High level heterologous expression in *E.coli* using the anaerobically-activated *nirB* promoter

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Received March 14, 1991; Accepted March 28, 1991

ABSTRACT

The anaerobically-regulated *nirB* promoter was used to express heterologous genes in *Escherichia coli*. Under anaerobic conditions the promoter was able to express tetanus toxin fragment C at approximately 20% total cell protein (tcp) and the *Bordetella pertussis* antigen pertactin at greater than 30% tcp. These levels are comparable to those obtained for the same products using the *tac* promoter. The *nirB* promoter is very well regulated, giving almost two orders of magnitude increase in fragment C on complete removal of oxygen. The use of this anaerobically-induced promoter in the production of recombinant proteins in *E. coli* is discussed.

INTRODUCTION

The large scale production of foreign proteins in *Escherichia coli* requires the use of a strong promoter that is well regulated, allowing the growth phase and the induction phase to be separated. Without this regulation a highly expressed gene can place a constraint on cell growth and plasmid stability, even if its product is not actually toxic. Of the many *E. coli* or coliphage promoters studied, only a few satisfy these requirements: e.g. p_L from lambda (1), *lac* (2) and *trp* (3, 4) from *E. coli*, the hybrid *trp-lac* (*tac*) promoter (5) and the T7 RNA polymerase promoter (6). All of these rely on either a temperature shift or the addition of a chemical to induce their activity. A temperature shift may cause the recombinant protein to form inclusion bodies (7), and furthermore by activating the heat-shock response may lead to increased proteolysis (8). Chemical agents are often expensive and require subsequent removal at the purification stage.

An attractive alternative to the standard choice of promoters appears to exist in the *nirB* promoter. This has been isolated from *E. coli*, where it directs expression of an operon which includes the nitrite reductase gene *nirB* (9), and *nirD, nirC* and *cysG* (10). It is regulated both by nitrite and by changes in the oxygen tension of the environment, becoming active under anaerobic conditions (11). Response to anaerobiosis is mediated through the protein FNR, acting as a transcriptional activator, in a mechanism common to many anaerobic respiratory genes (9). By deletion and mutational analysis the part of the promoter which responds solely to anaerobiosis has been isolated and by comparison with other anaerobically-regulated promoters a consensus FNR-binding site was identified (12, 13). It was also shown that the distance between the putative FNR-binding site and the −10 homology region is critical (14).

Some other bacterial oxygen-regulated promoters have been shown to require very low levels of oxygen for full transcriptional activation. The *nifA* promoter of *Rhizobium meliloti* (15) and the *Vitreoscilla* sp. haemoglobin (VHb) promoter (16) are examples of promoters with such a microaerobic optimum. Indeed these promote relatively little transcription under fully anaerobic conditions. However, it is not known whether the *nirB* promoter has a microaerobic optimum. We investigate the application to heterologous expression of a form of the *nirB* promoter which lacks the nitrite-responsive regions. We show how its strength and simple regulation by oxygen can be used to synthesise very high levels of both tetanus toxin fragment C and *Bordetella pertussis* pertactin—proteins that are both potential components of future sub-unit vaccines against tetanus and whooping cough respectively.

MATERIALS AND METHODS

Strain

E. coli strain MM294 (17) was used throughout this work.

Construction of plasmids

Expression plasmids pTETnir15 and pPERnir36 were constructed from pTETtac115 (18) and pPERtac36 (19) by replacing in each the *Eco*RI-*Apa*I region (1354 bp and 1294 bp respectively) containing the *lac*I gene and *tac* promoter with the oligonucleotide pair containing the *nirB* promoter (oligos 1 and 2 in Figure 1). The oligonucleotides were synthesized on a Pharmacia Gene Assembler and the resulting plasmids confirmed by sequencing as previously described (20). Plasmid pTETnir215 was constructed by ligating the 1530 bp *Aat*II-*Bam*HI fragment of pTETnir15, containing the *nirB* promoter and fragment C gene, with the 1983 bp *Bam*HI-*Aat*II fragment from pTETlac5 (21), containing the pUC-based origin of replication and β-lactamase gene. The construction of plasmid pTETtac315 has previously been described (21).

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Capped-tube inductions

Overnight cultures of *E. coli* (A_{650} approximately 2.0) containing the above plasmids were diluted 30-fold in fresh L-Broth containing 4 mg ml^{-1} glucose and 100 μg ml^{-1} ampicillin and used to completely fill 7 ml Bijou bottles. These were then capped tightly and incubated at 37°C for 4–6 h before being harvested and analysed for expression.

Fermenter inductions

All fermentations were carried out in a Braun Biolab 21 working-volume fermenter equipped with control systems for pH, temperature, and dissolved oxygen. The polarographic probe for measuring dissolved oxygen tension (DOT) was zero-calibrated by sparging the fermenter with oxygen free nitrogen. 1.5l of L-Broth containing 10 mg ml^{-1} glucose and 100 μg ml^{-1} ampicillin was inoculated with 45 ml of overnight culture of *E. coli* containing either pTETnir215 or pPERnir36. This was grown aerobically for a period of 4–5 h at pH7.2, stirrer rate of 750 rpm and air input at up to 1.3 vvm to maintain a DOT value in excess of 75% of saturation, after which time the A_{650} was typically 1.5–3.0. The culture was then induced in one of three ways:- (a) nitrogen-sparged, (b) low air input, (c) no air input. In (a) the air supply was switched off, the stirrer rate was lowered to 400 rpm and the culture sparged with oxygen-free nitrogen at 1.3 vvm until the DOT reading had reached a plateau, and then for a further 2 min before being reduced to 0.25 vvm for the remainder of the induction. In (b) the air supply was lowered to an average rate of 0.2 vvm, stirrer rate reduced to 300–400 rpm and the DOT reading maintained at 2–5% above the background probe reading. For (c) the air supply was switched off but stirring maintained at the same rate throughout. In all three cases, samples of the culture were taken at 1 h intervals, their A_{650} determined and 1 ml aliquots pelleted in a microfuge for 2 min, to be analysed for expression.

Analysis of expression

Cell pellets from the above inductions were resuspended in Laemmli loading buffer and the equivalent of 0.1 A_{650} units of each sample was run on a 7.5% or 6% (w/v) SDS–polyacrylamide gel (22). The gels were either stained with Coomassie-blue and expression levels quantitated by densitometer scanning using a Joyce-Loebl Chromoscan 3, or blotted. Western blots were detected as described previously (18, 20).

RESULTS

Capped-tube experiments

The plasmid pTETnir15 contains the gene for tetanus toxin fragment C under *nirB* promoter control (Figure 1). The version of the *nirB* promoter used was based on the F2 DR25X variant described by Bell and coworkers (14), the *Bam*HI (GGATCC) site of which was replaced by a *Kpn*I (GGTACC) site, involving only a minimal sequence change. An equivalent plasmid, pPERnir36, had the gene for *B. pertussis* pertactin [also referred to as P69 (19)] in place of the fragment C gene.

Preliminary experiments using these expression vectors in capped-tube cultures (see Materials and Methods) showed that in the absence of air, the *nirB* promoter sequence induced synthesis of either fragment C or pertactin, both readily visible on stained SDS–polyacrylamide gels (data not shown). We have previously shown the production of fragment C to be mRNA-limited, over a wide range of mRNA levels, and consequently a good indicator of promoter strength (21). In contrast, the production of pertactin becomes mRNA-saturated at relatively low concentrations. Fragment C synthesis was therefore chosen as a model system for studying promoter strength.

Fragment C levels were increased about two-fold when the pAT153 replicon of pTETnir15 was replaced by the higher copy-number pUC19 replicon, a similar improvement to that observed in *lac* (unpublished observations) and *tac* expression vectors (18, 21). The improved vector, pTETnir215, was used for later experiments as the higher fragment C levels could be more accurately estimated.

Anaerobic induction in the fermenter

The above capped-tube experiments could only be relatively crude, with little control over, or facility to measure, the DOT during the induction. A bench-top stirred-tank fermenter was therefore used for subsequent experiments.

We carried out inductions in which a culture of *E. coli* containing pTETnir215, having been grown aerobically at a DOT of greater than 75% of saturation, was continuously sparged with oxygen-free nitrogen to displace any air in the fermenter. Analysis of samples showed that recombinant protein accumulated following anaerobiosis, demonstrating that the *nirB* promoter is active under completely anaerobic conditions (Figure 2). Figure 3 shows accumulation of fragment C as a function of time in such an experiment. The fragment C levels produced under these conditions were very high, approximately 20% tcp, comparable to those obtained by pTETtac315 which has a shortened form of the *tac* promoter (Table 1). As was found with all previous production of fragment C in *E. coli* (18, 20), the recombinant protein was fully soluble (data not shown).

An anaerobic or microaerobic optimum?

As already noted, some bacterial oxygen-regulated promoters, such as the *Vitreoscilla* haemoglobin promoter, have a microaerobic optimum. Interestingly, as shown in Figure 1,

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(a)
  oligo-1  5'-AATTCAGGTAAATTTGATGTACATCAAATGGTACCCCTTGCTGAATCGTTAAGGTAGGCGGTAGGGCC-3'
      oligo-2  3'-GTCCATTTAAACTACATGTAGTTTACCATGGGGAACGACTTAGCAATTCCATCCGCCATC-5'

(b)          EcoRI              FNR          KpnI                -10          ApaI BglII              SD          Met....
pTETnir15....GAATTCAGGTAAATTTGATGTACATCAAATGGTACCCCTTGCTGAATCGTTAAGGTAGGCGGTAGGGCCCAGATCTTAATCATCCACAGGAGACTTTCTGATG....

                                        BamHI                                                    SD      Met....
F2 DR25X.....AGGTAAATTTGATGTACATCAAATGGATCCCCTTGCTGAATCGTTAAGGTAGGCGGTAATAGAAAAGAAATCGAGGCAAAAATG....
FNR consensus      AAATTTGATATATATCAAATTT
E. coli nirB.....GAATTTGATTTACATCAATAAGCGGGGTTGCTGAATCGTTAAGGT....
Vitreoscilla Hb.....AGTTTTGATGTGGATTAAGTTTTAAGAGGCAATAAAGATTATAAT....
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Figure 1. (a) oligonucleotides used in the construction of pTETnir15 and pPERnir36. (b) Comparison of part of the *nirB* promoter sequences in pTETnir15, F2 DR25X (14), the FNR consensus sequence (12), wild-type *nirB* promoter sequence (9) and the *Vitreoscilla* haemoglobin (Hb) promoter sequence (16). Restriction enzyme sites, −10 homology regions and Shine–Dalgarno sequences (SD) are underlined. Conserved nucleotides of the FNR consensus sequence are shown in bold italics.

inspection of the *Vitreoscilla* promoter sequence (16), upstream of the −10 region, revealed a run of nucleotides in which 15 out of 22 matched the consensus FNR-binding site (12). Furthermore, the distance between this sequence and the −10 region is consistent with the critical distance from the putative FNR-binding site to the −10 homology sequence in the *nirB* promoter (14). This finding prompted us to investigate whether the *nirB* promoter also had a microaerobic optimum.

We carried out further inductions involving two alternative modifications to the above experimental design. In the first of these, the oxygen tension of aerobic cultures was allowed to fall to a low level (approximately 2−5% DOT) by reducing the air supply to a slow rate. This led to a much lower accumulation of fragment C, giving a maximum yield of only 6−7% tcp (Table 1). In the second modification, the air supply was turned off during the logarithmic growth phase, allowing the growing culture to use up the remaining dissolved oxygen. In this way, the oxygen tension was rapidly reduced to undetectable levels. Because the fermenter used in this induction was open to the atmosphere, it is possible that an immeasurably small amount of oxygen was present in the culture. Under these conditions we achieved fragment C levels of 11−12% tcp (Table 1). Clearly expression levels achieved during nitrogen-sparging were significantly better than those in all other experiments in which oxygen was not rigorously excluded. It is therefore very unlikely that the *nirB* promoter has a microaerobic optimum.

Regulation of induction

In order to determine the degree of regulation of the *nirB* promoter, we estimated the background fragment C content of a preinduction sample on a Western blot using the method described previously (23). For comparison, we also estimated the uninduced level resulting from a *tac*-driven plasmid, pTETtac315, which expresses the fragment C gene at a similar level when induced (21). As shown in Table 1, using our culture conditions, the induction ratio of the *nirB* promoter closely matches that of the tightly regulated *tac* promoter.

Induction of pertactin synthesis by the *nirB* promoter

Production of *B. pertussis* pertactin in *E. coli* at low to moderate levels has previously resulted in a severe reduction in growth of the culture; paradoxically this does not occur at very high expression levels (19). Because of this, a high background promoter activity would be expected to compromise growth of a pertactin-producing culture prior to induction, possibly leading to plasmid loss. We investigated the expression of the pertactin gene under *nirB* promoter control as a further check on the regulation of the promoter. For this experiment we used a culture containing the plasmid pPERnir36 and nitrogen-sparged induction conditions. We found no evidence for any growth problems before induction and successfully produced high levels of pertactin (Figure 2). Densitometer scanning of the gel showed that expression was greater than 30% tcp — the same level as we achieved using the *tac* promoter (19) and probably represents the maximum achievable in this host. We also induced a culture of pPERnir36 by reducing the DOT to 2−5%. As we found with pTETnir215, the level of expression was reduced when compared to the level after nitrogen-sparging, and in this case led to pertactin at 17% tcp (data not shown).

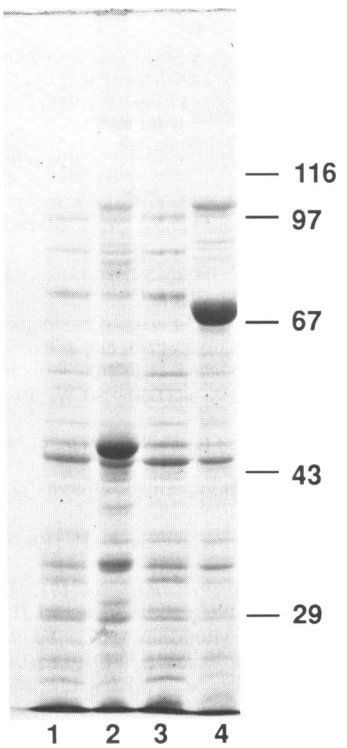

Figure 2. Coomassie blue stained SDS−polyacrylamide gel of *E. coli* extracts containing pTETnir215 or pPERnir36. Extracts from pTETnir215: pre-induction (lane 1) and 19 hours post-induction (lane 2); from pPERnir36: pre-induction (lane 3), and 20 hours post-induction (lane 4). Molecular weight markers are in kDa.

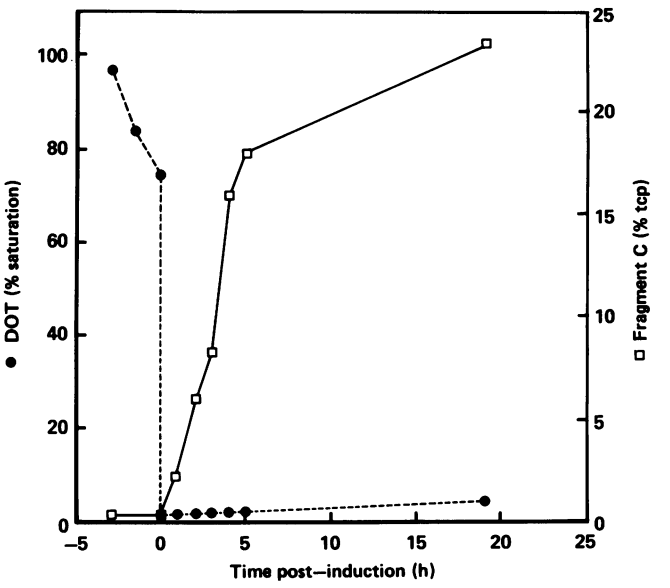

Figure 3. Accumulation of fragment C after nitrogen sparging.

Table I.

Plasmid	Induction conditions	Induced	Repressed	Ratio
pTETtac315	IPTG	21.5%	0.4%	54
	N_2-sparge	23.2%	0.3%	77
	N_2-sparge	18.3%	0.3%	61
pTETnir215	minimal air-sparge	7.2%	ND	–
	minimal air-sparge	6.7%	ND	–
	no air-sparge	11.6%	ND	–

DISCUSSION

We have demonstrated that under anaerobic conditions the *nirB* promoter is able to express very efficiently genes for both tetanus toxin fragment C and *B. pertussis* pertactin. We found that the *nirB* promoter produced similar levels of fragment C as a shortened version of the *tac* promoter. We have previously shown that this version produces approximately 50% of the steady-state mRNA level of the full-length *tac* promoter (18, 21). Since we have been able to use fragment C synthesis to discriminate between promoters with activities up to that of the full-length *tac* promoter (21), it is likely that under the conditions described in this paper, the *nirB* promoter has approximately half the activity of the full *tac* promoter. However, this conclusion assumes that in changing from aerobic to anaerobic culture conditions the effect on expression is only by activation of the *nirB* promoter. It is possible that pleiotropic changes brought about by removal of oxygen could affect other parameters of gene expression, such as plasmid copy number, mRNA or protein turnover, or efficiency of translation.

As already noted, the usefulness of a promoter for production systems depends on its regulation as well as its strength. In order to investigate the regulation of *nirB*, we estimated its induction ratio by measuring fragment C levels before and after induction. We found that the ratio is very similar to the value obtained for the *tac* promoter. We would expect these values to approximate to the corresponding mRNA ratios because, over most of this range, fragment C levels are directly proportional to mRNA levels (21). As a further check on the regulation of the *nirB* promoter we examined expression of the *B. pertussis* pertactin gene, which we would expect to be particularly sensitive to a poorly regulated promoter. Before induction, the growth rate of a culture containing pPERnir36 was comparable to growth rates obtained using pTETnir215 (data not shown). After induction, production of pertactin resulted in levels similar to those obtained using the *tac* promoter (19). Both these observations are consistent with good regulation of *nirB* promoter activity.

Because the *nirB* promoter is induced under different physiological conditions from those used in more conventional expression systems, it is possible that the nature of the product might be affected. Protein solubility in particular can sometimes be sensitive to physiological changes such as elevated temperature (7). It is of interest that expression of the fragment C gene under anaerobic conditions still results in a product which is fully soluble. Another possible consequence of the use of anaerobic conditions during induction might be a difference in the repertoire of proteases active *in vivo*. The similar pattern of lower molecular weight bands on Western blots of both fragment C and pertactin, produced under aerobic conditions using the *tac* promoter or anaerobic conditions using the *nirB* promoter (data not shown), would suggest a similar range of proteolytic activity.

One other oxygen-regulated promoter that has been used for heterologous expression in *E. coli* is the *Vitreoscilla* haemoglobin (VHb) promoter (24). This is optimally active under microaerobic conditions. In contrast, we have found no evidence for the *nirB* promoter requiring low levels of oxygen for maximum activity; its optimum appears to be genuinely anaerobic. This presumably relates to the fact that whereas *E. coli* is a facultative anaerobe, *Vitreoscilla* sp. is an obligate aerobe. However both promoters appear to have very similar FNR-binding sites, suggesting a common mechanism at least involving FNR. It is likely that some other feature of the *Vitreoscilla* promoter prevents it from functioning optimally under anaerobic conditions. Of possible relevance to this is the observation that the *nirB* FNR binding site gave rise to an altered ratio of anaerobic to aerobic FNR-dependent promoter activity when placed within a different promoter sequence (14).

An anaerobic induction optimum is clearly advantageous since, in an actively growing fermenter culture, such conditions are much more easily maintained than microaerobic conditions. In all induction systems, separation of the growth and induction phases allows high biomass to be achieved prior to induction. It is particularly important in the case of the *nirB* promoter that a high oxygen tension be maintained throughout this initial growth phase, to prevent premature activation of the promoter. This may be achieved by controlling the rate of feed of carbon source to limit the growth rate, and therefore the oxygen consumption rate, of the culture. Additionally, improvements to the oxygen transfer to the culture could be made by increasing sparge and stirrer rates, and by using oxygen-enriched air or pure oxygen as the sparge gas.

ACKNOWLEDGEMENTS

We would like to thank Dr J.A.Cole and Dr S.J.W.Busby for their helpful comments and encouragement at the start of this work, and for generously providing us with sequence information and expression data prior to publication. We would also like to thank Mr F.B.Rayment for his help in setting up the bench-top fermenter.

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