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Competition between NarL-dependent activation and Fis-dependent repression controls expression from the *Escherichia coli* *yeaR* and *ogt* promoters

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ABSTRACT

The *Escherichia coli* NarL protein is a global gene regulatory factor that activates transcription at many target promoters in response to nitrate and nitrite ions. Although most NarL-dependent promoters are also co-dependent on a second transcription factor, FNR, two targets, the *yeaR* and *ogt* promoters are activated by NarL alone with no involvement of FNR. Biochemical and genetic studies presented here show that activation of the *yeaR* promoter is dependent on the binding of NarL to a single target centred at position -43.5, whilst activation at the *ogt* promoter requires NarL binding to tandem DNA targets centred at position -45.5 and -78.5. NarL-dependent activation at both the *yeaR* and *ogt* promoters is decreased in rich medium and this depends on Fis, a nucleoid-associated protein. DNase I footprinting studies identified Fis binding sites that overlap the *yeaR* promoter NarL site at position -43.5, and the *ogt* promoter NarL site at position -78.5, and suggest that Fis represses both promoters by displacing NarL. The *ogt* gene encodes an O⁶-alkylguanine DNA alkyltransferase and, hence, this is the first report of expression of a DNA repair function being controlled by nitrate ions.

INTRODUCTION

Transcription in *Escherichia coli* is orchestrated by a complex network of protein factors that regulate promoter activity in response to different external signals. Many factors interact at multiple promoters, and transcription initiation at many promoters is regulated by two or more factors [1,2]. The *E. coli* NarL protein is a typical response-regulator family transcription factor that controls responses to nitrate and nitrite ions in the environment. Its activity is induced by phosphorylation by two inner membrane-bound sensor kinases, NarX and NarQ, which are activated by nitrate and nitrite ions in the periplasmic space [3-7]. NarL controls the expression of many proteins involved in anaerobic respiration and, at most of its known promoter targets, it co-regulates transcription together with another global transcription factor, FNR, the 'master' regulator of anaerobic adaptation [2,8-10]. However, a study of the NarL regulon based on transcriptome analysis [11] identified some genes that appeared to be activated by NarL without FNR, and a thorough genetic analysis of the *yeaR*-*yoaG* operon regulatory region by Valley Stewart and colleagues proved that NarL activates the *yeaR* promoter independently of FNR [12]. Here we describe the first biochemical study of the *E. coli* *yeaR* promoter and we show that NarL-dependent promoter activation can be reproduced *in vitro*. During our studies, we noticed that *yeaR* promoter activity is sharply reduced during bacterial growth in rich media and we present evidence that this is due to the action of Fis, a well-characterised DNA binding protein that is thought to play a key role in compaction of the *E. coli* chromosome [13]. In the second part of this study, we selected another *E. coli* promoter, the *ogt* promoter, that transcriptome analysis [11] had predicted to be activated by NarL alone, and we have used biochemical and genetic analysis to compare its expression and organisation with the *yeaR* promoter.

EXPERIMENTAL PROCEDURES

Strains, plasmids, promoter fragments and oligodeoxynucleotide primers

E. coli K-12 strains, plasmids and primers used in this study are listed in Table 1. The JCB387 Δ *fnr* derivative was made by P1 transduction of a Δ *fnr* (*cm*^R) mutation that had been constructed in strain MG1655 by Constantinidou *et al.* [11]. Standard techniques for recombinant DNA manipulation were used throughout.

Promoter fragments were amplified by PCR using primers that introduced flanking *Eco*RI and *Hind*III sites. By convention, promoter sequences are numbered with respect to the transcription start point (+1) and with upstream and downstream locations denoted by "-" and "+" prefixes, respectively. The *E. coli* K-12 *yeaR*-*yoaG* promoter fragment, *yeaR*100, was amplified from genomic DNA using primers *pyeaR*fw and *pyeaR*rev, and consisted of nucleotide sequences from positions -294 to +96. The *ogt*100 promoter fragment was amplified using primers *pogt*fw and *pogt*rev, and consists of nucleotide sequences from

position -269 to +51. For the generation of DNA fragments for EMSA and footprinting, the *yeaR100* and *ogt100* fragments were cloned into plasmid pSR, encoding resistance to ampicillin [14]. To assay promoter activities, fragments were cloned into the low-copy number *lac* expression vector plasmid, pRW50, encoding resistance to tetracycline [15].

The *ogt102* and *ogt104* promoter fragments, in which the NarL I or NarL II binding sites were disrupted, were constructed using two-step megaprimer PCR. Promoter DNA was amplified using primer pSRdown and either primer *ogt102* or primer *ogt104* with pSR/*ogt100* as template. The purified PCR products were used in a second round of PCR with primer D5431 and pSR/*ogt100* as template. Purified products were restricted with *EcoRI* and *HindIII* and sub-cloned into pRW50.

β-galactosidase activity assays

Plasmids containing either the *yeaR::lacZ* or *ogt::lacZ* promoter fusions were transformed into relevant strains. β-galactosidase activities were measured using the Miller [16] protocol. Cells were grown either in rich medium: Lennox broth (2% (w/v) peptone (Oxoid), 1% (w/v) yeast extract (Oxoid) and 170 mM NaCl) plus 0.4% glucose, or in minimal medium: minimal salts with 0.4% glycerol, 5% Lennox broth, 20 mM fumarate and 20 mM trimethylamine *N* oxide [11,17]. Where indicated, sodium nitrate or sodium nitrite was added to cultures to final concentrations of 20 mM and 2.5 mM respectively. Cultures were grown in static sealed test tubes to an optical density between 0.4 and 0.6 (at 650 nm). Activities were calculated as nmol ONPG hydrolysed min⁻¹ mg⁻¹ dry cell mass and represent the average of three independent experiments.

Protein preparations

Preparation and purification of a fusion of maltose binding protein to NarL (MBP-NarL) was as described by Li *et al.* [18]. In all experiments, the mature native NarL protein was used after the MBP moiety had been cleaved from MBP-NarL using protease factor Xa (New England Biolabs) [18]. Purified Fis protein, that had been prepared according to Osuna *et al.* [19], was donated by Rick Gourse, and purified *E. coli* RNA polymerase holoenzyme was purchased from Epicentre technologies (Madison). Purified DA154 FNR was prepared as in Wing *et al.* [20].

Electromobility shift assays

Electromobility shift assays (EMSA) using purified NarL were as described by Browning *et al.* [21]. *EcoRI-HindIII* digested *yeaR100* or *ogt100* promoter fragments were end-labelled with [γ -³²P]-ATP, and approximately 0.5 ng of each fragment was incubated with different amounts of NarL. The reaction buffer contained 10 mM potassium phosphate (pH 7.5), 100 mM potassium glutamate, 1 mM EDTA, 50 μM DTT, 5% glycerol, 25 μg ml⁻¹ herring sperm DNA and 50 mM acetyl phosphate (final reaction volume was 10 μl). After incubation at 37°C for 30 minutes, samples were run in 0.25 x TBE on a 6% polyacrylamide gel (12 V cm⁻¹) containing 2% glycerol and analysed using Bio-Rad Molecular Imager FX and Quantity One software (Bio-Rad). EMSA experiments with DA154 FNR were run as described by Browning *et al.* [22].

DNase I and potassium permanganate footprinting experiments

The protocols of Savery *et al.* [23] were used for footprinting experiments with ³²P-end-labeled *AatII-HindIII* fragments carrying the *yeaR* or *ogt* promoters. Each reaction (20 μl) contained a final concentration of 1.35 nM template DNA. The buffer composition was 20 mM HEPES (pH 8.0), 50 mM potassium glutamate, 5 mM MgCl₂, 1 mM DTT, 500 μg ml⁻¹ bovine serum albumin and 25 μg ml⁻¹ herring sperm DNA. NarL protein was pre-incubated with 50 mM acetyl phosphate for 45 min at 37°C, to allow for NarL phosphorylation and DNA binding [24]. In the competition experiments shown in Figures 2B and 5B, NarL and Fis, were premixed prior to incubation with the labeled DNA fragment.

For potassium permanganate footprint experiments, herring sperm DNA was omitted and *E. coli* RNA polymerase holoenzyme was used at a final concentration of 50 nM. Samples were analyzed by electrophoresis in denaturing gels that were calibrated with the products of Maxam-Gilbert 'G+A' sequencing reactions of the labelled fragment. Data were captured using a Bio-Rad Molecular Imager FX and Quantity One software (Bio-Rad).

RESULTS

Analysis of the *yeaR* promoter

The starting point of this work was the *yeaR100* DNA fragment that carries the DNA sequence from position -294 to position +96 with respect to the *yeaR* promoter transcript start site. NarL recognises 7 base pair sequence elements (the NarL heptamer) and, at many promoters, two of these elements are organised as an inverted repeat, separated by 2 base pairs (the 7-2-7 site) [4]. Figure 1A shows the DNA sequence of the *yeaR* promoter region, highlighting the 7-2-7 NarL target centred at position -43.5 that was identified by Lin *et al.* [12]. Using mutational analysis, Lin *et al.* showed that NarL binding at this target activates the *yeaR* promoter. To confirm this, the *yeaR100* fragment was cloned into pRW50, a low copy number *lacZ* expression vector to generate a *yeaR::lac* fusion, and Figure 1B shows measurements of the expression of this fusion. In the JCB387 *narL*⁺ strain, expression is strongly induced by both nitrate and nitrite ions and this induction is not found in the JCB3883 *narL* background. To measure binding of NarL to the *yeaR100* fragment directly, we used EMSA and DNase I footprinting with purified NarL. The EMSA experiment, illustrated in Figure 1C, shows that NarL causes a single clear shifted band, consistent with NarL binding a single target site. The DNase I experiment, illustrated in Figure 1D, shows that this NarL binding site corresponds to the 7-2-7 site centred at position -43.5.

A role for Fis at the *yeaR* promoter

Whilst studying the expression of the *yeaR::lac* fusion, we noticed that nitrate-dependent induction was greater in minimal medium than in more nutrient-rich media. In our previous work, we had discovered that the nucleoid-associated protein, Fis, plays a direct role in the repression of several promoters in response to nutrient abundance and rapid growth [22,25,26]. Thus, to investigate any role of Fis at the *yeaR* promoter, expression of the *yeaR::lac* fusion was compared in strain JCB387 and a *fis* mutant derivative, JCB3871. Results illustrated in Figure 2A show that nitrate-induced expression is sharply suppressed during growth in L broth supplemented with glucose, and that this suppression is greatly reduced in the JCB3871 *fis* strain. DNase I footprinting with purified Fis protein was then used to investigate whether Fis interacts directly with the *yeaR* promoter. The results (Figure 2B) reveal that Fis binds to a target (Fis I) that overlaps the upstream end of the DNA site for NarL and, at higher Fis concentrations, a second site (Fis II), that overlaps the downstream end of the DNA site for NarL, is occupied. Furthermore, footprinting experiments with both Fis and NarL (Figure 2B and 2C) show that Fis prevents NarL binding to its target.

To investigate the effects of NarL and Fis on transcription initiation at the *yeaR* promoter, we used potassium permanganate footprinting, which can be exploited to monitor DNA unwinding during open complex formation [23]. Results in Figure 3A show that incubation of the *yeaR* promoter fragment with holo RNA polymerase results in permanganate-induced DNA cleavage, indicating unwinding just upstream of the transcription startpoint. As expected, preincubation with NarL increases this cleavage, most likely as NarL promotes the formation of a transcriptionally competent complex at the *yeaR* promoter. The experiment illustrated in Figure 3B shows that this unwinding is totally suppressed by Fis, both in the presence and absence of NarL.

Analysis of the *ogt* promoter

A previous transcriptome analysis had predicted that expression of the *ogt* gene is also activated by NarL independently of FNR [11]. To investigate whether the *ogt* and *yeaR*

regulatory regions share common features, we generated the *ogt100* DNA fragment that carries the DNA sequence from position -269 to position +51 with respect to the *ogt* transcript start site, and cloned it into pRW50, to give an *ogt::lac* fusion. Figure 4A shows the DNA sequence around the *ogt* promoter transcript start point and Figure 4B illustrates measurements of the expression of the fusion in the JCB387 *narL*⁺ and JCB3883 *narL* strains. The data show that expression is strongly induced by nitrate ions and that this induction is not observed in the *narL* background. In contrast to the *yeaR* promoter, induction by nitrite is minimal.

An EMSA experiment showed that NarL binds to at least two sites at the *ogt* promoter (Figure 4C) and DNase I footprinting (Figure 4D) revealed two binding targets for NarL that appear to correspond to two 7-2-7 sites, centred at position -78.5 (NarL I) and -45.5 (NarL II) (highlighted in Figure 4A). To investigate the function of these two targets, derivatives of the *ogt100* fragment, *ogt102* and *ogt104*, were constructed in which the NarL I or NarL II sites respectively were inactivated by mutations (shown in Figure 4A). Each fragment was cloned into pRW50 and Table 2 lists measurements of the expression of the resulting *ogt::lac* fusions. The data show that nitrate-dependent activation of the *ogt* promoter is suppressed by mutations in either of the two DNA sites for NarL.

In a further EMSA experiment, we found that purified FNR gave no clear shifted band with the *ogt100* DNA fragment, suggesting that the *ogt* region contains no specific DNA site for FNR (data not shown). To check directly for any involvement of FNR in regulating the *ogt* promoter, nitrate-dependent induction of the *ogt::lac* fusion in strain JCB387 was compared with induction in a Δ *fnr* derivative. Results presented in Figure 4E show that FNR has no significant effect on expression from the *ogt* promoter.

Fis represses the *ogt* promoter by displacing upstream-bound NarL

To investigate any role of Fis at the *ogt* promoter, expression of the *ogt::lac* fusion was compared in strain JCB387 and in the *fis* mutant derivative, JCB3871. Results illustrated in Figure 5A show that nitrate-induced expression is sharply suppressed during growth in L broth supplemented with glucose, and that this suppression is greatly reduced in the JCB3871 *fis* strain. DNase I footprinting with purified Fis protein was then used to investigate whether Fis interacts directly at the *ogt* promoter. The results, illustrated in Figure 5B, identify a target for Fis that overlaps the upstream site for NarL. Footprinting experiments with both Fis and NarL show that Fis prevents NarL binding to its upstream target at position -78.5, whilst not affecting binding at the downstream target (Figure 5B and 5C).

DISCUSSION

Most *E. coli* transcription activators function by binding to specific sites at target promoters and then recruiting RNA polymerase via a direct contact, and there are three major classes of activator-dependent promoter [27]. At Class I promoters, the activator binds upstream of the promoter -35 element and recruits RNA polymerase by contacting the C-terminal domain of its α subunit. At Class II promoters, the activator overlaps the promoter -35 element and can contact both the RNA polymerase α and σ subunits. At some promoters, recruitment of RNA polymerase requires more than one contact. At these promoters, known as Class III promoters, two or more activators make independent contacts with different targets in RNA polymerase [28]. Although NarL appears to be a typical transcription activator belonging to the response-regulator family, at most of its previously studied target promoters [4], it coactivates promoter activity in conjunction with FNR, the master regulator for the adaptation of *E. coli* to growth in the absence of oxygen [8]. The likely explanation for this is that most of the genes controlled by these promoters encode products that play a role in anaerobic respiration of nitrate and nitrite, and thus their expression is fully induced only in both the absence of oxygen (signalled via FNR) and the presence of nitrate or nitrite (signalled by NarL) [4,29,30]. However, the transcriptomics study of Constantinidou *et al.* [11] predicted that a small number of *E. coli* promoters are activated by NarL without FNR, and the

comprehensive genetic analysis of the *yeaR* promoter by Lin *et al.* [12] showed that binding of NarL to a site that overlaps the promoter -35 element is sufficient to activate transcription without FNR. Here, using biochemical methods, we confirmed that purified NarL binds to this target and can activate open complex formation, most likely functioning as a Class II activator. The lack of induction of the *yeaR* promoter in a *narL* mutant background argues that NarP, which is a homologue of NarL, plays little or no role here. Recall that NarP is also activated in response to nitrate and can bind to 7-2-7 NarL binding targets [4,24]. In supplementary experiments, we found that the introduction of a *narP* mutation into either JCB387 or JCB3883 had very little effect on *yeaR* promoter activity (D.J.P.S., unpublished results).

Although the function of the products of the *yeaR-yaog* operon is unknown, an important clue comes from the observation that the *yeaR* promoter is repressed by the binding of NsrR to a target that overlaps the -10 hexamer element (Figure 1A)[12,31,32]. Hence, even if NarL activity is triggered by nitrate or nitrite ions, the *yeaR-yaog* operon will not be expressed unless repression by NsrR is lifted. Recent studies have identified NsrR as a transcriptional repressor that controls the expression of a regulon concerned with bacterial responses to reactive nitrogen species (RNS), such as nitric oxide [32-34]. NsrR contains an iron-sulphur cluster that senses RNS, which convert NsrR to a form that is unable to bind at its targets, and hence unable to function as a repressor [35-37]. Our best guess is that most of the genes in the NsrR regulon encode products needed for RNS detoxification and the repair of damage caused by RNS. It is known that RNS are generated during anaerobic respiration of nitrate and nitrite [38-40], and hence it is easy to envisage that the *yeaR-yaog* operon helps manage some aspect of RNS stress, after its expression is co-induced by external nitrate or nitrite ions (sensed by NarL) and RNS (sensed by NsrR). If this is the case, it makes sense for the *yeaR* promoter to be independent of FNR, since some RNS inactivate FNR [38,41].

During our experiments we noticed that nitrate-dependent induction at the *yeaR* promoter is greatly reduced during growth in rich media and we propose that this is due to the action of Fis protein. Recall that Fis is a sequence-specific DNA binding protein that plays many roles in *E. coli* including contributing to compaction of the folded chromosome and regulating promoter activity [13,42]. Crucially, levels of Fis fluctuate dramatically, with over 50,000 molecules per cell in rapidly growing cells in rich media, and greatly decreased levels during slow growth, for example in poor media or during starvation [43,44]. Whole genome studies using chromatin immunoprecipitation have shown that Fis can interact at hundreds of targets in the *E. coli* genome, and that over 60% of these targets are in gene regulatory regions [45,46]. Our *in vitro* studies (Figure 2) show that purified Fis binds to two targets at the *yeaR* promoter that flank the DNA site for NarL. Fis binding can displace NarL and this stops open complex formation (Figure 3). Thus, we propose that, in conditions where Fis levels are raised, Fis binds at the *yeaR* promoter and prevents nitrate-dependent induction by blocking NarL binding to its target (illustrated in Figure 6A). This explains why the reduction of nitrate-dependent induction at the *yeaR* promoter, found in rich media, is lost in a *fis* mutant strain.

In the second part of our work, we investigated the *E. coli ogt* promoter, which Constantinidou *et al.* [11] had also found to be induced by NarL independently of FNR. Note that a previous study [47] had identified the transcription start of the *ogt* promoter and its -10 element but did not investigate its regulation. Our results confirm that the *ogt* promoter is induced by NarL in response to nitrate ions, without help from FNR. However, in contrast to the situation at the *yeaR* promoter, induction requires the binding of NarL to two 7-2-7 sites, and the *ogt* promoter appears to be a Class III promoter. A possible explanation for this is that NarL binding at the *ogt* promoter is ~10 times weaker than binding at the *yeaR* promoter, and this may also account for its lack of induction by nitrite ions, which are known to result in lower levels of active NarL [4]. As at the *yeaR* promoter, the *ogt* promoter is not induced in a *narL* mutant background, suggesting that NarP also plays little or no role here.

In agreement, we found that the introduction of a *narP* mutation into either JCB387 or JCB3883 had very little effect on *ogt* promoter activity (M.X., unpublished results).

In contrast to the *yeaR* promoter, the *ogt* promoter is not a target for NsrR [32]. A clue to understanding the rationale for this comes from the work of Margison and colleagues [47,48], who were the first to clone and sequence the *E. coli ogt* gene and to investigate its promoter. These authors identified the *ogt* gene product as an O⁶-alkylguanine DNA alkyltransferase that removes alkyl groups from chemically damaged guanine residues in DNA. We suggest that the key to understanding why the expression of this DNA repair gene product is induced by nitrate ions is that RNS cause damage to DNA as well as to proteins [39,49-52]. In particular, RNS can cause lysine sidechains in proteins and some free aminoacids to become potent DNA methylating agents. We suggest that the induction of *ogt* by NarL alone in response to external nitrate provides a prophylactic insurance policy against possible genotoxic effects arising from nitrate metabolism. As at the *yeaR* promoter, nitrate-dependent induction can be repressed by Fis that binds to a single site overlapping the upstream DNA site for NarL. We propose that, in conditions where Fis levels are raised, Fis binds at this target and prevents nitrate-dependent induction by blocking NarL binding to its target (illustrated in Figure 6B).

It is known that the expression of many non-essential genes is repressed in rapidly growing cells, where a large proportion of the available RNA polymerase is channelled to the essential genes for protein synthesis and cell growth, and in some cases, this repression is due to Fis [22,25,26]. Although there is no simple rationale for why the *yeaR* and *ogt* promoters are also repressed by Fis, we suggest that rapidly growing cells may opt out of certain stress responses, and we speculate that RNS may be a small risk in these conditions compared to other stresses. Remarkably, at both promoters, the mechanism of action of Fis is similar, with Fis binding to targets that overlap a DNA site for NarL, thereby preventing binding of an essential activator.

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FIGURE LEGENDS

Figure 1. NarL regulates the *Escherichia coli yeaR* promoter.

(A) The panel shows the *Escherichia coli* K-12 *yeaR* promoter sequence from positions -64 to +86 with respect to the transcription start site (+1) (12). The -10 hexamer element is bold and underlined, the transcription start point is designated by a lower case letter and a bent arrow, and the translation start ATG codon is underlined. The DNA sites for NarL and NsrR are identified by shaded arrows and a rectangle, respectively, and the centre of each site is numbered with respect to the transcription start site. (B) The panel shows measured β -galactosidase activities in JCB387 and JCB3883 (*narL*) cells carrying pRW50, containing the *yeaR100* promoter fragment. Cells were grown in minimal medium (MM) and, where indicated, sodium nitrate or nitrite was added to a final concentration of 20 mM and 2.5 mM, respectively. β -galactosidase activities are expressed as nmol of ONPG hydrolysed $\text{min}^{-1} \text{mg}^{-1}$ dry cell mass. (C) The panel shows EMSA experiments with purified NarL protein. End-labelled *yeaR100* promoter fragment was incubated with increasing concentrations of NarL and protein-DNA complexes were separated by polyacrylamide gel electrophoresis. The concentration of NarL was: lane 1, no protein; lane 2, 0.2 μM NarL; lane 3, 0.4 μM NarL; lane 4, 0.8 μM NarL; lane 5, 1.6 μM NarL. (D) The panel shows an *in vitro* DNase I footprint experiment with purified NarL. End-labelled *yeaR100 AatII-HindIII* fragment was incubated with increasing concentrations of NarL and subjected to DNase I footprint analysis. The concentration of NarL was: lane 1, no protein; lane 2, 0.8 μM ; lane 3, 1.6 μM ; lane 4, 3.2 μM . The gel was calibrated using a Maxam-Gilbert 'G+A' sequencing reaction and relevant positions are indicated. The location of the DNA site for NarL is shown by a box and hypersensitive sites due to NarL binding are starred.

Figure 2. Fis represses transcription at the *yeaR* promoter.

(A) The panel shows β -galactosidase activities in JCB387 and JCB3871 (*fis*) cells carrying pRW50, containing the *yeaR100* promoter fragment. Cells were grown in either minimal or rich media, which was supplemented with 20 mM nitrate where indicated. β -galactosidase activities are expressed as nmol of ONPG hydrolyzed $\text{min}^{-1} \text{mg}^{-1}$ dry cell mass. (B) The panel shows an *in vitro* DNase I footprint experiment. End-labelled *yeaR100 AatII-HindIII* fragment was incubated with increasing concentrations of Fis in combination with NarL, as indicated, and subjected to DNase I footprinting. The concentration of Fis was: lanes 1 and 6, no protein; lanes 2 and 7, 0.22 μM ; lanes 3 and 8, 0.45 μM ; lanes 4 and 9, 0.90 μM ; lanes 5 and 10, 1.8 μM . The concentration of NarL was 1.6 μM in lanes 6-10. The gel was calibrated using a Maxam-Gilbert 'G+A' sequencing reaction and relevant positions are indicated. The locations of DNA sites for NarL and Fis are indicated by vertical boxes. (C) Quantification of Fis and NarL binding. The binding of Fis and NarL to the *yeaR100* promoter fragment was analysed using data from lane 5 (Fis only), lane 6 (NarL only) and lane 10 (Fis and NarL) in panel (B) with Quantity One Software (BioRad). Inverted arrows and rectangular boxes indicate the NarL and Fis binding sites, respectively, and selected locations are shown. In panels (B) and (C), hypersensitive sites at positions -52 and -40, which are induced by NarL binding, are starred, whilst the hypersensitive site at position -29, which is induced by Fis binding to Fis II, is marked by a circle.

Figure 3. DNA opening at the *yeaR* promoter.

The figure shows results of *in vitro* potassium permanganate footprint experiments at the *yeaR* promoter. Gels were calibrated using Maxam-Gilbert 'G+A' sequencing reactions and the locations of some cleavage sites are shown. (A) End-labelled *yeaR* promoter *AatII-HindIII* fragment was incubated with 50 nM RNA polymerase and NarL protein, as indicated, before permanganate treatment. NarL concentrations were: lanes 1 and 2, no NarL; lane 3, 0.1 μM ; lane 4, 0.2 μM ; lane 5, 0.4 μM ; lane 6, 0.8 μM . (B) End-labelled *yeaR* promoter *AatII-HindIII* fragment was incubated with 50 nM RNA polymerase, 0.4 μM NarL and 0.45 μM Fis protein, as indicated, before permanganate treatment.

Figure 4. NarL regulates the *Escherichia coli* *ogt* promoter.

(A) The panel shows the *Escherichia coli* K-12 *ogt* promoter sequence from position -106 to +57 with respect to the transcription start site (+1) [47]. The -10 hexamer element is bold and underlined, the transcription start point is designated by a lower case letter and a bent arrow, and the translation start ATG codon is underlined. The DNA sites for NarL are identified by shaded arrows and the centre of each site is numbered with respect to the transcription start site. The base substitutions used to disrupt NarL I and NarL II are indicated above each site. (B) The panel shows measured β -galactosidase activities in JCB387 and JCB3883 (*narL*) cells carrying pRW50, containing the *ogt100* promoter fragment. Cells were grown in minimal medium (MM) and, where indicated, sodium nitrate or nitrite was added to a final concentration of 20 mM and 2.5 mM, respectively. β -galactosidase activities are expressed as nmol of ONPG hydrolysed $\text{min}^{-1} \text{mg}^{-1}$ dry cell mass. (C) The panel shows EMSA experiments with purified NarL protein. End-labelled *ogt100* promoter fragment was incubated with increasing concentrations of NarL and protein-DNA complexes were separated by polyacrylamide gel electrophoresis. The concentration of NarL was: lane 1, no protein; lane 2, 0.2 μM NarL; lane 3, 0.8 μM NarL; lane 4, 1.6 μM NarL; lane 5, 3.2 μM NarL. (D) The panel shows an *in vitro* DNase I footprint experiment with purified NarL. End-labelled *ogt100* *AatII-HindIII* fragment was incubated with increasing concentrations of NarL and subjected to DNase I footprint analysis. The concentration of NarL was: lane 1, no protein; lane 2, 0.4 μM ; lane 3, 0.8 μM ; lane 4, 1.6 μM ; lane 5, 3.2 μM . The gel was calibrated using a Maxam-Gilbert 'G+A' sequencing reaction and relevant positions are indicated. The location of NarL binding sites is shown by boxes and a hypersensitive site due to NarL binding is starred. (E) The panel shows measured β -galactosidase activities in JCB387 and a Δ *fnr* derivative, carrying pRW50 containing the *ogt100* promoter fragment. Cells were grown in minimal medium (MM) and, where indicated, 20 mM sodium nitrate was added.

Figure 5. Fis represses transcription at the *ogt* promoter.

(A) The panel shows β -galactosidase activities in JCB387 and JCB3871 (*fis*) cells carrying pRW50, containing the *ogt100* promoter fragment. Cells were grown in either minimal or rich media, which was supplemented with 20 mM nitrate where indicated. β -galactosidase activities are expressed as nmol of ONPG hydrolyzed $\text{min}^{-1} \text{mg}^{-1}$ dry cell mass. (B) The panel shows an *in vitro* DNase I footprint experiment. End-labelled *ogt100* *AatII-HindIII* fragment was incubated with increasing concentrations of Fis in combination with NarL, as indicated, and subjected to DNase I footprinting. The concentration of Fis was: lanes 1 and 6, no protein; lanes 2 and 7, 0.45 μM ; lanes 3 and 8, 0.89 μM ; lanes 4 and 9, 1.8 μM ; lanes 5 and 10, 3.8 μM . The concentration of NarL was 3.2 μM in lanes 6-10. The gel was calibrated with a Maxam-Gilbert 'G+A' sequencing reaction and relevant positions are indicated. The locations of DNA sites for NarL and Fis are indicated by vertical boxes. (C) Quantification of Fis and NarL binding. The binding of Fis and NarL to the *ogt100* promoter fragment was analysed using data from lane 5 (Fis only), lane 6 (NarL only) and lane 10 (Fis and NarL) in panel (B) and Quantity One Software (BioRad). The inverted arrows and the rectangular box indicate the NarL and Fis binding sites, respectively, and selected locations are shown. In panels (B) and (C), the hypersensitive site at position -76 that is induced by NarL binding to NarL I is starred, and the Fis-induced hypersensitive site at position -90 is marked by a filled circle.

Figure 6. Regulation at the *yeaR* and *ogt* promoters by NarL and Fis.

The figure illustrates the juxtaposition of DNA sites for NarL and Fis at the *Escherichia coli* *yeaR* and *ogt* promoter regions. DNA sites for NarL are shown as convergent arrows that represent 7-2-7 sites [4], and DNA sites for Fis are shown as horizontal shaded bars that identify the core 15 base pair target at each site [13]. Note that, because Fis induces sharp DNA bending [42], DNase I footprints of Fis extend beyond the core target. (A) When nitrate is present in minimal medium, transcription from the *yeaR* promoter is activated by NarL binding to the NarL site. In richer media, Fis represses transcription by binding to the Fis I and Fis II sites, thereby displacing NarL. (B) When nitrate is present in minimal medium,

transcription from the *ogt* promoter is activated by NarL binding to the NarL I and NarL II sites. In richer media, Fis represses transcription by binding to Fis I, thereby displacing NarL from the upstream Fis I site.

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Table 1. Strains, plasmids, promoter fragments and primers used in this work.

Name	Details	Source
<i>Bacterial strains.</i>		
JCB387	$\Delta nir \Delta lac$	[53]
JCB3883	JCB387 $\Delta narL$	[54]
JCB3871	JCB387 <i>fis985</i> (str / spc^R)	[55]
JCB3911	JCB387 Δfnr (cm^R)	This work
<i>Bacterial plasmids</i>		
pSR	pBR322 derivative containing transcription terminator	[14]
pRW50	Broad-host-range <i>lacZ</i> fusion vector for cloning promoters on <i>EcoRI-HindIII</i> fragments: contains the RK2 origin of replication and encodes Tc^R	[15]
<i>Promoter fragments (all EcoRI-HindIII fragments)</i>		
<i>yeaR100</i>	<i>E. coli yeaR</i> promoter fragment carrying nucleotide sequences from -294 to +96.	This work
<i>ogt100</i>	<i>E. coli ogt</i> promoter fragment carrying nucleotide sequences from -269 to +51.	This work
<i>ogt102</i>	Fragment <i>ogt100</i> carrying C to G and T to C substitutions at positions -84 and -73.	This work
<i>ogt104</i>	Fragment <i>ogt100</i> carrying T to G and G to C substitutions at positions -51 and -40.	This work
<i>Oligodeoxynucleotide Primers (all are shown 5'-3')</i>		
D5431 pSRDown	ACCTGACGTCTAAGAAACC ATCCAGATGGAGTTCTGAGG	
pyeaRfw pyeaRrev	ACCTGTGAATTCGCGACGCTGGAAGTGGTG GTGAACAAGCTTCAGAAAGGCGTTGAGCGCG	
pogtfw pogtrev	GCAGAATTC CAATCTGGTCGATTCTCGCC GCAAAGCTTCATCCGTTCTCTTAAGACAAACGTG	
pogt102	CGCGAAACTGGGTAGTTACTATTTCGCTAGTCTTGCCCT ATCC	
pogt104	GCCCTATCCACTTAGCTTTTTGGTGCTATGGCTGCTGA TG	

Table 2. Effects of mutations in the NarL I and NarL II sites at the *ogt* promoter.

The table lists β -galactosidase activities measured in extracts of JCB387 cells carrying derivatives of pRW50 with different promoter fragments. The *ogt100* fragment contains DNA from positions -269 to +52 from the *ogt* promoter transcript start point. The *ogt102* fragment carries substitutions at positions -84 and -73, which disrupt the NarL I binding site, and the *ogt104* fragment carries substitutions at positions -51 and -40, which disrupt the NarL II binding site (Figure 4A). Cells were grown in the absence or presence of 20 mM nitrate and the fold induction due to nitrate is listed.

Promoter	Mutated Site	β -Galactosidase activity		Fold Induction
		minus NO ₃ ⁻	plus NO ₃ ⁻	
<i>ogt100</i>		93 ± 1	439 ± 12	4.7
<i>ogt102</i> 1.2	NarL I ↓		92 ± 5	107 ± 6
<i>ogt104</i>	NarL II ↓	56 ± 2	87 ± 3	1.6

Figure 1. Squire *et al.*, 2009

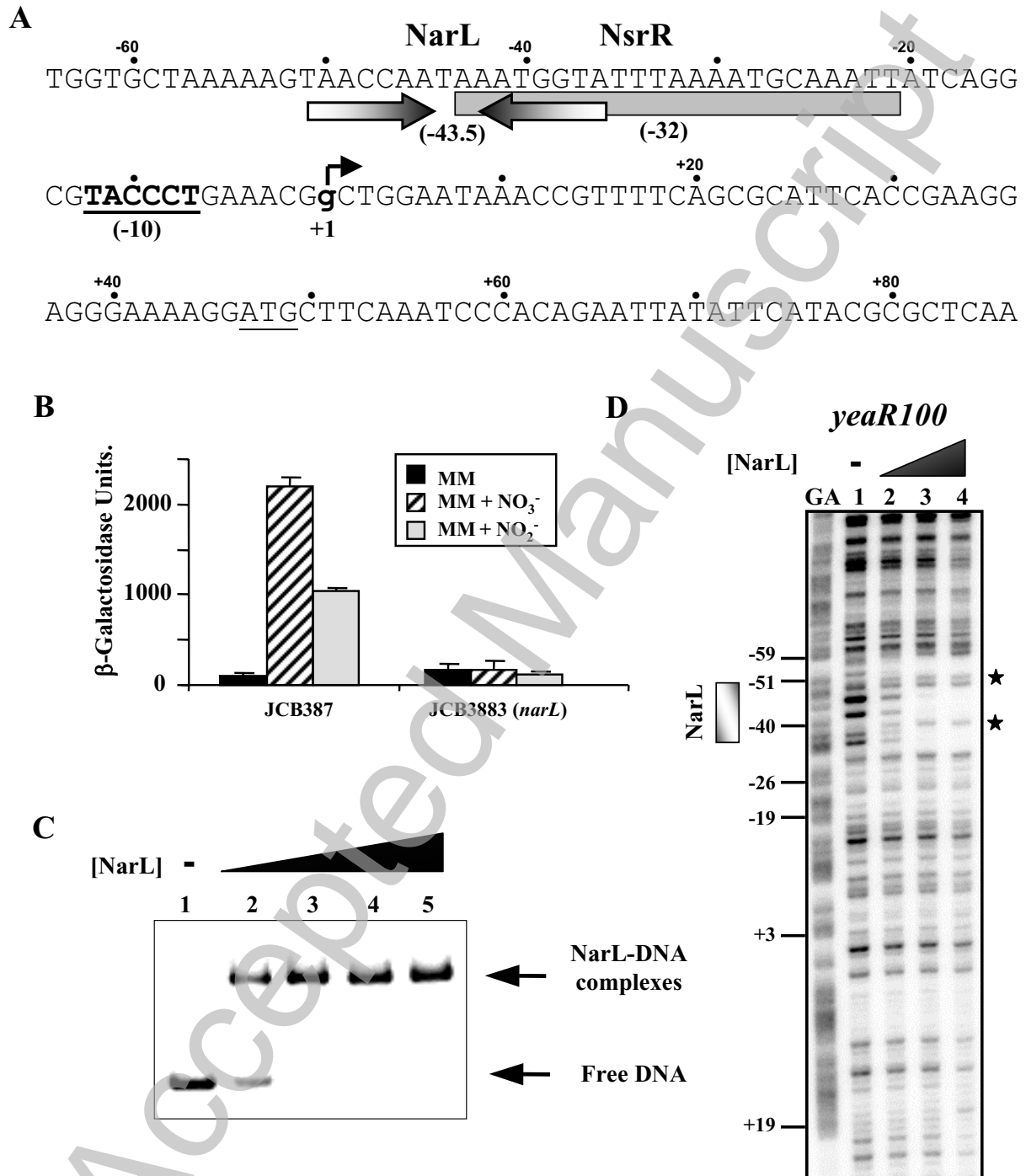


Figure 2. Squire *et al.*, 2009

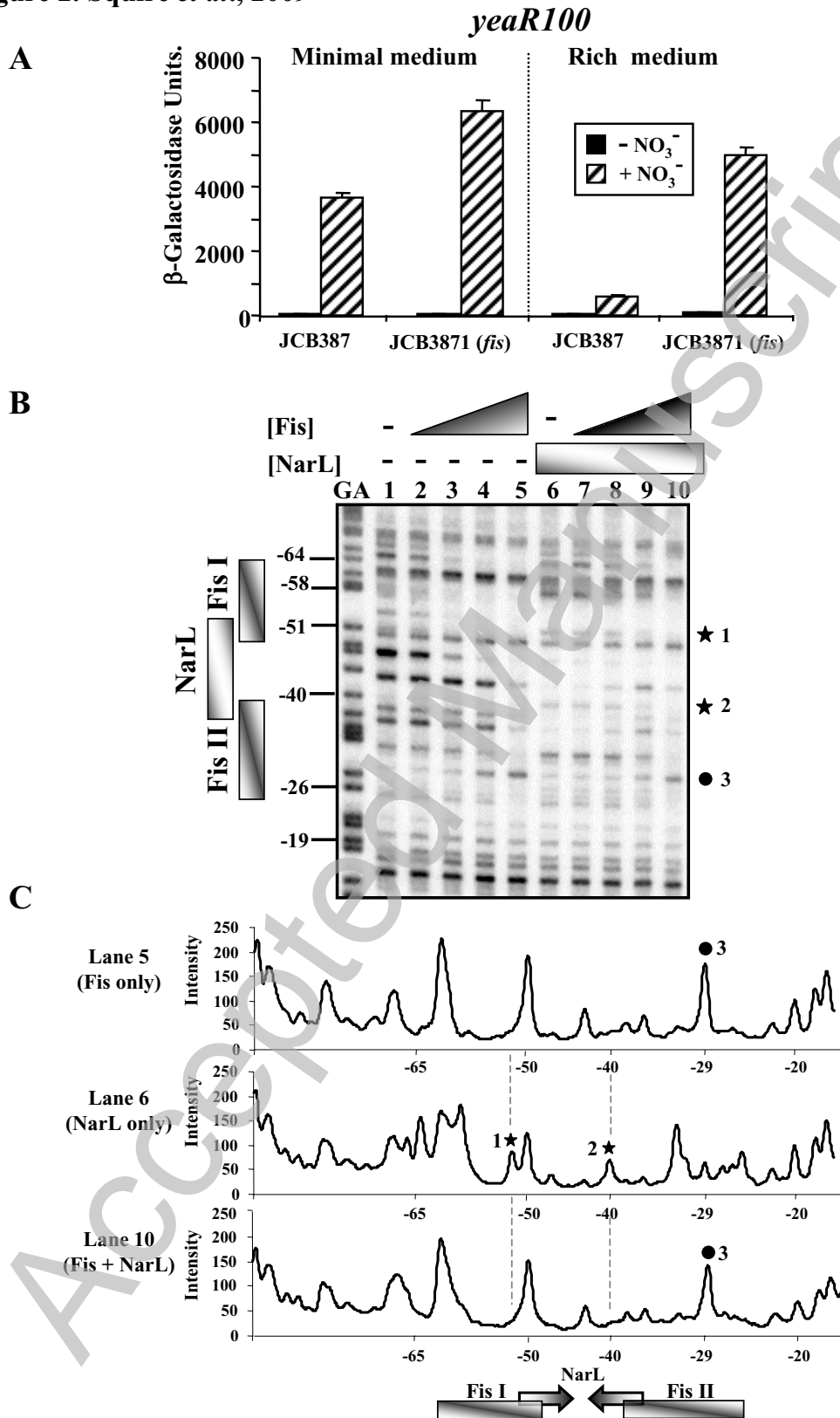
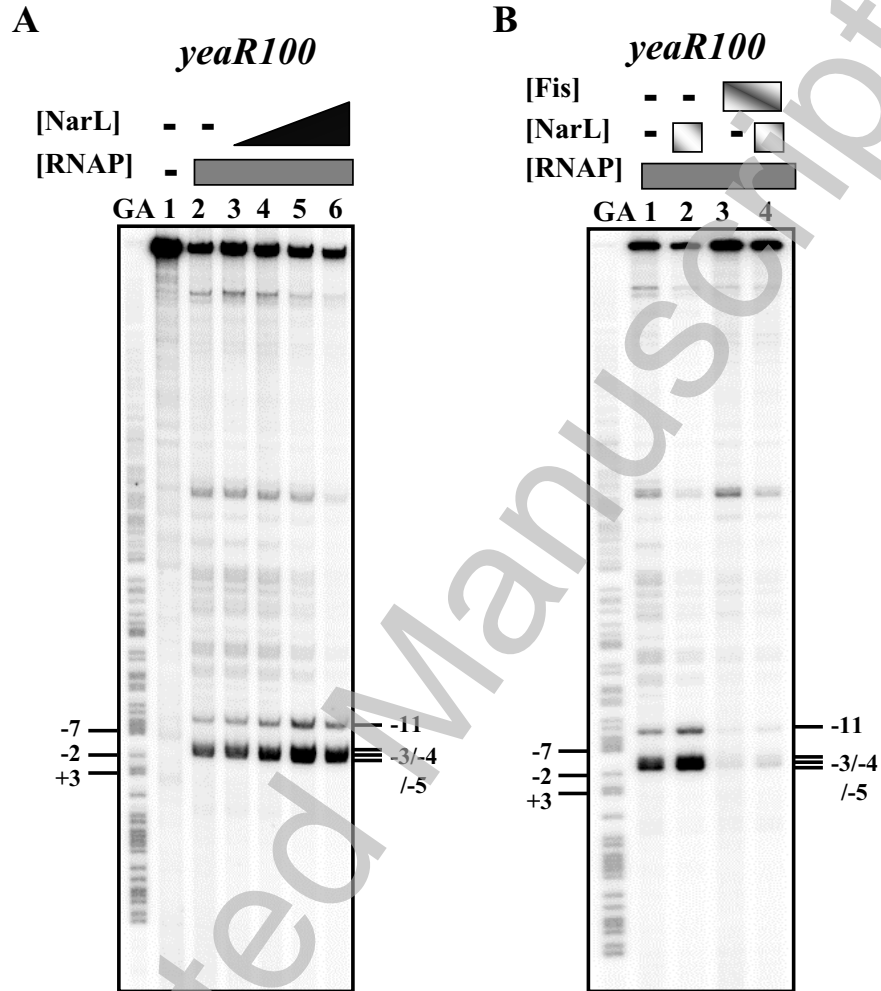
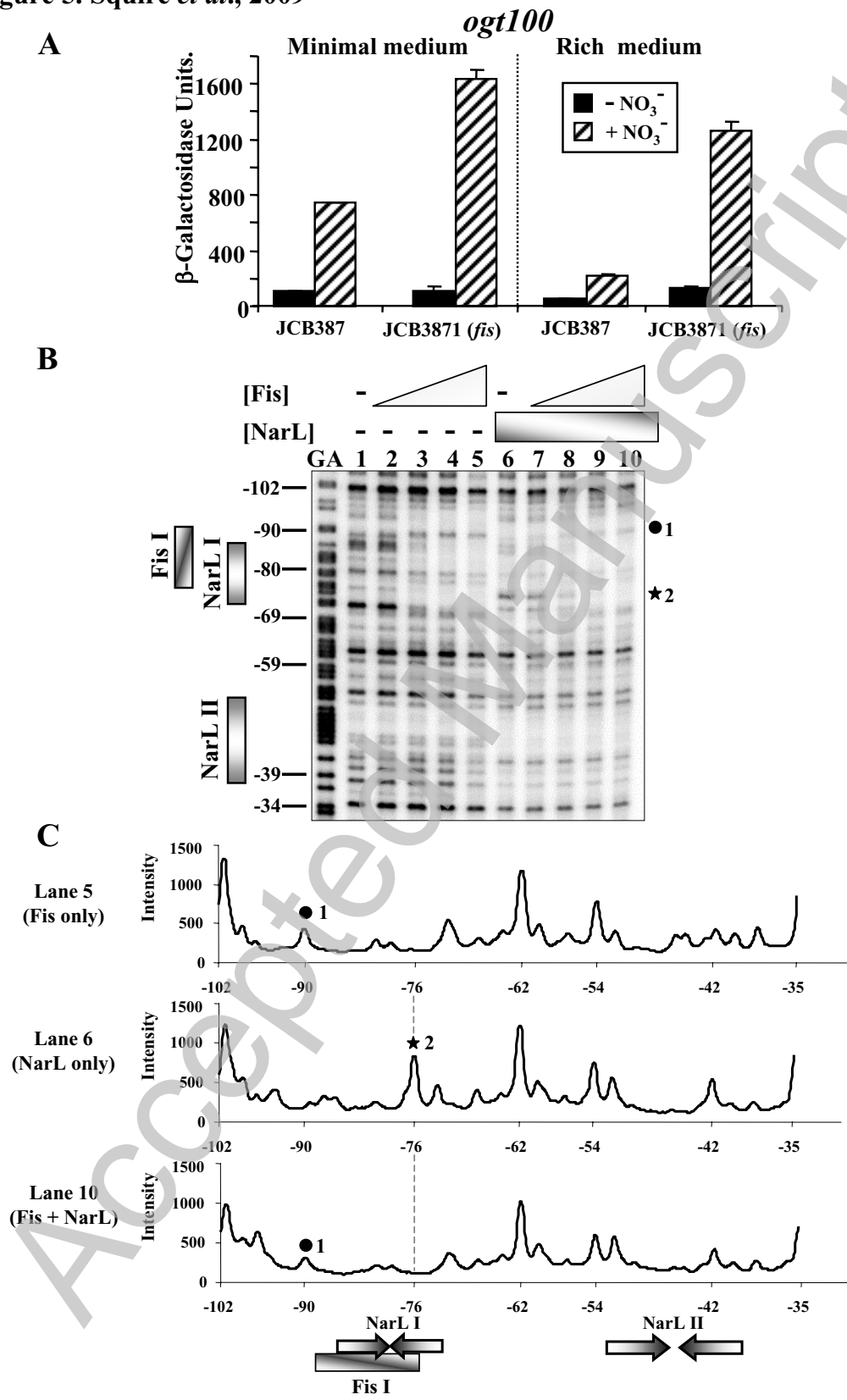


Figure 3. Squire *et al.*, 2009.

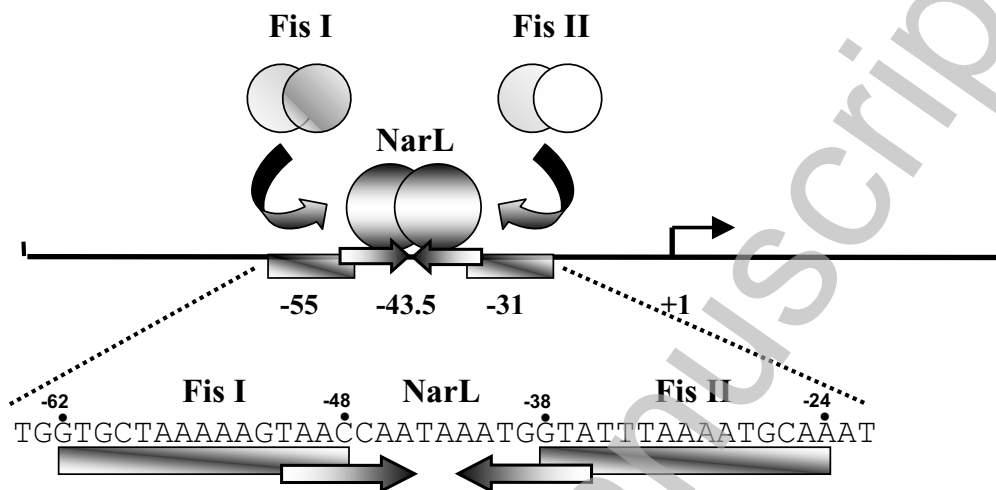


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Figure 5. Squire *et al.*, 2009



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Figure 6. Squire *et al.*, 2009A: the *yeaR* promoterB: the *ogt* promoter