Isolation of the *Thiobacillus ferrooxidans ntrBC* genes using a *T. ferrooxidans nifH–lacZ* fusion

Carol A. Kilkenny, David K. Berger and Douglas E. Rawlings

Author for correspondence: Douglas E. Rawlings. Tel: +27 21 650 3621. Fax: +27 21 650 4023.

e-mail: doug@micro.uct.ac.za

INTRODUCTION

*Thiobacillus ferrooxidans* is a Gram-negative, diazotrophic, obligately chemolithoautotrophic bacterium that grows optimally in acid (pH 1.8–2.0) conditions. It is usually the dominant member of mixed cultures of bacteria that are used for the commercial recovery of metals such as copper, uranium and more recently gold (Lundgren & Silver, 1980; van Aswegen *et al.*, 1991). In the highly aerobic conditions of commercial biooxidation tanks, nitrogen fixation is inhibited and nitrogen is added in the form of ammonium sulfate.

Several genes involved in the nitrogen metabolism of *T. ferrooxidans* have been isolated and sequenced. These include the *glnA* (Barros *et al.*, 1985; Rawlings *et al.*, 1987), *nifHDK* (Pretorius *et al.*, 1987; Rawlings, 1988) and *ntrA* (Berger *et al.*, 1990) genes. We wished to isolate other nitrogen genes and regulators as part of an investigation of the nitrogen metabolism of *T. ferrooxidans*. NtrC (also called GlnG or NR1), the product of the *ntrC* gene, is a transcriptional regulator at the heart of the nitrogen regulatory system. In enteric bacteria, NtrC is converted to a transcriptional activator by the binding of a phosphate group to an aspartate residue within the N-terminal domain (Ninfa & Magasanik, 1986; Keener & Kustu, 1988; Sanders *et al.*, 1992). Phosphorylation of NtrC occurs under nitrogen-limiting conditions and is carried out by NtrB, a member of the histidine kinase family. Under conditions of nitrogen sufficiency, NtrB acts as a phosphoprotein phosphatase to remove the covalently bound phosphate (Ninfa & Magasanik, 1986). NtrB and NtrC are members of the two-component regulatory systems which respond to environmental stimuli and share strongly conserved domains (Nixon *et al.*, 1986).

*ntrBC* genes have been cloned from bacteria by virtue of their linkage to the *glnA* gene (De Brujin & Ausubel, 1981; Steglich-Mörsdorf *et al.*, 1993), through the use of heterologous *ntrC* probes (Nixon *et al.*, 1986; Szeto *et al.*, 1987) or by complementation of *Escherichia coli ntrC* mutants for growth on minimal medium with arginine as a sole source of nitrogen (Toukdarian & Kennedy, 1986). We had previously isolated and sequenced the *glnA* gene from *T. ferrooxidans* (Barros *et al.*, 1985), but there was no equivalent of the *ntrBC* genes in the region immediately downstream of *glnA* (Rawlings *et al.*, 1987). We therefore attempted to use the complementation procedure that Toukdarian & Kennedy (1986) had used to isolate the *Azotobacter vinelandii ntrC* gene. Our attempts to isolate the *T. ferrooxidans ntrC* gene by this method were unsuccessful.
A feature of NtrCP activation is that they are of the sigma-54 or (σ54 dependent type (Popham et al., 1989). Promoters recognized by the RNA polymerase (enzyme that catalyzes the synthesis of RNA) are characterized by having GG and GC doublets at positions -24 and -12 from the transcription start. NifA is an activator of the σ54-dependent promoters and transcriptions from the nifH promoter of Klebsiella pneumoniae are normally dependent on the binding of NifA at the upstream activator sequence. However, transcription from some mutants still require NtrC. In contrast, the wild-type K. pneumoniae nifH promoter has a stringent requirement not to be activated by NtrCP. Ray et al. (1980) have shown by transcript mapping both in vitro and in vivo that the K. pneumoniae nifH promoter requires NtrCP at high intracellular concentration for activation, while NtrCP was unable to activate transcription from the wild-type nifH promoter.

**Fig. 1. **σ54-dependent promoter regions of several nitrogen metabolism genes. The conserved GG- and GC-doublents at positions equivalent to -24 to -12 from the start site are blocked. NtrC is putative whether the promoters are activated by NtrCP. Bold and underlined letters indicate the C to T transitions constructed in the K. pneumoniae nifH promoter (Ow et al., 1985). Sequences are those identified by transcript mapping: Ec glnA, E. coli glnA promoter (Reiterzer & Magasanik, 1985); Kp nifL, K. pneumoniae nifL promoter (Drummond et al., 1983); Rm nifH, Rhizobium meliloti nifH promoter (Sundaresan et al., 1983); Kp nifH, K. pneumoniae nifH promoter (Sundaresan et al., 1983). The T. ferrooxidans nifH promoter (Tf nifH) is putative.

**METHODS**

**Bacterial strains, plasmids and media.** The relevant properties of the strains and plasmids used in this study are shown in Table 1. Bacteria were grown using Luria broth and glucose minimal medium (GMM) supplemented with nitrogen sources as de-
Table 2. Expression of the *T. ferrooxidans* nifH-lacZ fusion in *E. coli* in the presence and absence of ntrC genes

<table>
<thead>
<tr>
<th>Strain and relevant genotype</th>
<th>( \beta )-Galactosidase activity (Miller units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YMC10 ntrBC( +) (pHlac) <em>T. ferrooxidans</em> nifH-lacZ</td>
<td>30204( +) (4220)</td>
</tr>
<tr>
<td>ET8556 ntrC (pHlac) <em>T. ferrooxidans</em> nifH-lacZ</td>
<td>214( +) (38)</td>
</tr>
<tr>
<td>ET8556 ntrC (pETB514, pHlac) <em>Kp</em> ntrBC( +), <em>T. ferrooxidans</em> nifH-lacZ</td>
<td>13326( +) (1952)</td>
</tr>
<tr>
<td>ET8556 ntrC (pHlac20, pHC79) <em>T. ferrooxidans</em> nifH-lacZ</td>
<td>(26) (42)</td>
</tr>
<tr>
<td>ET8556 ntrC (pT101, pHlac20) <em>T. ferrooxidans</em> nifH-lacZ</td>
<td>1048 (125) 553 (74)</td>
</tr>
<tr>
<td>ET8556 ntrC (pT130, pHlac20) <em>T. ferrooxidans</em> nifH-lacZ</td>
<td>2035 (420) 483 (65)</td>
</tr>
<tr>
<td>ET8556 ntrC (pHlac20) <em>T. ferrooxidans</em> nifH-lacZ</td>
<td>654 (77) 413 (58)</td>
</tr>
<tr>
<td>ET8556 ntrC (pK70, pHlac20) <em>Kp</em> ntrBC( +), <em>T. ferrooxidans</em> nifH-lacZ</td>
<td>2185 (415) 452 (61)</td>
</tr>
<tr>
<td>YMC10 ntrBC( +) (pHET79, pHlac20) <em>T. ferrooxidans</em> nifH-lacZ,</td>
<td>7634 (1015) 464 (62)</td>
</tr>
<tr>
<td>ET8051 ntrBC (pHlac20) <em>T. ferrooxidans</em> nifH-lacZ</td>
<td>341 (49) 418 (55)</td>
</tr>
<tr>
<td>ET8051 ntrBC (pT130, pHlac20) <em>T. ferrooxidans</em> nifH-lacZ</td>
<td>1252 (325) 980 (157)</td>
</tr>
<tr>
<td>ET8051 ntrBC (p804, pHlac20) Ec ntrBC( +), <em>T. ferrooxidans</em> nifH-lacZ</td>
<td>2816 (633) 1577 (139)</td>
</tr>
</tbody>
</table>

* *T. ferrooxidans*; Ec, *E. coli*; Kp, *Klebsiella pneumoniae*; host genotype follows strain designation, plasmid genotype follows plasmid name.

\( \dagger \) Strains were grown aerobically under nitrogen-limiting conditions.

\( \ddagger \) Strains were grown in GMM supplemented with either low-nitrogen (Low N) or high-nitrogen (High N) as described in Methods. \( \beta \)-Galactosidase data are the means of three assays, with standard deviations in parentheses.

Plasmid constructions. The construction of the *T. ferrooxidans* nifH-lacZ fusion plasmid, pHlac, has been described previously (Berger et al., 1990). This plasmid contains the pMC1403 vector, while the cosmid gene library that was screened was based on the vector pHC79. Both vectors contain the same pBR322-derived replicon and ampicillin-resistance marker. The *T. ferrooxidans* nifH-lacZ fusion was therefore subcloned into the compatible pACYC184 vector as follows. An 8.4 kb EcoRI–SalI fragment from pHlac (containing the *T. ferrooxidans* nifH-lacZ fusion and the lacYZ genes) was ligated to pACYC184 that had been digested with BamHI and SalI. After ligation of the SalI ends, the remaining EcoRI and BamHI ends were filled-in with the Klenow fragment of DNA polymerase and these blunt ends ligated. This gave plasmid pHlac10, containing a pACYC184-compatible pACYC184 replicon and a chloramphenicol-resistance marker. As the *T. ferrooxidans* nifH-lacZ fusion had been cloned into the pACYC184 tetracycline-resistance gene behind its promoter, this promoter was deleted by digestion of pHlac10 with XbaI and EcoRV (both unique sites upstream and downstream of the tet promoter), the XbaI sticky end was filled in and the flushed ends ligated to give plasmid pHlac20.

Development of a screening technique and the isolation of the ntrC gene. An agar plate medium was developed whereby it was possible to distinguish single *E. coli* colonies which exhibited low-level expression of the *T. ferrooxidans* nifH-lacZ fusion from those that exhibited high-level expression. Several media were tried before a successful medium and procedure was...
Fig. 3. For legend see facing page.
developed. The optimal procedure was to plate transformed or transduced *E. coli* cells onto GMM containing 20 g glucose l-1, 0.02% Casamino acids, 0.2% yeast extract and 16 g agar l-1. Plates were incubated for 48 h at 37°C, cooled to 4°C and then flooded with 2 ml ONPG (4 mg ml⁻¹). 

Plates were incubated for 48 h at 37°C, cooled to 4°C and then flooded with 2 ml ONPG (4 mg ml⁻¹). *E. coli* YMC10(pHlac10) or 20) and *E. coli* ET8556(pHlac20, pK70) colonies which contained either the *trnBC* or *trnD* gene produced bright yellow colonies within 5-15 min. A problem with colony dislodgement. The addition of the chromogenic dye 5-bromo-4-chloro-indolyl β-D-galactopyranoside (X-Gal) instead of ONPG to the medium was too sensitive and most wash-off on flooding with ONPG was experienced initially. 

Protein gels. Translation products were identified by using a prokaryotic DNA-directed transcription—translation kit (*E. coli* S30 System, Promega). Polypeptides were separated on sodium dodecyl sulfate (SDS)-12% (w/v) polyacrylamide gels.

β-Galactosidase assays. *E. coli* cells containing the pHlac or pHlac20 fusion constructs were transformed with the test plasmids. Cells were grown in a preculture as described above and inoculated at an OD₆₀₀ of 0.2 units into the assay culture media. To assay β-galactosidase activity in nitrogen-rich media, GMM supplemented with (w/v) 0.2% glutamine, 0.2% (NH₄)₂SO₄, 0.2% Casamino acids and 0.2% yeast extract was used. Nitrogen-poor medium consisted of GN containing (w/v) 0.02% glutamine, 0.02% Casamino acids and 0.02% yeast extract. Appropriate antibiotic selection was provided at all times and cultures were grown in their respective media for at least 4 h before β-galactosidase assay.

RESULTS

Cloning of the *T. ferrooxidans* ntrC gene by activation of expression of a *T. ferrooxidans* nifH–lacZ fusion

In order to test the isolation strategy, experiments were carried out to determine whether there was an increase in expression from the *T. ferrooxidans* nifH promoter in the presence of a cloned *K. pneumoniae* ntrC gene. The *E. coli* ntrC mutant ET8556 containing the *T. ferrooxidans* nifH–lacZ fusion plasmid, pHlac, produced low basal levels of β-galactosidase activity when grown in nitrogen-
limiting conditions (Table 2). In contrast, E. coli YMC10- (ntrC*+)(pHlac) cells had 150-fold greater levels of β-galactosidase activity. The presence of a cloned K. pneumoniae ntrC gene was able to partially complement the ntrC mutation, as E. coli ET8556(pHlac, pFB514) cells had 60-fold greater levels of β-galactosidase activity than ET8556(pHlac) cells (Table 2). These results formed the rationale behind the strategy to isolate the T. ferrooxidans ntrC gene by activation of expression of the T. ferrooxidans nifH–lacZ fusion.

The T. ferrooxidans cosmid bank was transduced into the E. coli ET8556 ntrC mutant containing the T. ferrooxidans nifH–lacZ fusion. After growth on GMM low-nitrogen plates and flooding with ONPG, six positive clones were identified and purified. Cosmid DNA was extracted from cultures of each clone and retransformed into the same strain as used to screen the bank. Each clone retested positive for β-galactosidase activity. The cosmids had contain overlapping pieces of the same region of the T. ferrooxidans genome. One cosmid clone, pT101, was chosen for further study.

Localization of the T. ferrooxidans ntrC gene

A 13.5 kb HindIII fragment of cosmid pT101, which was subcloned into the vector pEcoR252 to give pT110, was still able to activate T. ferrooxidans nifH–lacZ expression (Fig. 2). A restriction map of pT110 was constructed and 5.2 kb HindIII-KpnI and 4.47 kb EcoRI-HindIII fragments (pT120 and pT130 respectively) were subcloned into the vector Bluescript SK. Both fragments retained the ability to activate the pHlac fusion and the nucleotide sequence of the pT130 insert was determined from both strands.

Nucleotide sequence of the T. ferrooxidans ntrBC genes

Analysis of the sequence data revealed three ORFs (Figs 2 and 3), each starting with an ATG and each preceded by a short sequence that could serve as a ribosome-binding site. The first and second ORFs coded for proteins with calculated Mr values of 40,278 and 51,894 respectively. The ntrBC genes overlapped slightly, with the TGA stop codon of ntrB providing part of the ATG start codon of ntrC. Using the tFASTA subroutine of the GCG package, the predicted amino acid sequence of the first ORF clearly aligned with the NtrB sequences and the amino acid sequence of the second ORF with the NtrC sequences of the GenBank (release 76) database (Fig. 4a, b). This indicated that the first ORF encoded the T. ferrooxidans NtrB protein and the second ORF the NtrC protein. The amino acid sequences of T. ferrooxidans NtrB and NtrC proteins were most similar to those of the y proteobacteria. The T. ferrooxidans NtrB protein had 39 to 40% sequence identity with the NtrB proteins of E. coli, K. pneumoniae, Proteus vulgaris and Vibrio alginolyticus, while the NtrC protein had 63, 61 and 59% sequence identity to the NtrC proteins of K. pneumoniae, E. coli and P. vulgaris respectively.

A putative promoter sequence, -CTGGTGGCCTTTT-TGCTT-, which has the conserved GG and GC bases, the correct spacing and an 8 out of 11 bp match with the consensus sequence (CTGGCAC-N5-TTGCAC) of σ40-dependent promoters, was found immediately upstream of the ntrB gene (Fig. 3). This putative promoter had a poly(T) tract in position -14 to -18, which is thought to facilitate high-affinity binding between the promoter and σ40 (Buck & Cannon, 1992). At least two potential NtrC-binding sites were located upstream of the ntrB translation start (Fig. 3). One site, approximately 190 bp upstream of ntrB, had a one base pair mismatch to the conserved sequences of a -TGCACC-N5-TGTCAC-consensus NtrC-binding site (Ames Ferro-Luzzi & Nikaido, 1985). There were several other sequences which matched one or other, but not both of the elements of a consensus NtrC-binding site.

A third ORF which could encode a protein of calculated Mr 35106 was located immediately downstream of the ntrC gene. A potential ribosome-binding site, GGGG, was situated 5 bp upstream of the ORF. Codon preference analysis with a codon usage table derived from previously sequenced T. ferrooxidans genes (Rawlings et al., 1991) indicated that this ORF was likely to be protein-encoding. A search of the GenBank/EMBL database (release 76) for proteins with homology to this ORF using the predicted amino acid of the ORF and the BLASTNCBI subroutine (National Center for Biotechnology Information, National Institutes of Health, New Bethesda, USA) failed to indicate any similarity to the translated products of the deposited DNA sequences or proteins.

Analysis of polypeptide synthesis

An E. coli-derived in vitro transcription–translation system was used to identify polypeptides produced from the T. ferrooxidans clones (Fig. 5). A comparison between the proteins produced by the vector pEcoR252 (lane 2) and clones pT120 and pT130 (lanes 3 and 4 respectively)
indicated that three polypeptides, of 51 kDa, 41 kDa and 40 kDa, were synthesized from both clones. The 51 kDa band corresponded closely to the predicted Mr of 51 894 for NtrC. When DNA encoding NtrC and the third ORF was deleted but the DNA encoding NtrB remained, only the 40 kDa band was synthesized (pT128; Fig. 5, lane 6). This identified the 40 kDa band as NtrB (predicted Mr, 40 278). When only the third ORF was deleted (construct pT132, Fig. 2), all three of the proteins were still produced (results not shown). This indicated that the 41 kDa protein was a breakdown product of the 51 kDa NtrC protein. No polypeptide corresponding to the predicted size of the product of the third ORF (35 kDa) was detected. It is possible that a promoter which is required for expression of this third ORF but which is not recognized in the E. coli in vitro transcription–translation system is located in the region between the ntrC gene and the third ORF. When the ClaI–PstI fragment of pT130 was deleted and only the ClaI–HindIII fragment containing NtrC and the third ORF remained, no proteins were detected (pT127; Fig. 5, lane 5).

Besides NtrB, NtrC, and the 41 kDa protein, an additional polypeptide of 42 kDa was produced from pT110 in the E. coli–derived transcription–translation system (Fig. 5, lane 7). All four proteins were also produced from pT101, the cosmid clone that was the source of all other subclones used in this study (Fig. 5, lane 1). This unidentified protein appears to be synthesized from the region of pT110 and pT101 that lies upstream of the ntrB gene.

The T. ferrooxidans ntrBC genes and the glnA gene are not linked

No ntrB gene had been located in the 350 bp region downstream of the previously sequenced T. ferrooxidans glnA gene (Rawlings et al., 1987). Likewise no portion of the glnA gene was found in the approximately 900 bp upstream of the ntrB gene (Fig. 3). In order to investigate further the lack of linkage between the T. ferrooxidans glnA and ntrBC genes, Southern hybridization experiments were carried out. Chromosomal DNA isolated from T. ferrooxidans ATCC 33020 was digested with PstI and hybridized to labelled pT120. The 3.6 kb and 300 bp fragments internal to the pT120 insert hybridized to T. ferrooxidans chromosomal fragments of the same size (Fig. 6, lanes 4 and 5). The 300 bp fragment was slightly retarded due to the overloading of T. ferrooxidans chromosomal DNA required to give a signal from the small fragment. Analysis of the sequence data indicated that the ntrBC genes were located completely within the 3.6 kb PstI fragment. In contrast, the T. ferrooxidans glnA gene is located on a 2.35 kb PstI chromosomal fragment (Rawlings et al., 1987), the uppermost band of the glnA pMEB100 clone (Fig. 6a, lane 3). No T. ferrooxidans chromosomal fragment of this size hybridized to the pT120 probe. The two fragments of pMEB100 that hybridized to the pT120 probe were the 2.1 and 1.75 kb fragments which contained pEcoR251 vector DNA sequences that are homologous to the pBluescript SK vector of pT120. Furthermore, PstI restriction digests of pT110 did not indicate the presence of a 2.35 kb band which would have been predicted had the glnA gene been located within the 6 kb region upstream of the ntrB genes (data not shown).

Expression of the T. ferrooxidans nifH–lacZ fusion in the presence of the cloned ntrBC genes

The effect of the presence of the cloned T. ferrooxidans ntrB and ntrC genes on expression of the T. ferrooxidans nifH–lacZ fusion was investigated. β-Galactosidase assays of E. coli cells grown in liquid culture were carried out in nitrogen-limiting and nitrogen-rich media. Low levels of β-galactosidase activity were obtained from ET8556 (pHlac20, pHC79) ntrC control cells grown with high or

Fig. 5. SDS-PAGE analysis of proteins expressed from pT101 and subclones in an E. coli–derived in vitro transcription–translation system. Lanes: 1, pT101; 2, pECoR252; 3, pT120; 4, pT130; 5, pT127; 6, pT128; 7, pT110; 8, pBluescript SK. Maps of all constructs except cosmids clone pT101 are shown in Fig. 2. The 51, 41 and 40 kDa bands indicated by means of arrows are the products of ntrC, its cleavage product and ntrB respectively.

Fig. 6. Hybridization of labelled pT120 DNA to T. ferrooxidans chromosomal DNA and plasmid digests. (a) Photograph of an agarose gel containing PstI digests of (in lanes) 1, λ DNA; 2, 500 ng of pMEB100; 3, 100 ng of pMEB100; 4, 15 mg of T. ferrooxidans chromosomal DNA; 5, 100 ng of pT120; 6, 500 ng of pT120. (b) Autoradiograph of panel (a).
low levels of nitrogen (Table 2). ET8556(pHlac20, pT101) cells, which contained the T. ferrooxidans ntrBC genes as a cosmid insert in the vector pHC79, produced levels of β-galactosidase activity that were threefold greater than control levels when grown under nitrogen-limiting conditions. In nitrogen-rich media there was a twofold repression of β-galactosidase activity compared with nitrogen-poor media. ET8556(pHlac20, pT130) cells, which contained the T. ferrooxidans ntrBC genes on the high copy pBluescript SK vector, had approximately twofold higher β-galactosidase levels compared with ET8556(pHlac20, pT101) cosmid-containing cells when grown in nitrogen-poor media. The cloned K. pneumoniae ntrBC genes on pK70 were capable of inducing expression from the T. ferrooxidans nifH-lacZ fusion to the same level as the cloned T. ferrooxidans ntrBC genes (Table 2, rows 6 and 8). The chromosomal E. coli ntrBC genes present in YMC10 were, however, far more efficient at inducing expression from the T. ferrooxidans nifH-lacZ fusion than the cloned genes. In high-nitrogen growth conditions β-galactosidase activity was repressed to approximately equal levels irrespective of the source of the ntrBC genes. Activation of the T. ferrooxidans nifH-lacZ fusion was also examined in the E. coli ntrB deletion strain ET8051. ET8051(pHlac20, pT130) produced fourfold greater levels of β-galactosidase than the ET8051(pHlac20) control when grown under low-nitrogen conditions. The β-galactosidase activity of ET8051(pHlac20, pT130) was lowered in high-nitrogen medium, but not to the extent that was observed in YMC10(pHlac20, pHC79). A similar trend was observed for ET8051(pHlac20, p804) containing cloned E. coli ntrBC genes, although its β-galactosidase levels were approximately twofold higher than those of ET8051(pHlac20, pT130), which contained the T. ferrooxidans ntrBC genes.

**DISCUSSION**

The K. pneumoniae nifH promoter is activated by NifA, but not activated by NtrC-PO4 in K. pneumoniae (Merrick, 1983). This is consistent with the two-tiered model of regulation of the K. pneumoniae nif genes in which NtrC is responsible for regulation of expression of nif/LA at the first level only. The unresponsiveness of the K. pneumoniae nifH promoter to NtrC-PO4 is thought to prevent expression of nif genes under inappropriate circumstances, such as in aerobic nitrogen-limiting conditions. Although the K. pneumoniae nifH promoter was also not activated in an E. coli background (Merrick, 1983), the expression of nif genes cloned from other bacteria may not necessarily mimic that in the original organism. The T. ferrooxidans nifH1 regulatory region contains two canonical NifA upstream activator sequences (Pretorius et al., 1987), which indicates that a NifA-like protein is likely to function as activator of the nifH gene in T. ferrooxidans. We set out to isolate the T. ferrooxidans nifA gene by screening for transformants in which expression of a T. ferrooxidans nifH-lacZ fusion had been activated. Because of the similarity of the T. ferrooxidans nifH promoter sequence with the Rhizobium meliloti nifH promoter and certain K. pneumoniae nifH mutant promoters (from which transcription may be mediated by NtrC-PO4; Fig. 1), we tested whether transcription of the T. ferrooxidans nifH-lacZ fusion could also be NtrC-mediated in E. coli. Since NtrC-mediated activation of the T. ferrooxidans nifH-lacZ promoter did occur, we thought it possible that either the T. ferrooxidans nifA or ntrC genes or both could be isolated by screening for β-galactosidase production in an E. coli ntrC mutant. Some screening experiments were carried out under anaerobic conditions at 30°C (Forma Scientific anaerobic glove box, N2:CO2:H2 ratio 85:15:5) to allow for the possibility that the expression of nifA, or the activity of NifA from T. ferrooxidans, might be affected by oxygen or temperature. The T. ferrooxidans ntrC gene was reisolated on several occasions during the course of both the aerobic and anaerobic screenings, but to date the T. ferrooxidans nifA gene has not been isolated (unpublished data).

Activation of the T. ferrooxidans nifH promoter by NtrC in E. coli may have been due to sequences upstream of the T. ferrooxidans nifH gene which resemble NtrC-binding sites. Although no complete NtrC-binding sites were apparent, examination of the nucleotide sequence upstream of the T. ferrooxidans nifH gene (Pretorius et al., 1987) revealed sequences with identity to one or other half of a NtrC consensus binding site. Reitzes et al. (1989) have provided evidence that NtrC-PO4 can bind weakly to a partial NtrC-binding site. Alternatively, positions of certain bases within the T. ferrooxidans nifH promoter (Fig. 1) could affect the stability of the cloned promoter complex compared to that of the K. pneumoniae nifH promoter and allow the activation by NtrC-PO4 from solution in the absence of a specific NtrC-binding site. Buck & Cannon (1989) demonstrated that transcription from a K. pneumoniae nifH promoter which contained transition mutations in the −15 to −17 region could be increased due to activation from solution by a form of NifA protein which was unable to bind to an upstream activator sequence. Whatever the method of activation of the T. ferrooxidans nifH gene in the E. coli system used, it provided a convenient colorimetric plate screening method for identifying the T. ferrooxidans ntrBC genes.

We developed the above method because we were unable to isolate the T. ferrooxidans ntrBC genes by using their ability to complement E. coli ntrC mutants for growth on minimal medium plus arginine. Having cloned and confirmed the presence and expression of the T. ferrooxidans ntrBC genes in E. coli, we again tested whether the cloned genes were able to complement the E. coli ntrC mutant ET8556 for growth on minimal medium plus arginine. No growth was obtained with ET8556(pT101) or ET8556(pT110). ET8556(pT130) cells in which the ntrBC genes were present on a high-copy-number pBluescript SK vector did form very small colonies after at least 72 h of incubation at 30°C.

The results of the complementation experiments showed that the T. ferrooxidans ntrB and ntrC genes together activated NtrC-directed expression of the T. ferrooxidans nifH-lacZ fusion to produce β-galactosidase levels clearly above basal levels. Regulation of this expression in response to nitrogen was, however, not as clear as for the
YMC10 ntrBC control. The lack of efficient regulation of the T. ferrooxidans nifH–lacZ fusion by cloned T. ferrooxidans ntrBC genes compared to the E. coli ntrBC chromosomal equivalent is probably due to the cloned ntrBC genes being carried on a multicopy plasmid. Overexpression would result in concentrations of both proteins bearing no relation to the normal stoichiometry within the cell, resulting in poor nitrogen regulation.

Unlike those of the enteric bacteria and A. vinelandii, the T. ferrooxidans ntrBC genes are not located immediately downstream of the glnA gene. It is interesting to note the distribution of the gnhALG or gnhA/ntrBC operon structure amongst bacteria in which this has been investigated. Bacteria have been divided into a number of phylogenetic divisions on the basis of 16S rRNA sequence data (Woese, 1987). All the bacteria for which ntrBC sequence information is available belong to the division proteobacteria (previously called purple bacteria). The proteobacteria have again been divided into four subdivisions, α, β, γ and δ. E. coli, K. pneumoniae, Proteus vulgaris, Vibrio alginolyticus and A. vinelandii all belong to the γ subdivision (Woese, 1987). In each of these bacteria, the ntrB and ntrC genes are located downstream of the glnA gene. T. ferrooxidans has been placed in the β subdivision of proteobacteria (Lane et al., 1991) whereas Rhizobium meliloti and Rhodobacter capsulatus are members of the α subdivision. In these bacteria the ntrBC genes (or their r. capsulatus homologues, nifR2 and nifRF) do not appear to be downstream of the glnA gene. Bradyrhizobium parasponia, Azospirillum brasilense and Agrobacterium tumefaciens are also members of the α subdivision but the physical proximity of the B. parasponia and A. brasilense ntrBC genes and the A. tumefaciens ntrC gene to their glnA genes has not been reported. As other ntrBC genes are studied, it will be interesting to find out whether the linkage of glnA to the ntrBC genes is a characteristic of members of the γ proteobacteria only, or whether this linkage also applies to any other groups of bacteria.

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