*† in pSU19	This work
pVJS4098	Cm^{r}; *narP*† in pSU19	This work
pVJS4505	Cm^{r}; *narL-CTD* in pSU18	This work
pVJS4506	Cm^{r}; *narP-CTD* in pSU18	This work
pVJS5259	Ap^{r}; His$_{6}$-*narP-CTD* in pQE30	This work
pVJS5265	Ap^{r}; His$_{6}$-*narL-CTD* in pQE30	This work

presence of nitrate were 13 and 160 ($napF_{Hi}$), 88 and 1730 ($napF_{Ec}$ P2$^-$), and 200 and 8040 (*yeaR*).

O1-*lac* substitution reporters have Nar 7–2–7 binding sites in place of the primary operator. The O1-*nirB*, O1-*napF* and O1-*nrfA* versions have been described previously (Stewart & Bledsoe, 2003). Identical methods were used to construct the O1-*nirB* (−74/−74) (5′-AATACCCATATATGGGTATT-3′) version. Activities (Miller units) after growth in the absence and presence of nitrate were 1930 and 74 (O1-*nirB*), 1730 and 330 (O1-*napF*), 6360 and 150 (O1-*nrfA*), and 1240 and 200 (O1-*nirB* −74/−74).

LacZ assay. β-Galactosidase activities were determined as described by Miller (1972). All cultures were assayed in duplicate, and reported values were averaged from at least two independent experiments. Relative activation or repression as percentages of the corresponding wild-type values were calculated as described elsewhere (Zhang *et al.*, 1992).

Proteins and their analysis

Purification. Isolation of His_6-NarL and His_6-NarP has been described previously (Noriega *et al.*, 2010); essentially identical methods were used to prepare His_6-NarL-CTD and His_6-NarP-CTD. Expression constructs were made by cloning *narL* or *narP* sequence from the introduced *Sph*I site (within the interdomain linker coding region) into plasmid pQE30. The amino termini were MRGSH$_6$GSA*CTERD*... for His_6-NarL-CTD, and MRGSH$_6$GSA*EDPF*... for His_6-NarP-CTD (NarL and NarP sequence is in italic type). This His_6-NarL-CTD protein is virtually identical to that used for X-ray analysis of NarL–DNA interaction, which has an amino terminus of MRGSH$_6$GS*ATTERD*... (Maris *et al.*, 2002).

Electrophoretic mobility shift assays (EMSAs). Templates were prepared from plasmid pVJS3253 constructs in which the *lac* operon primary operator O1-*lac* has been substituted with Nar-binding sites (Stewart & Bledsoe, 2003). Primers AVL2478 and AVL2479 (5′-GACGCCCGCCATAAACTGCCAGGAATTG-3′ and 5′-CGCCAGGGTTTTCCCAGTCACGACG-3′, respectively), which anneal upstream and downstream of O1-*lac*, respectively, generate 332 bp products. These yielded 296 bp fragments after digestion with *Eco*RI, which cleaves at a site introduced near the end of the *lacI* gene. The O1-*lac* substitutions are at the centre of the resulting fragments, which were end-labelled with [α-^{32}P]dATP (Perkin Elmer) by using DNA polymerase I large fragment (Klenow) (New England Biolabs).

EMSA followed the procedure of Maris *et al.* (2002). Briefly, proteins were incubated with 2 nM ^{32}P-labelled DNA for 10 min at room temperature in reaction buffer containing 10 mM Tris (pH 7.5), 50 mM KCl, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 15 mg poly-dIdC ml^{-1} and 10 % glycerol (v/v). Acetyl phosphate at 100 mM was added for phosphorylation of His_6-NarL and His_6-NarP; control assays established that this concentration did not influence the EMSA. The reaction mixture was loaded immediately onto a 6 % non-denaturing polyacrylamide gel running at 100 V, and allowed to electrophorese for 1 h at 4 °C. Radiolabelled bands were analysed using a Storm PhosphorImager Scanner with ImageQuant Software (Molecular Dynamics).

RESULTS

NarL and NarP DNA binding

The affinities of NarL-CTD for several 7–2–7 target sites *in vitro* have been determined by using EMSA (Maris *et al.*, 2005). We chose three of these sites to measure affinities for NarP-CTD, also by using EMSA. Target sequences were the native sites from the *nirB* and *napF* operons, and an artificial site consisting of two copies of *nirB* heptamer −74 in inverted orientation. Our version of the *nirB* (−74/−74) site (central AT) is subtly different from that used earlier (central TA) (Maris *et al.*, 2005).

The K_D for NarL-CTD binding to the *nirB* site (Table 2) was similar to that reported earlier: 0.45 μM (Maris *et al.*, 2005). By constrast, we measured substantially higher K_D values for the *nirB* (−74/−74) and *napF* sites, which were reported as 0.65 and 0.45 μM, respectively. The reason(s) for these differences is unknown. NarL-dependent repression at *lac* O1-substitution constructs was much stronger for the *nirB* site than for the *nirB* (−74/−74) and *napF* sites (Stewart & Bledsoe, 2003), so relative affinities *in vitro* and *in vivo* were broadly correlated.

Full-length NarL, phosphorylated by incubation with acetyl phosphate, exhibited affinities similar to those measured for NarL-CTD (Table 2), extending the observation made with the *narG* (−89/−89) site (Maris *et al.*, 2002). By contrast, full-length NarP exhibited very weak binding even after incubation with a high concentration of acetyl phosphate

Table 2. DNA-binding affinities

Protein	Affinity at site [K_D (μM)]*		
	O1-*nirB* (−74/−74)	O1-*nirB*	O1-*napF*
His_6-NarL†	1.0 ± 0.05	0.3 ± 0.1	3.2 ± 0.08
His_6-NarL-CTD	9 ± 2	0.1 ± 0.06	2.3 ± 0.5
His_6-NarP-CTD	4 ± 1	0.2 ± 0.06	6 ± 1
His_6-NarP-CTD (K186A)	1.0 ± 0.2	–‡	–
His_6-NarP-CTD (V187A)	>30	–	–
His_6-NarP-CTD (R190A)	>30	–	–

*EMSA with binding site amplified from the indicated O1-substitution constructs.
†Reaction performed with 100 mM acetyl phosphate.
‡–, Not determined.

(Lin, 2009). We hypothesize that acetyl phosphate is a relatively poor substrate for NarP autophosphorylation, but have not pursued this point further. Nevertheless, NarP-CTD exhibited strong binding to all three sites (Table 2). This confirms that the NarP receiver inhibits binding, similar to NarL, and that NarP-CTD binds target sites *in vitro* as avidly as NarL-CTD.

The NarL-CTD–DNA X-ray structure shows recognition helix residues Lys-188, Val-189 and Lys-192 in direct contact with major groove base pairs (Maris *et al.*, 2005, 2002) (Fig. 1). We made Ala substitutions at each of these positions, and measured effects on NarL-CTD affinity for DNA *in vitro* and on NarL repression at O1-*lac* substitution constructs *in vivo*. The V189A substitution substantially decreased binding in both assays (Table 3). Similar results were obtained with the corresponding mutants TraR V207A *in vivo* and *in vitro* (White & Winans, 2007), and NarP V187A *in vitro* (Table 2).

The NarL K192A substitution abolished binding in both assays, as expected from the previous observation that the K192C substitution eliminates binding *in vitro* (Xiao *et al.*, 2002). Similar results were obtained with the NarP R190A mutant *in vitro*, although the TraR R210A mutant exhibited 15–20 % of wild-type binding both *in vivo* and *in vitro* (White & Winans, 2007).

Finally, the NarL K188A and NarP K186A substitutions reduced binding *in vitro* by only two- to threefold (Tables 2 and 3). NarL residue Lys-188 contacts DNA positions 5, 6 and 7, which are less conserved in NarL heptamer sequences and therefore may be less critical for overall affinity. Nevertheless, the NarL K188A mutant exhibited very weak repression *in vivo* (Table 3), hinting that additional parameters (such as supercoiling) influence binding-site recognition. By contrast, the TraR R206A mutant displayed undetectable binding in both assays (White & Winans, 2007).

NarL-CTD and NarP-CTD transcription control

It has been reported that NarL-CTD 'is not sufficient for transcriptional activation ... (data not shown)' (Maris *et al.*, 2002). Because Nar-dependent control regions are complex and varied (Fig. 2), we wished to revisit this conclusion. Accordingly, we monitored target operon expression in *narL narP* double null strains expressing either full-length or CTD versions of NarL and NarP from medium-copy-number plasmids.

The NarL-CTD and NarP-CTD proteins activated Φ(*napF*$_{Hi}$–*lacZ*) transcription to the same extent as their full-length counterparts (Table 4). Similarly, NarP and NarP-CTD were equally effective activators of expression from the wild-type Φ(*napF*$_{Ec}$–*lacZ*) reporter (Lin, 2009), which is not activated by NarL (Stewart *et al.*, 2003). Finally, the CTD proteins were about 20–25 % as effective as their full-length counterparts for stimulating Φ(*yeaR*–*lacZ*) expression. Thus, the CTD proteins were competent for activating transcription from Nar class II control regions.

By contrast, the CTD proteins were very weak repressors of transcription from the *lac* O1-substitution constructs O1-*napF* (Table 4), O1-*nirB* and O1-*nrfA* (Lin, 2009), even though the CTD proteins bound these sites well *in vitro* (Table 2). Moreover, neither CTD protein activated Φ(*narG*–*lacZ*) or Φ(*fdnG*–*lacZ*) expression (Lin, 2009). These results imply that the receiver domain is important for these processes *in vivo*.

NarL positive control (PC) mutants

Specific side-chain determinants of transcription activation are identified by PC missense substitution alleles whose products display near-normal DNA binding but are defective in transcription activation (Browning *et al.*, 2002). In order to identify NarL PC substitutions, we used site-specific mutagenesis to substitute Ala for nine different surface-exposed residues (Fig. 3), focusing on those identified as conferring the PC phenotype for TraR (Qin *et al.*, 2009; White & Winans, 2005). (A tenth mutant, D180A, yielded conflicting results in different assays and so was excluded.)

Table 3. Effects of recognition helix alterations on the NarL–DNA interaction

narL allele*	Affinity [K_D (μM)]† O1-*nirB* (−74/−74)	Percentage of wild-type repression‡ in strain§		
		O1-*nirB* (−74/−74)	O1-*nirB*	O1-*napF*
None	–	0	0	0
Wild-type	0.9 ± 0.2	100	100	100
K188A	1.8 ± 0.7	26	3.4	15
V189A	11 ± 2	46	58	57
K192A	>30	<0.1	17	21

*Indicated His_6-NarL-CTD protein or *narL* allele integrated at *att* Φ80.

†EMSA with binding site amplified from the indicated O1-substitution construct.

‡Percentage repression or activation relative to wild-type value during growth with nitrate (Zhang *et al.*, 1992).

§VJS10054 [O1-*nirB* (−74/−74)], VJS10030 (O1-*nirB*) and VJS10665 (O1-*napF*).

Table 4. Transcription control by NarL-CTD and NarP-CTD

Plasmid	Activator	Percentage of wild-type regulation* in strain†		
		Repression O1-*napF*	Class II activation	
			Φ(*napF$_{Hi}$–lacZ*)	Φ(*yeaR–lacZ*)
pACYC184	None	0	0	0
pVJS4095	NarL	100	100	100
pVJS4505	NarL-CTD	66	98	21
pVJS4098	NarP	99	155	76
pVJS4506	NarP-CTD	63	154	20

*Percentage repression or activation relative to wild-type value during growth with nitrate (Zhang *et al.*, 1992).
†VJS9284 [λΦ(*napF$_{Hi}$–lacZ*)], VJS10983 [λΦ(*yeaR–lacZ*)] and VJS9719 (O1-*napF*).

We assayed DNA binding *in vivo* by measuring repression at the *lac* O1-substitution constructs O1-*nrfA* and O1-*napF*, and we tested for activation from the class I Φ(*narG–lacZ*) and Φ(*fdnG–lacZ*) reporters, and from the class II Φ(*napF$_{Ec}$–lacZ*) and Φ(*napF$_{Hi}$–lacZ*) reporters (Table 5). Most of the mutants exhibited close to 100 % of the wild-type repression value for both of the O1-substitution constructs. The two exceptions were the L171A and K199A mutants, which exhibited less than 90 % repression with at least one construct (Table 5). These mutants also displayed the strongest defects in activation from both the class I and the class II reporters. Therefore, we classify these mutants as deficient for functions in addition to transcription activation.

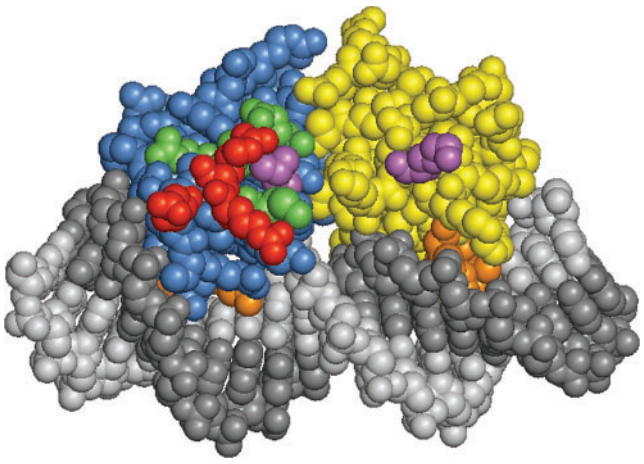

Fig. 3. X-ray model of a NarL-CTD dimer in complex with its DNA-binding site from the *nirB* operon control region; from Maris *et al.* (2005). The two protomers are coloured blue and gold, and the two DNA strands are shaded light and dark grey. Residues are highlighted according to their phenotype: PC, red (Arg-178, Arg-179, Asp-181); functional, green (Asp-162, Leu-166, Gln-169, Met-175); deficient, violet (Leu-171, Lys-199); DNA-binding, orange (Lys-188, Val-189, Lys-192).

Four other mutants, denoted as functional (D162A, M175A, Q169A and L166A), displayed 70 % or more of the wild-type activation value for at least one reporter from each class. The remaining three mutants, denoted as PC (R178A, R179A and D181A), exhibited 60 % or less of the wild-type value for both class I reporters (Table 5). However, all had 85 % or more of the wild-type value for both class II reporters.

To examine these mutants further, we made double substitutions. The R178A + D181A mutant yielded conflicting results in different assays, and so was excluded. The other two double mutants exhibited robust repression, but defective transcription activation of all reporters including Φ(*napF$_{Ec}$–lacZ*) and Φ(*napF$_{Hi}$–lacZ*). In particular, the R178A + R179A double mutant displayed a synergistic effect, with sharply reduced activation of the Φ(*narG–lacZ*) and Φ(*fdnG–lacZ*) reporters (Table 5).

The R178A mutant had the strongest PC phenotype. To examine this mutant further, we tested for activation of the Fnr-independent, class II Φ(*yeaR–lacZ*) reporter. Indeed, the R178A mutant exhibited a strong defect in transcription activation (29 %), whereas the R179A mutant was near-normal (79 %). Therefore, the class II PC phenotype of the R178A mutant apparently was masked by synergy with Fnr at the *napF* control regions.

DISCUSSION

The receiver domain of response regulators controls activity depending on its phosphorylation state. GerE-family CTDs define one of the three major categories of DNA-binding response regulators (Galperin, 2006), for which the NarL protein provides a well-studied model. This investigation evaluated NarL-CTD involvement in transcription control and DNA binding. Results are congruent with those for other GerE-family proteins, including the well-understood TraR regulator.

Transcription from most Nar-regulated operons is activated by Fnr, so NarL and NarP impose nitrate-responsive

Table 5. Phenotypes of NarL missense mutants

narL allele*	Percentage of wild-type regulation† in strain‡					
	Repression		Class I activation		Class II activation	
	O1-*nrfA*	O1-*napF*	Φ(*narG–lacZ*)	Φ(*fdnG–lacZ*)	Φ($napF_{Ec}$–*lacZ*)	Φ($napF_{Hi}$–*lacZ*)
None	0	0	0	0	0	0
Wild-type	100	100	100	100	100	100
Deficient						
L171A	85	68	1.2	24	48	56
K199A	94	86	1.0	26	40	43
Functional						
D162A	96	97	70	52	52	86
M175A	100	101	86	92	103	185
Q169A	99	102	98	96	57	313
L166A	98	99	119	85	70	242
Positive control						
R178A	101	99	18	33	86	86
R179A	100	101	61	52	226	100
D181A	98	102	52	41	146	93
R178A + R179A	101	105	<0.1	5.5	8	28
R179A + D181A	101	81	39	14	20	31

*Indicated allele integrated at *att* Φ80.

†Percentage repression or activation relative to wild-type value during growth with nitrate (Zhang *et al.*, 1992).

‡VJS10461 (O1-*nrfA*), VJS9719 (O1-*napF*), VJS10649 [λΦ(*napF–lacZ*)], VJS10247 [λΦ($napF_{Hi}$–*lacZ*)], VJS10258 [λΦ(*narG–lacZ*)] and VJS10248 [λΦ(*fdnG–lacZ*)].

control on top of Fnr-dependent anaerobic induction. Activation is synergistic, because expression in wild-type strains is much higher than that in either *fnr* or *nar* null strains. Synergy is revealed further by studies of *narG* operon expression in *fnr* PC mutants (Lamberg & Kiley, 2000). Although basal anaerobic expression is low in these mutants, expression during growth with nitrate is near-normal. Thus, NarL bypasses the Fnr PC defect by providing additional contacts to Fnr and/or RNA polymerase (Barnard *et al.*, 2004; Browning *et al.*, 2002).

Liberated Nar CTDs activate transcription from Nar class II control regions

NarL-CTD and NarP-CTD, liberated from their receiver domains (Morrison & Parkinson, 1994) and expressed from plasmids, fully activated transcription from the Fnr-dependent $napF_{Hi}$ control region, but they were less effective at the Fnr-independent *yeaR* operon control region (Table 4). This difference between the two control regions may reflect Fnr stabilization of CTD binding at the former, or it may result from inefficient nucleoprotein remodelling at the latter (Squire *et al.*, 2009). Nevertheless, overall results demonstrate that NarL-CTD and NarP-CTD can activate transcription from class II control regions, consistent with prior studies of GerE, LuxR-CTD and FixJ-CTD (Choi & Greenberg, 1991; Kahn & Ditta, 1991; Zheng *et al.*, 1992).

NarL PC mutants

Following the strategy employed by others (Crater & Moran, 2002; Egland & Greenberg, 2001; Qin *et al.*, 2009; White & Winans, 2005), we substituted Ala for surface-exposed residues on NarL-CTD and evaluated the resulting mutants for PC phenotypes. All were studied in the context of full-length protein expressed from chromosomal monocopy constructs. We examined all of the residues that correspond to PC substitutions identified for TraR-CTD (Qin *et al.*, 2009; White & Winans, 2005).

Ala substitutions at three positions (Arg-178, Arg-179 and Asp-181) yielded unambiguous PC phenotypes for expression from the Nar class I *narG* and *fdnG* operon control regions (Table 5, Fig. 1). These residues form a cluster around the end of the scaffold helix, and therefore are positioned appropriately for contact to RNA polymerase (Fig. 3).

Like TraR Gly-199 (Qin *et al.*, 2009), Ala substitution for NarL Asp-181 resulted in a class I-specific PC phenotype. Ala substitutions for NarL Arg-178 and Arg-179 also appeared to be class I-specific PC mutants, unlike their TraR counterparts (Asp-196 and Val-197). However, the R178A + R179A and R179A + D181A double mutants exhibited strong class II PC phenotypes. Moreover, the R178A mutant exhibited a strong PC phenotype for activation from the Fnr-independent Nar class II *yeaR* operon control region (Lin, 2009). Therefore, Fnr–NarL

synergy may obscure NarL PC phenotypes in addition to Fnr PC phentoypes (Lamberg & Kiley, 2000).

Substitutions at four other positions resulted in functional phenotypes. Two of these, Leu-166 and Met-175, correspond to TraR residues Trp-184 and Glu-193, for which Ala substitutions yielded PC phenotypes (Qin *et al.*, 2009). The third NarL position, Gln-169, corresponds to TraR Val-187, for which Glu and Ile but not Ala substitution resulted in PC phenotypes (White & Winans, 2005).

Ala substitutions at Leu-171 and Lys-199 strongly affected transcription activation from all reporters tested (Table 5). However, these mutants also exhibited relatively weak repression, and therefore are classified as deficient.

Activation from class I and class II control regions involves interaction with RNA polymerase subunits α-CTD, and with α and σ, respectively (Barnard *et al.*, 2004). However, since NarL works in synergy with Fnr, these NarL PC mutants might be defective in interaction with Fnr in addition to (or instead of) RNA polymerase. We attempted to study this by examining phenotypes of *narL fnr* double PC mutants. Initial results were inconclusive (Lin, 2009), so this point was not pursued further.

Possible roles for the NarL and NarP receiver domains

Both CTD and phosphorylated full-length Nar proteins bind DNA *in vitro* with equal affinities (Maris *et al.*, 2005, 2002) (Table 2). We therefore were surprised to find that the CTD proteins mediated inefficient repression at *lacO1*-substitution constructs (Table 4) (Lin, 2009), because repression is a function of DNA-binding affinity (Schlax *et al.*, 1995). This implies that, *in vivo*, the receiver domain stabilizes the CTD, enhances DNA binding or enables repression per se.

By contrast, we were not surprised to find that the CTD proteins did not activate transcription from the Nar class I control regions for the *narG* and *fdnG* operons (Lin, 2009), because we hypothesize that the NarL receiver mediates cooperative binding to the multiple sites in these control regions (Stewart & Bledsoe, 2008). Additionally, the receiver may make protein–protein contacts necessary for transcription activation, as shown by isolation of PC substitutions in the FixJ receiver and TraR amino-terminal ligand-binding domain (Costa *et al.*, 2009; Qin *et al.*, 2009; Ton-Hoang *et al.*, 2001).

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