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David L. Chismon, Douglas F Browning, Gregory K. Farrant, Stephen J.W.  
Busby

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## Unusual organisation, complexity and redundancy at the *Escherichia coli* *hcp-hcr* operon promoter

David L. Chismon, Douglas F. Browning,  
Gregory K. Farrant and Stephen J.W. Busby\*

School of Biosciences, University of Birmingham,  
Edgbaston, Birmingham B15 2TT, UK

\*To whom correspondence should be addressed [s.j.w.busby@bham.ac.uk](mailto:s.j.w.busby@bham.ac.uk)  
+44 (0)121 414 5439

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## ABSTRACT

Expression from the *Escherichia coli hcp-hcr* operon promoter is optimally induced during anaerobic conditions in the presence of nitrite. This expression depends on transcription activation by FNR, which binds to a target centred at position -72.5 upstream of the transcript start site. Mutational analysis was exploited to identify the corresponding -10 and -35 hexamer elements. A DNA site for NarL and NarP, located at position -104.5, plays only a minor role, whilst NsrR binding to a DNA target centred at position +6 plays a major role in induction of the *hcp-hcr* operon promoter. Electromobility shift assays show that NsrR binds to this target. The consequences of this for the kinetics of induction of the *hcp-hcr* operon are discussed.

## INTRODUCTION

The expression of many *E. coli* transcripts is tightly controlled in response to environmental cues, and, in many instances, this control is exercised at the level of the promoter by the regulation of the initiation of transcript formation [1]. Two very important signals are the absence of oxygen and the presence of nitrate or nitrite ions, and many promoters are activated only when these are combined [2]. These signals are 'transmitted' to promoters by transcription factors that can either activate or repress transcription initiation [3]. Hence, absence of oxygen triggers the activity of FNR, a well-characterised transcription factor that is the master regulator of *E. coli* adaptation to growth in anaerobic conditions [4]. FNR binds to ~25 base pair sequences at target promoters and recruits RNA polymerase, thereby activating transcription. Similarly, the presence of nitrate or nitrite ions leads to the activation of NarL and its homologue, NarP, that belong to the response regulator family of transcription factors. Activation of both NarL and NarP is mediated by the membrane-bound NarX and NarQ sensor kinases that are triggered by nitrate or nitrite ions in the environment (reviewed in [5,6]). Upon activation, NarL and NarP bind to identical heptamer sequences and, at many target promoters, two heptamers are found as a 'tail-to-tail' inverted repeat (often referred to as the 7-2-7 arrangement). These heptamers are found at a variety of locations at different target promoters and NarL and NarP have been found to modulate transcription by a variety of mechanisms. Many of these target promoters control the expression of gene products involved in the reduction of nitrate or nitrite and, hence, NarL and NarP are thought to be the principal regulators of nitrate and nitrite metabolism. However, recently, another transcription factor, NsrR, has been found to play a role at some promoters that are controlled by oxygen and by nitrate or nitrite ions (reviewed in [7,8]). NsrR appears to bind to 23 base pair sequences at many target promoters and it functions as a repressor [9]. The activity of NsrR is regulated by nitric oxide or reactive nitrogen species that convert NsrR to a form that is unable to bind to its targets and hence unable to repress transcription. Recall that nitric oxide and reactive nitrogen species are produced as by-products of nitrate and nitrite metabolism, and hence, nitrate- or nitrite-induced changes in transcription can be mediated by NsrR as well as by NarL and NarP.

The *E. coli hcp-hcr* operon encodes an iron-sulphur cluster containing protein (*hcp* is locus b0873 in cmr.jcvi.org) and an NADH oxidoreductase (*hcr* is locus b0872 in cmr.jcvi.org). The *in vivo* function of these proteins is not clear, but it is well established that their expression is induced by the absence of oxygen and the presence of nitrate or nitrite ions [10,11]. Studies of the *hcp-hcr* operon regulatory region have identified a single promoter and mapped the transcript start [10]. Expression from this promoter was shown to be completely dependent on FNR, and a single DNA site for FNR, centred at position -72.5 (i.e. between base pairs 72 and 73 upstream of the transcript start) was identified [10]. An upstream 7-2-7 NarL/NarP-binding site at position -104.5 was also identified and it was suggested that this site is essential for stimulation of *hcp-hcr* operon expression by nitrate or nitrite ions [11]. However, recent studies also found a 23 base pair DNA site for NsrR centred at position +6, and showed that the *hcp-hcr* promoter can be repressed by NsrR [9,11]. Hence, the major aim of this study was to quantify the relative contributions of NarL/NarP and NsrR to the regulation of the *hcp-hcr* promoter by nitrate and nitrite ions. A second aim was to confirm the location of the DNA site for FNR at position -72.5 and to identify other functional determinants. To achieve these aims, we performed a mutational and deletion analysis of the *hcp-hcr* operon promoter, and we report here that NsrR plays a major role whilst the effects of NarL and NarP are minimal.

## EXPERIMENTAL

### Bacterial strains, plasmids, promoter fragments and primers

The bacterial strains, plasmids, promoter fragments and oligodeoxynucleotide primers used in this work are listed in Table 1. *E. coli* K-12 strain JCB387 and derivatives were used throughout this work. Strains JCB387  $\Delta nsrR$  and JCB38849S  $\Delta nsrR$  were constructed by P1 transduction of the  $\Delta nsrR$  mutation from strain JCB5010 [11].

The low-copy number *lac* expression vector, pRW50 [12], was used as the main cloning vector during this work and it was maintained in host cells using media supplemented with 35  $\mu\text{g}\cdot\text{ml}^{-1}$  tetracycline. Plasmids pNF383, pSTBlue-1 and pGIT9 were maintained in host cells using media supplemented with 80  $\mu\text{g}\cdot\text{ml}^{-1}$  ampicillin.

All the promoter fragments used on this work carry an upstream EcoRI site and a downstream HindIII site and were cloned into pRW50. By convention, positions at the *hcp-hcr* operon promoter are labelled with the reported transcript start point [10] designated as +1 and upstream and downstream locations prefixed with '-' and '+' respectively.

The hcp383-1, hcp383-2 and hcp383-3 fragments, which encode *hcp-hcr* promoter sequences from positions -125, -92 and -54 respectively, to position +98, were constructed by PCR. DNA fragments were amplified using primers D10527 and either hcp-1 FWD, hcp-2 FWD or hcp-3 FWD using pNF383 as template. PCR products were restricted with EcoRI and HindIII and cloned into pRW50. Different mutations were generated in these fragments using different PCR methods.

Single base substitutions in promoter fragments are denoted NX, where N is the position of the substitution relative to the transcript start, and X is the substituted base in the non-template strand of the promoter. The hcp383-1n fragment, containing the +9G and +10G substitutions, was constructed by two step megaprimer PCR [13]. In the first step, a megaprimer was generated by PCR using primers hcp-n FWD and D10527 with hcp383-1 cloned in pRW50 as template. Purified megaprimer product was then used in a second PCR, together with primer D10520 and the same template. This generated the hcp383-1n fragment which was then cloned into pRW50. This recombinant plasmid was then used as a template in a further PCR to generate the hcp383-1ns fragment using hcp-s REV and D10520 as primers. Further point mutations were introduced into the hcp383-1ns fragment by megaprimer PCR using the hcp P9V FWD, hcp P13G FWD, hcp P14V FWD, hcp P35R FWD, and hcp P37V FWD primers.

The hcp383-3s fragment was constructed by PCR using primers D10520 and hcp-s REV using pRW50 carrying hcp383-3 as template. The hcp383-3ns fragment was constructed by PCR using primers hcp-3 FWD and hcp-s REV with pRW50 carrying hcp383-1ns as template.

### Promoter activity assays

The pRW50 *lac* expression vector plasmid was exploited to measure the activity of cloned promoters. Hence, relevant  $\Delta lac$  strains were transformed with plasmids encoding either *hcp-hcr* operon promoter::*lacZ* or *ogt* promoter::*lacZ* fusions and  $\beta$ -galactosidase activities were measured using the Miller method [14]. Cells were grown in minimal medium (minimal salts with 0.4% glycerol, 10% Lennox broth, 40 mM fumarate [15]) at 37°C. For aerobic growth, cells were shaken vigorously, whilst, for anaerobic growth, they were held static in growth tubes (150 mm long and 15 mm in diameter). Aerobic cultures were grown to an OD<sub>650</sub> of 0.2 to 0.3, anaerobic cultures were grown to an OD<sub>650</sub> of 0.4 to 0.6 and assayed exactly as described previously [16]. Note that aerobically grown cells were harvested at a lower cell density when it is known that cultures are fully oxygenated. Throughout this study, secondary effects due to differences in metabolism between different culture conditions were minimised by comparing  $\beta$ -galactosidase expression controlled by different derivatives of the *hcp-hcr* operon promoter.  $\beta$ -galactosidase activities are reported as nmol of ONPG hydrolysed in our assay conditions  $\text{min}^{-1} \text{mg}^{-1}$  dry cell mass and each activity is the average of three independent determinations.

### *In vitro* experiments

DNase I footprinting of FNR at the *hcp-hcr* operon promoter region was performed as in Browning *et al.* [16], using purified Ala-154 FNR and purified AatII-HindIII *hcp383-1* fragment that had been <sup>32</sup>P-end labelled at the HindIII site using polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]-ATP.

Electromobility shift assays (EMSA) using crude protein extracts containing NsrR were run as in Browning *et al.* [17]. Extracts were made from a  $\Delta$ *nsrR* derivative of JCB387 carrying either pGIT9, which expresses NsrR, or empty vector pSTBlue-1. Cells were grown anaerobically at 37°C in 50 ml of minimum medium to OD<sub>650</sub> = 0.6. Each culture was centrifuged in an Eppendorf 5810R centrifuge at 3000 x g for 15 minutes at 4°C and the pellet was resuspended in 5 ml of ice cold wash buffer (20 mM Tris-HCl (pH 8.0), 5% glycerol, 1 mM DTT, 200  $\mu$ g.ml<sup>-1</sup> phenylmethylsulfonyl fluoride and 4  $\mu$ g.ml<sup>-1</sup> pepstatin). Suspensions were recentrifuged and pellets were resuspended in 2 ml of wash buffer. Cells were disrupted by sonication using a Misonix Ultrasonic Processor XL for three 20 second bursts with 30 seconds cooling between each sonication step. Cell debris was removed by centrifuging 1 ml samples in an Eppendorf 5417R centrifuge at 20800 x g for 30 minutes at 4°C. Cell lysates were stored at 4°C and used within in 24 hours of preparation.

For the EMSA assays, purified EcoRI-HindIII *hcp383-3s* and *hcp383-3ns* promoter fragments were <sup>32</sup>P-end labelled using polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]-ATP. Approximately 0.5 ng of each fragment was incubated with varying amounts of lysate (0 to 9  $\mu$ g of protein) in buffer containing 10 mM potassium phosphate (pH 7.5), 100 mM potassium glutamate, 1 mM EDTA, 50  $\mu$ M DTT, 5% glycerol and 25  $\mu$ g.ml<sup>-1</sup> herring sperm DNA. The final reaction volume was 10  $\mu$ l. After incubation at 37°C for 20 minutes, samples were run in 0.25 x TBE on a 6% polyacrylamide gel containing 2% glycerol at 12 V.cm<sup>-1</sup> and analysed using a Bio-Rad Molecular Imager FX and Quantity One software (Bio-Rad).

## RESULTS

### Analysis of the *hcp-hcr* operon promoter

The *hcp383-1* EcoRI-HindIII DNA fragment, illustrated in Figure 1, covers the *hcp-hcr* operon regulatory region that includes the *hcp-hcr* promoter. The figure shows the base sequence from position -125 to position +98 with respect to the reported transcript start, and the previously proposed DNA sites for NarL and NarP, for FNR and for NsrR [9-11]. Note that the sequence 5'-TCTGCGTAA-3' from position -121 to position -113 corresponds to the last three codons of the preceding gene, *ybjE* (locus b0874 in cmr.jcvi.org) and thus the *hcp383-1* DNA fragment contains the complete *ybjE-hcp* intergenic region and is likely to carry all the sequence elements needed for *hcp-hcr* promoter activity and regulation. Furthermore, extension of the *hcp383-1* DNA fragment to position -285 to position +98 with respect to the *hcp-hcr* transcript start had no measurable effects in the assays described below (unpublished results).

In order to assess the importance of different elements for the activity of the *hcp-hcr* operon promoter, the *hcp383-1* fragment was cloned into plasmid pRW50, a low copy number *lac* expression vector, and this fuses the promoter to the *lacZ* gene that encodes  $\beta$ -galactosidase. *E. coli* K-12 strain JCB387 containing the recombinant plasmid gives rise to Lac<sup>+</sup> red colonies during growth on Macconkey indicator plates. In contrast, with empty pRW50, Lac<sup>-</sup> white colonies are observed, since the JCB387 chromosome carries a deletion of the *lac* operon.

In a preliminary experiment to identify sequence elements essential for the *hcp-hcr* operon promoter, error prone PCR was used to generate several independent preparations of EcoRI-HindIII promoter fragment carrying random point mutations. The fragments were then cloned into pRW50, the mixture of resulting recombinant plasmids was transformed into *E. coli* strain JCB387, and transformants were grown on Macconkey indicator plates. As expected, the majority of colonies scored as Lac<sup>+</sup>, but, after screening over 500 transformants, we identified 7 Lac<sup>-</sup> colonies. Sequence analysis showed that each of these colonies contained pRW50 carrying the *hcp-hcr* operon promoter fragment with different mutations. Three of the recombinant plasmids carried a point mutation at position -67 of the *hcp-hcr* promoter, two carried a mutation at position -69, one carried a mutation at position -78, and one carried a mutation at position -36. The different mutations are shown in Figure 1. Strikingly, six out of seven of



these changes fall in the proposed DNA site for FNR and would be expected to reduce FNR binding at this target. This is consistent with FNR being essential for expression from the *hcp-hcr* operon promoter. DNase I footprinting was then used to provide a biochemical assay to confirm FNR binding at the proposed DNA site, and the result presented in Figure 2 shows that purified FNR creates a clear single ~25 base pair footprint covering the target centred at position -72.5.

Surprisingly, none of the 'down' mutations identified in the preliminary analysis fell in the upstream DNA site for NarL and NarP. Since this target was thought to be important for promoter activity [10,11], we constructed two derivatives of the *hcp383-1* fragment, *hcp383-2* and *hcp383-3*, to investigate directly the role of the DNA site for NarL and NarP. The *hcp383-2* fragment carries a deletion that removed just this site whilst, in the *hcp383-3* fragment, both the DNA site for FNR and the DNA site for NarL and NarP are deleted (Figure 1). The upper panel of Figure 3 illustrates measurements of  $\beta$ -galactosidase activity in JCB387 cells containing pRW50 carrying the *hcp383-1*, *hcp383-2* or *hcp383-3* fragments, grown in different conditions. The data show that, with *hcp383-1*, *hcp-hcr* operon promoter expression, as measured by  $\beta$ -galactosidase expression, is induced by anaerobiosis. Some further induction is found with added sodium nitrate, whilst added sodium nitrite results in optimal induction. Removal of the DNA site for NarL and NarP in the *hcp383-2* fragment causes a decrease in nitrate-dependent induction whilst having a minimal effect on the greater nitrite-dependent induction. In contrast, as expected, removal of the DNA site for FNR in the *hcp383-3* fragment prevented all induction.

In a complementary experiment, we constructed derivatives of the *hcp383-1* fragment carrying point mutations in the proposed DNA site for NarL and NarP (*hcp383-101*) or in the DNA site for FNR (*hcp383-102*). The lower panel of Figure 3 illustrates measurements of  $\beta$ -galactosidase activity in JCB387 cells containing pRW50 carrying the *hcp383-1*, *hcp383-101* or *hcp383-102* fragments. The data underscore the importance of FNR for induction of the *hcp-hcr* operon promoter and corroborate the deletion analysis.

#### Effects of NsrR at the *hcp-hcr* promoter

The data presented in Figure 3 argue that NarL and NarP play but a small role in the induction of the *hcp-hcr* promoter. To quantify effects due to NsrR, a derivative of the *hcp383-1* fragment, carrying substitutions at positions +9 and +10, in the suggested DNA site for NsrR was constructed (see Figure 1). The resulting fragment, *hcp383-1n* was cloned into pRW50. The upper panel of Figure 4 illustrates measurements of  $\beta$ -galactosidase in JCB387 cells containing pRW50 carrying either the *hcp383-1* or *hcp383-1n* fragment, grown in different conditions. The data show that, with the *hcp383-1n* fragment, promoter expression is increased in anaerobic conditions without the addition of nitrate or nitrite, and that induction by nitrate and nitrite is greatly decreased. These results argue that nitrate- and nitrite-dependent induction of the *hcp-hcr* promoter is principally due to relief of repression by NsrR. This conclusion was confirmed by comparison of the activity of the *hcp-hcr* promoter, carried by the *hcp383-1* fragment, in an *nsrR*<sup>+</sup> and a  $\Delta$ *nsrR* background. In the  $\Delta$ *nsrR* background, full induction is observed in anaerobic conditions without the addition of nitrate or nitrite (Figure 4).

Figure 5 illustrates an EMSA to measure the binding of NsrR to the *hcp-hcr* promoter region. In this experiment, a shorter derivative of the *hcp383-3* fragment, *hcp383-3s*, carrying *hcp-hcr* promoter sequences from positions -54 to +46, was end-labelled with <sup>32</sup>P and incubated with cell extracts from  $\Delta$ *nsrR* cells that carried either a plasmid encoding NsrR or a control empty vector plasmid. DNA-protein complexes were separated using polyacrylamide electrophoresis. With extracts containing NsrR, the EMSA shows a unique NsrR-DNA complex, but this complex is absent with the control extract. Furthermore, the formation of the complex was greatly decreased when the fragment carried the substitutions at positions +9 and +10, in the DNA site for NsrR. Note that the residual binding of NsrR to the mutated DNA site likely accounts for the small NsrR-dependent nitrite-dependent induction of the *hcp-hcr* promoter seen with the *hcp383-1n* fragment (Figure 4).

#### Identification of -10 and -35 elements at the *hcp-hcr* operon promoter

It is well established that the two principal sequence elements required for the activity of most bacterial promoters are the -10 and -35 hexamers that interact with the RNA polymerase  $\sigma$  subunit [1]. Inspection of the base sequence upstream of the *hcp-hcr* promoter transcript start suggested that the promoter -10

hexamer element is 5'-TAACCT-3', corresponding to the consensus 5'-TATAAT-3' at 3/6 positions. If this were the case, the likely corresponding -35 hexamer element would be 5'-TTTTAT-3' (Figure 1). Although this corresponds to the 5'-TTGACA-3' consensus at only 2/6 positions, it is consistent with the observation that one of the seven 'down' mutations in the *hcp-hcr* operon promoter, identified after random mutagenesis using error-prone PCR, fell at position -36, changing 5'-TTTTAT-3' to 5'-TCTTAT-3' (Figure 1). In order to confirm our assignment of the two hexamer elements, we used site-directed mutagenesis to make changes in each hexamer and then measured the effects of the different changes. For this experiment, we used the shorter *hcp383-1ns* fragment which carries the *hcp-hcr* promoter sequence from position -125 to position +46 and the substitutions at positions +9 and +10, in the DNA site for NsrR. Derivatives of the *hcp383-1ns* fragment carrying different mutations were cloned into pRW50, recombinant plasmids were transformed into strain JCB387 and the activity of the *hcp-hcr* promoter was deduced from measurements of  $\beta$ -galactosidase activities. Results in Table 2 show that mutations at positions -14, -13 and -9, that changed the putative -10 hexamer away from the consensus, all caused substantial decreases in promoter activity. Similarly, substitutions at positions -37 and -36 that altered the -35 hexamer element away from the consensus, also caused decreases. A substitution at position -35 that changed the hexamer to 5'-TTGTAT-3', which accords better with the consensus, led to an increase in activity. Taken together, these data support the assignment of 5'-TAACCT-3' and 5'-TTTTAT-3' as the *hcp-hcr* operon promoter -10 and -35 hexamer elements respectively.

### Kinetics of activation of promoters in response to nitrate

The induction of promoter activity by NarL/NarP follows from the direct sensing of external nitrate or nitrite ions by the NarX/NarQ transmembrane sensor kinases [5,6]. In contrast, the induction of a promoter that is repressed by NsrR in response to nitrate or nitrite ions depends on the build up of either nitric oxide or other reactive nitrogen species that result from the metabolism of nitrate or nitrite [7,8]. We reasoned that, upon addition of nitrate ions to a culture of *E. coli*, induction that was dependent on NarL/NarP would be more rapid than induction that was mainly dependent on NsrR. To test this, we compared the kinetics of induction of the *hcp-hcr* promoter by a pulse of sodium nitrate with the induction of the *ogt* promoter, which is known to be directly activated solely by NarL [18]. Figure 6 shows the results of this experiment using the pRW50 *lac* expression vector carrying either the *hcp383-1* promoter fragment or the *ogt100* fragment that carries the *ogt* promoter. The data show a clear lag in the induction of the *hcp-hcr* operon promoter in response to nitrate compared to the *ogt* promoter.

### DISCUSSION

Many *E. coli* transcription factors interact at more than one promoter, and most promoter targets are regulated by more than one transcription factor [3]. This results in a complex transcriptional regulatory network that permits responses to different combinations of environmental inputs [19]. The promoter of the *E. coli hcp-hcr* operon is activated by two signals, anaerobiosis and nitrate or nitrite ions, which are mediated by two different transcription factors [2,10,11]. Hence this promoter is completely dependent on FNR, the principal regulator of anaerobic adaptation, which binds to a location centred at position -72.5 [10]. This location is unusual, since the DNA site for FNR is centred near position -41.5 at the vast majority of FNR-dependent promoters [20], but the biological significance, if any, is unclear. Concerning the mechanism of activation, the consequence of the unusual location is that activation must be mediated by an interaction between FNR and the RNA polymerase  $\alpha$  subunit, rather than an interaction between FNR and the RNA polymerase  $\sigma$  subunit, as at the majority of FNR-dependent promoters [20]. This is because the RNA polymerase  $\alpha$  subunit contains a flexible linker that permits contact with upstream-bound transcription factors, whilst the RNA polymerase  $\sigma$  factor can only interact directly to transcription factors that overlap the promoter -35 element [1].

The second functional transcription factor at the *hcp-hcr* operon is the NsrR repressor whose activity is modulated by some of the by-products of nitrate and nitrite metabolism [7,8]. The consequence of this is a lag in the induction of the *hcp-hcr* transcript in response to increases in nitrate, compared to transcripts whose induction is directly stimulated by nitrate. Again, the biological significance of this is unknown, and the situation is not helped by our lack of understanding of the function of the *hcp* and *hcr* gene products. Since their most likely function is some role in detoxification of reactive nitrogen species, it is not difficult

to rationalise the involvement of NsrR, which controls a network of gene products concerned with managing nitric oxide and other reactive nitrogen species.

Previous studies of the *E. coli hcp-hcr* operon promoter have focussed on possible roles for NarL and the DNA site for NarL and NarP located at position -104.5, and confirmed that purified NarL can bind to this site [10,11]. However, our results show that this site plays but a small role in the induction of the *hcp-hcr* operon promoter. We surmise that the previously reported lack of induction in *narL* mutants [10] was due to a failure to stop NsrR-dependent repression because of reduced nitrate and nitrite metabolism, rather than due to the absence of direct NarL-mediated effects. The apparent secondary role for the upstream DNA site for NarL and NarP at the *hcp-hcr* promoter is best explained as a vestige of evolution, though it is possible that the site either plays another role or comes into play in conditions that are yet to be discovered. In any case, it is now clear that binding to some targets by some *E. coli* transcription factors has no apparent effect on promoter activity. For example, in the case of the cyclic AMP receptor protein, up to 25% of hundreds of binding sites are likely to be redundant [21,22], whilst, for RutR, the majority of its 20 characterised binding targets play no role in controlling promoter activity [23]. The simplest explanation for this is that evolution drives the creation of new sites that are then eliminated if they have no function, and that genomes are bound to contain redundant sites that are 'awaiting' elimination. In the case of NarL, bioinformatics predicts over 300 binding targets throughout the *E. coli* chromosome [24] but transcriptomic experiments argue that NarL likely controls no more than 100 promoters directly [2]. Following analysis by chromatin immunoprecipitation several *bona fide* DNA sites for NarL that played little or no role in transcriptional regulation were identified [25]. We suggest that the upstream DNA site for NarL and NarP at the *E. coli* K-12 *hcp-hcr* promoter may also be moving towards redundancy. Consistent with this, comparison of the promoter sequence in several related bacteria show that core elements, including the DNA sites for FNR and NsrR, are better conserved than the upstream flanking sequences that contain the DNA site for NarL and NarP (Figure 7).

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**Table 1: Bacterial strains, plasmids, promoter fragments and oligodeoxynucleotide primers**

Name	Details	Source
<i>Bacterial strains</i>		
JCB5010	$\Delta lacU169 araD139 rpsL gyrA \Delta nsrR$	Filenko <i>et al.</i> [11]
JCB387	$\Delta nir \Delta lac$	Page <i>et al.</i> [26]
JCB387 $\Delta nsrR$	$\Delta nsrR$ derivative	This work
JCB38849S	Tet <sup>s</sup> version of JCB387 <i>narL narP253::Tn10dCm</i> <i>ΔhimA452::Tn10dTc</i> isolated as a fusaric acid resistant colony	Browning <i>et al.</i> [27]
JCB38849S $\Delta nsrR$	$\Delta nsrR$ derivative	This work
<i>Bacterial plasmids</i>		
pRW50	Broad host range <i>lacZ</i> fusion vector for cloning promoters on EcoRI-HindIII fragments: contains the RK2 origin of replication and encodes Tc <sup>R</sup>	Lodge <i>et al.</i> [12]
pNF383	pAA182 containing extended <i>E. coli hcp-hcr</i> promoter region on an EcoRI-HindIII fragment	Filenko <i>et al.</i> [10]
pSTBlue-1	Cloning vector containing the pUC origin of replication.	Novagen
pGIT9	pSTBlue-1 containing <i>E. coli nsrR</i>	Bodenmiller and Spiro [28]
<i>Promoter fragments (all EcoRI-HindIII fragments)</i>		
hcp383-1	<i>E. coli hcp-hcr</i> promoter fragment carrying nucleotide sequences from -125 to +98	This work
hcp383-2	<i>E. coli hcp-hcr</i> promoter fragment carrying nucleotide sequences from -92 to +98	This work
hcp383-3	<i>E. coli hcp-hcr</i> promoter fragment carrying nucleotide sequences from -54 to +98	This work
hcp383-1n	Fragment hcp383-1 carrying A to G and T to G mutations at positions +9 and +10	This work
hcp383-1ns	<i>hcp-hcr</i> promoter fragment carrying nucleotide sequences from -125 to +46 and carrying A to G and T to G mutations at positions +9 and +10	This work
hcp383-3s	<i>hcp-hcr</i> promoter fragment carrying nucleotide sequences from -54 to +46	This work
hcp383-3ns	Fragment hcp383-3s carrying A to G and T to G mutations at positions +9 and +10	This work

ogt100 *E. coli ogt* promoter fragment carrying nucleotide sequences from -269 to +51 Squire *et al.* [18]

*Oligodeoxynucleotide primers (all are shown 5' to 3')*

D10520	CCCTGCGGTGCCCTCAAG
D10527	GCAGGTCGTTGAACTGAGCCTGAAATTCAGG
hcp-1 FWD	CCCGAATTCTTTCTCTGCGTAATACCTCTCTGGCGGT
hcp-2 FWD	CCCGAATTCCTGCCGCCAAAATTGCGCTAAATC
hcp-3 FWD	CCCGAATTCGTTGCATGAAAAATCCCTTTTATCC
hcp-n FWD	CCTTAAACATGTATATTAAGGATAACTTTAAAAGG
hcp-s REV	CCCAAGCTTGCACACAAAACATGATCACACCTT
hcp P9V FWD	GCGTTAAGCGTCTTAACCVTAAACATGTATATTAA
hcp P13G FWD	GCGTTAAGCGTCTTGACCTTAAACATGTATATTAA
hcp P14V FWD	GCGTTAAGCGTCTVAACCTTAAACATGTATATTAA
hcp P35R FWD	GAAAAATCCCTTRTATCCCCGCGTTAAG
hcp P37V FWD	GAAAAATCCCVTTTATCCCCGCGTTAAG



Table 2: Effects of point mutations in the *hcp-hcr* operon promoter -10 and -35 elements

-35 Element (TTGACA)	-10 Element (TATAAT)	Mutation	Activity	
			Aerobic	Anaerobic
<sup>-37</sup> TTTAT <sup>-32</sup>	<sup>-14</sup> TAACCT <sup>-9</sup>	none	2.9	100.0
<i>Mutations in the -10 element</i>				
TTTTAT	TAACCA	-9A	0.4	6.2
TTTTAT	TAACCC	-9C	0.4	2.8
TTTTAT	TGACCT	-13G	0.5	2.9
TTTTAT	AAACCT	-14A	0.2	15.0
TTTTAT	GAACCT	-14G	0.4	3.6
TTTTAT	CAACCT	-14C	0.4	8.7
<i>Mutations in the -35 element</i>				
TTGTAT	TAACCT	-35G	20.0	246.0
TCATTAT	TAACCT	-36C	1.1	13.0
CTTTAT	TAACCT	-37C	0.4	6.1
ATTTAT	TAACCT	-37A	0.4	3.3

$\beta$ -galactosidase expression was measured in JCB387 cells carrying pRW50 containing the *hcp383-1ns* promoter fragment with single point mutations at different locations in the proposed -35 or -10 hexamer elements. The different base changes are shown in the first two columns and the consensus -35 and -10 hexamers for *E. coli* promoters are given at the head of each column. Cells were grown aerobically or anaerobically at 37°C in minimal medium. Each measurement is the average of three biological replicates and is expressed as a percentage of the measured  $\beta$ -galactosidase activity during anaerobic growth with the starting *hcp383-1ns* fragment.

## FIGURE LEGENDS

### Figure 1. Base sequence of the *hcp383-1* promoter DNA fragment

The *hcp383-1* fragment carries the *hcp-hcr* operon promoter sequence from position -125 to position +98 flanked by an upstream EcoRI site (boxed) and a downstream HindIII site (boxed). The previously proposed DNA targets [11] for NarL (and NarP), FNR and NsrR are indicated by shaded horizontal inverted arrows. Consensus binding sequences are shown in bold above each target, aligned with the actual sequence, and the centre of the site is noted. In the consensus sequences, Y denotes either C or T, M denotes A or C, K denotes G or T, R denotes A or G, W denotes A or T, and N denotes any base. The proposed promoter -35 and -10 hexamer elements are underlined and labelled. The reported transcription start site for the *hcp-hcr* operon promoter [10] is identified with a bent arrow and the *hcp* gene translation start codon (ATG) is in bold type. The upstream ends of the *hcp383-2* and *hcp383-3* fragments are indicated by solid lines that become dotted lines above the sequence. Base substitutions described in the text are indicated by vertical lines.

### Figure 2. Binding of FNR at the *hcp-hcr* promoter

The figure shows a DNase I footprint of purified FNR binding at the *hcp-hcr* operon promoter. End-labelled *hcp383-1* AatII-HindIII fragment was incubated with increasing concentrations of Ala154 FNR and subjected to DNase I footprint analysis. The concentration of FNR was: lane 1, no protein; lane 2, 0.5  $\mu\text{M}$ ; lane 3, 1.0  $\mu\text{M}$ ; lane 4, 2.0  $\mu\text{M}$ ; lane 5, 4.0  $\mu\text{M}$ . The gel was calibrated using a Maxam-Gilbert 'G+A' sequencing reaction and relevant positions are indicated. The location of the DNA site for FNR is shown by a box and hypersensitive sites due to FNR binding are starred.

### Figure 3. Regulation at the *hcp-hcr* promoter: effects of nested upstream deletions

The upper panel shows a bar chart that illustrates  $\beta$ -galactosidase activities measured in JCB387 cells carrying pRW50, containing either the *hcp383-1* fragment, the *hcp383-2* fragment, or the *hcp383-3* fragment. Cells were grown in minimal medium, aerobically or anaerobically at 37°C, and, where indicated, a supplement of 20 mM sodium nitrate or 2.5 mM sodium nitrite was added.  $\beta$ -galactosidase activities are expressed as nmol of ONPG hydrolysed  $\text{min}^{-1} \text{mg}^{-1}$  dry cell mass. Each activity is the average of three biological replicates and the error bars indicate the standard deviation of the replicates from the average. The lower panel shows a bar chart which illustrates an identical experiment that measured  $\beta$ -galactosidase activities in JCB387 cells carrying pRW50, containing either the *hcp383-1* fragment, the *hcp383-101* fragment, or the *hcp383-102* fragment. The *hcp383-101* fragment carries the -110G and -99C mutations in the proposed DNA site for NarL and NarP, and the *hcp383-102* fragment carries the -78C and -67G mutations in the DNA site for FNR.

### Figure 4. Regulation at the *hcp-hcr* promoter: effects of NsrR

The figure illustrates  $\beta$ -galactosidase activities measured in JCB387 cells (upper bar chart) or JCB387  $\Delta\text{nsrR}$  cells (lower bar chart) carrying pRW50, containing either the *hcp383-1* fragment or the *hcp383-1n* fragment with the +9G and +10G mutations that abrogate NsrR binding. Cells were grown in minimal medium, anaerobically at 37°C, and, where indicated, a supplement of 20 mM sodium nitrate or 2.5 mM sodium nitrite was added.  $\beta$ -galactosidase activities are expressed as nmol of ONPG hydrolysed  $\text{min}^{-1} \text{mg}^{-1}$  dry cell mass. Each activity is the average of three biological replicates and the error bars indicate the standard deviation of the replicates from the average.

### Figure 5. EMSA analysis of NsrR binding at *hcp-hcr* promoter

The figure shows an autoradiogram from an EMSA performed with  $^{32}\text{P}$ -labelled *hcp383-3s* fragment (lanes 10-19) or the derivative *hcp383-3ns* fragment with the +9G and +10G mutations that abrogate NsrR binding (lanes 1-9). These fragments carry *hcp-hcr* operon promoter sequences from position -54

to position +46. Labelled fragments were incubated with increasing amounts of soluble cell extract from strain JCB38849S  $\Delta nsrR$  carrying either pGIT9 which expresses NsrR (+NsrR; lanes 2-5,11-14), or empty vector pSTBlue-1 (-NsrR; lanes 7-9,16-19). The total amount of protein in the extract used in each reaction was: lanes 1, 6, 10 and 15, no protein; lanes 2, 7, 11 and 16, 1.5  $\mu$ g; lanes 3, 8, 12 and 17, 3  $\mu$ g; lanes 4, 9, 13 and 18, 6  $\mu$ g; lanes 5, 14 and 19, 9  $\mu$ g. The location of the band due to the specific binding of NsrR is indicated.

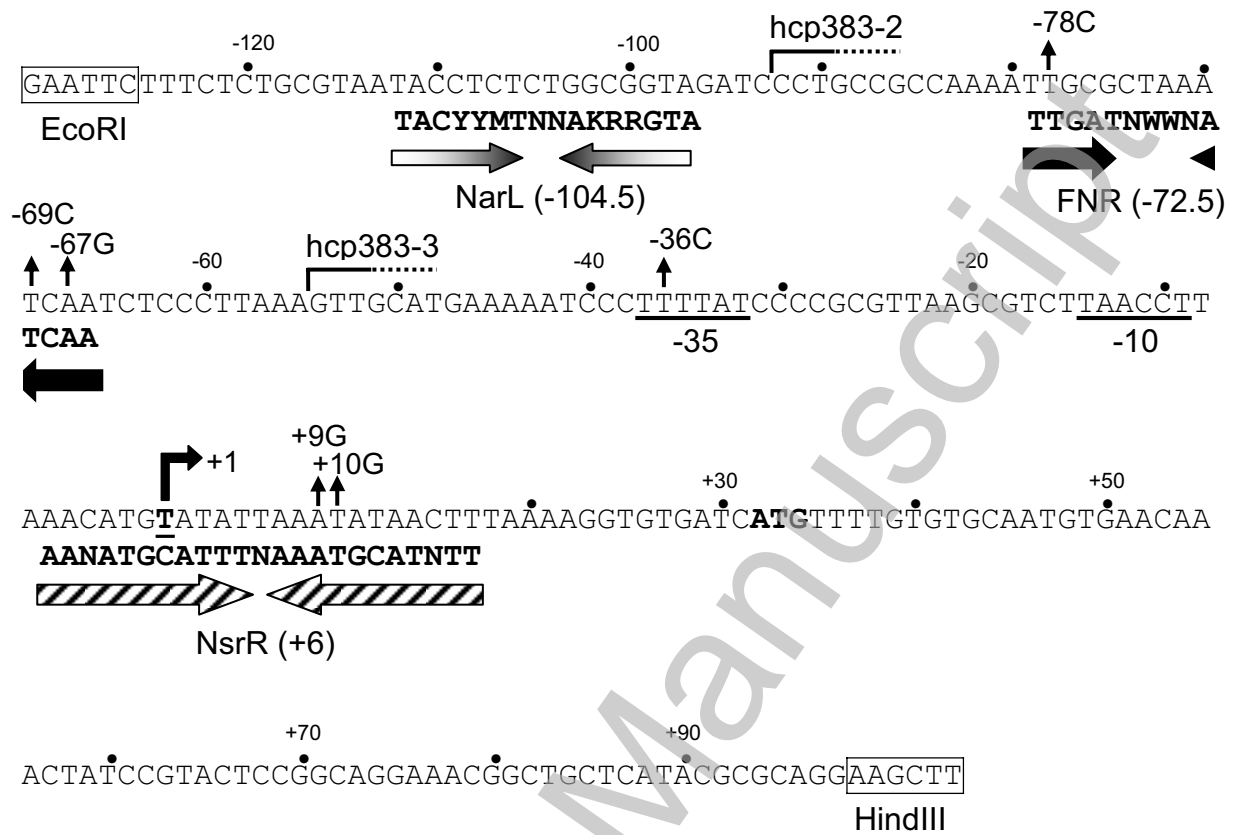
### Figure 6. Kinetics of induction of the *hcp-hcr* and *ogt* promoters

Cultures of strain JCB387 carrying pRW50 containing the *hcp383-1* or *ogt100* promoter fragments were grown anaerobically at 37 °C in minimal medium. 20 mM sodium nitrate was added when cells reached OD<sub>650</sub> of ~0.2.  $\beta$ -galactosidase activities were measured immediately prior to the addition of sodium nitrate (timepoint 0) and at 3 minutes intervals following addition. The figure shows the ratio of the measured activity relative to timepoint 0 plotted as a function of time after the addition of sodium nitrate.

### Figure 7. Phylogenetic analysis of *hcp-hcr* operon regulatory region sequences

The figure shows an alignment of the *E. coli* K-12 *hcp-hcr* operon promoter region sequence with corresponding sequences from related bacteria, downloaded from the xBASE database ([xbase.bham.ac.uk](http://xbase.bham.ac.uk))[29]. The different bacteria (and corresponding xbase Taxon ID) are: ECO, *E. coli* K-12 (83333); SEN, *Salmonella enterica* subspecies enterica serovar Choleraesuis str. SC-B67 (321314); STY, *Salmonella typhimurium* LT2 (99287); YEN, *Yersinia enterocolitica* (393305); YPE, *Yersinia pestis* (229193); YPS, sequence from *Yersinia pseudotuberculosis* (349747). DNA targets for NarL (and NarP), FNR and NsrR are indicated by shaded horizontal inverted arrows. For each target, the consensus binding sequence is shown in bold, aligned with the actual sequence, and the centre of the site is noted. In the consensus sequences, Y denotes either C or T, M denotes A or C, K denotes G or T, R denotes A or G, W denotes A or T, and N denotes any base. The transcription start point in the *E. coli* K-12 sequence is shown in lower case. Bases that are identical in each strain's genome are highlighted with black shading.

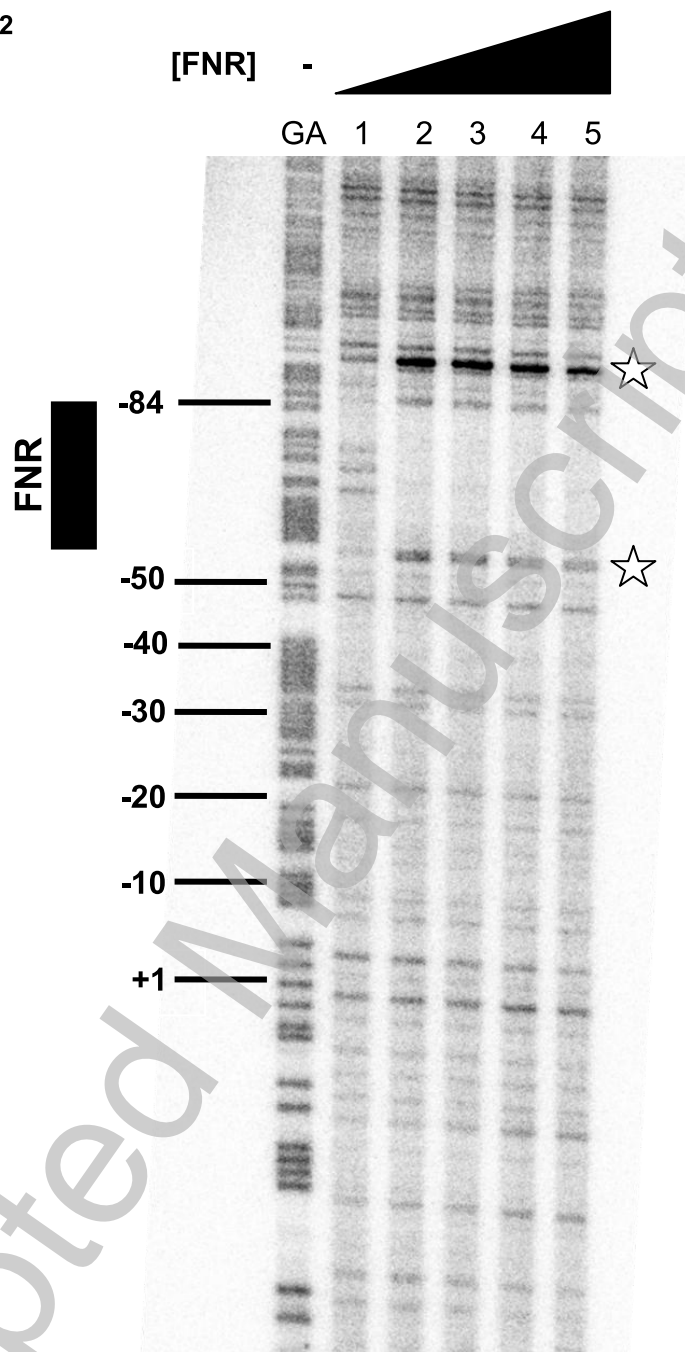
Chismon *et al.* (2010) Figure 1



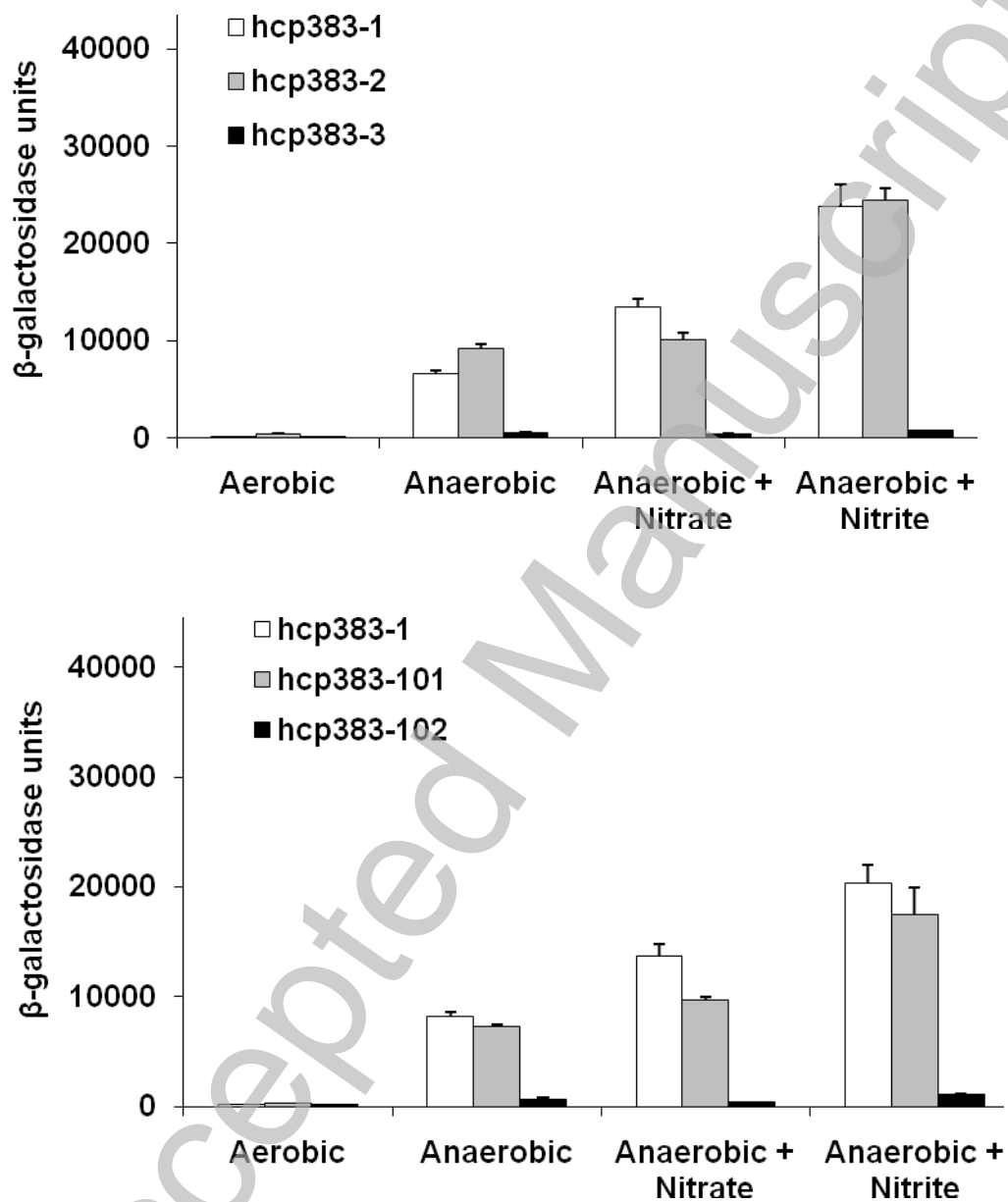
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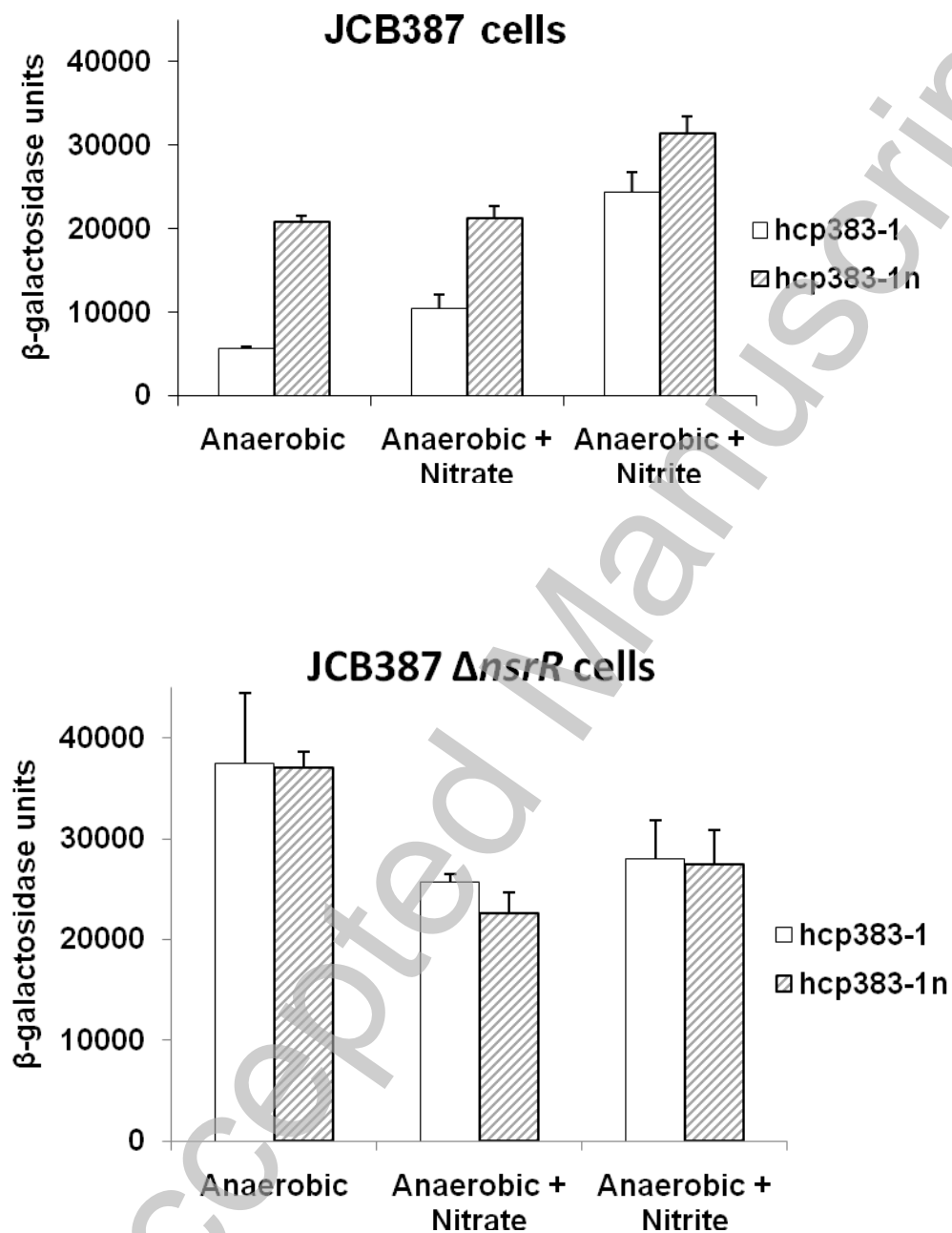
Chismon *et al.* (2010) Figure 2

Chismon *et al.* (2010) Figure 3

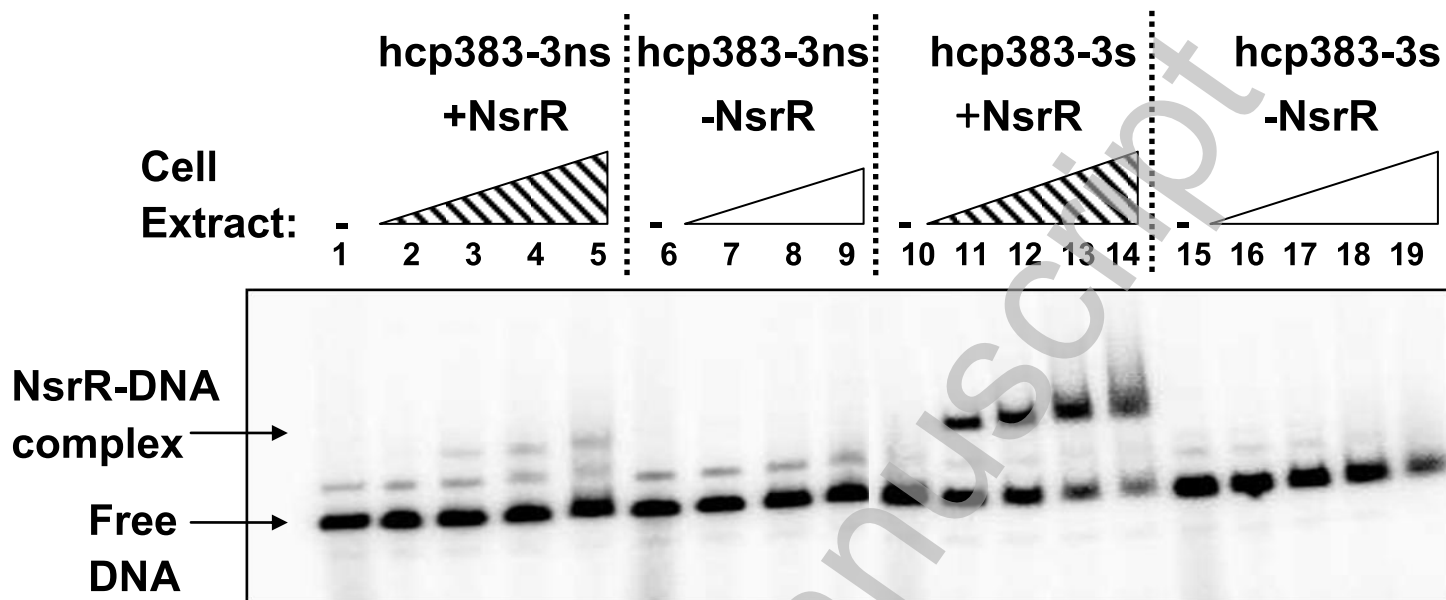


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Chismon *et al.* (2010) Figure 4

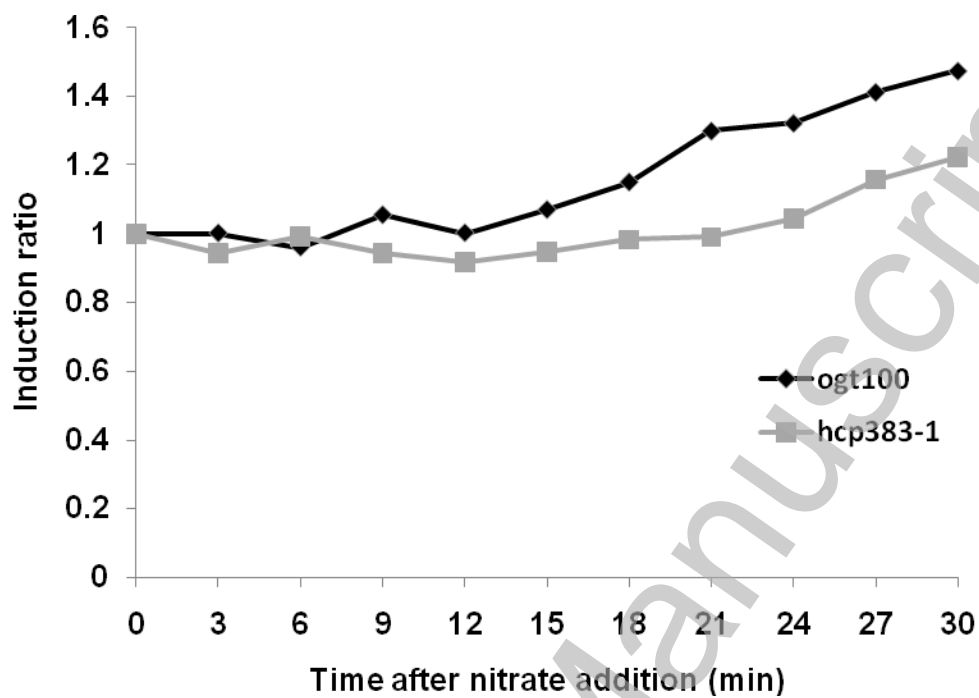
Chismon *et al.* (2010) Figure 5



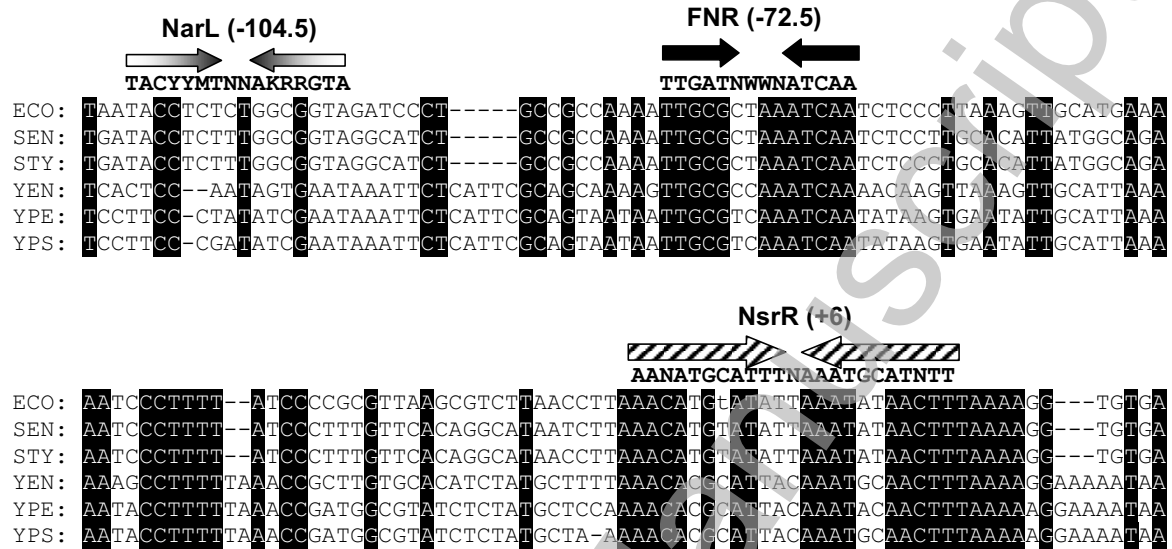
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