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

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An agar plating technique was developed in which the activation of expression of a *Thiobacillus ferrooxidans* *nifH-lacZ* gene fusion was used to isolate the *ntrBC* genes from a *T. ferrooxidans* gene library. An *Escherichia coli* *ntrC* mutant containing the *nifH-lacZ* fusion was transformed and plated on a low-nitrogen medium so that on flooding with ONPG, the production of yellow colonies indicated the presence of the cloned *T. ferrooxidans* *ntrBC* genes. A 4.47 kb region from the *T. ferrooxidans* chromosome was sequenced. Analysis of the sequence revealed that the *ntrB* and *ntrC* genes were closely linked to a third ORF of unknown function. Analysis of the 900 bp region upstream of the *T. ferrooxidans* *ntrBC* genes and Southern hybridization experiments confirmed that in *T. ferrooxidans* ATCC 33020, the *glnA* and *ntrBC* genes are unlinked. Expression of the *T. ferrooxidans* *nifH-lacZ* fusion in *E. coli* was activated in the presence of the *T. ferrooxidans* *ntrBC* genes and regulated by nitrogen.

Keywords: *Thiobacillus ferrooxidans*, *ntrBC* genes, *nifH-lacZ* fusion, nitrogen regulation

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 Bruijn & Ausubel, 1981; Steglitz-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.

The GenBank accession number for the sequence reported in this paper is L18975.

	-24												-12												NtrC ^a
<i>Tf nifH</i>	T	T	G	G	G	C	A	C	G	G	G	C	C	T	T	T	T	G	C	A	A	T	?		
<i>Ec glnA</i>	T	T	G	G	G	C	C	G	C	A	C	G	G	T	T	T	T	G	G	C	A	T	+		
<i>Kp nifL</i>	A	G	T	G	G	G	C	G	C	A	C	G	G	T	T	T	T	G	G	C	A	T	+		
<i>Rm nifH</i>	C	C	T	G	G	G	C	A	C	G	C	A	C	C	T	T	T	G	G	C	A	C	+		
<i>Kp nifH</i>	C	C	T	G	G	G	T	A	T	G	G	T	T	T	T	T	T	G	G	C	A	C	-		
<i>Kp nifH</i> B1	C	C	T	G	G	G	T	A	T	G	G	T	T	T	T	T	T	G	G	C	A	C	+		
<i>Kp nifH</i> B2	C	C	T	G	G	G	T	A	T	G	G	T	T	T	T	T	T	G	G	C	A	C	+		
<i>Kp nifH</i> B3	C	C	T	G	G	G	T	A	T	G	G	T	T	T	T	T	T	G	G	C	A	C	+		

Fig. 1. σ^{54} -dependent promoter regions of several nitrogen metabolism genes. The conserved -GG- and -GC- doublets at positions equivalent to -24 to -12 from the start site are blocked. NtrC^a indicates whether the promoters are activated by NtrC-PO₄. 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 (Reitzer & 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.

A feature of NtrC-PO₄ activated promoters is that they are of the sigma-54 (σ^{54})-dependent type (Popham *et al.*, 1989). Promoters recognized by the RNA polymerase ($E\sigma^{54}$) holoenzyme are characterized by having GG and GC doublets at positions -24 and -12 from the transcription start respectively. NifA is an activator of the σ^{54} -dependent promoters and transcription from the *nifH* promoter of *Klebsiella pneumoniae* is normally dependent on the binding of NifA at the upstream activator sequence (UAS). However, transcription from some mutant *K. pneumoniae nifH* promoters (Ow *et al.*, 1985) may be mediated by NtrC in the absence of NifA. The sequences in the region between the conserved GG and GC doublets within the σ^{54} -dependent promoter are important in distinguishing between promoters from which transcription may be mediated by NtrC-PO₄ and those requiring

NifA. Examination of σ^{54} -dependent promoters (Fig. 1) has indicated that the presence of T residues in positions -17 to -15 is characteristic of a promoter which could be activated by NtrC-PO₄ (Ow & Ausubel, 1983). In contrast, the wild-type *K. pneumoniae nifH* promoter, which has a stringent requirement not to be activated by NtrC-PO₄, has C residues at these positions (Benyon *et al.*, 1983). Ow *et al.* (1985) used *K. pneumoniae nifH-lacZ* fusions to study the effect of point mutations in the *K. pneumoniae nifH* promoter region. Three mutant promoters which were the result of C to T transitions at positions -17 to -15 (Fig. 1) showed activation by NtrC-PO₄. Ray *et al.* (1980) have shown by transcript mapping both *in vitro* and *in vivo* that the *K. pneumoniae nifH* B3 promoter mutant (Fig. 1) required NtrC-PO₄ at high intracellular concentration for activation, while NtrC-PO₄ was unable to activate transcription from the wild-type *nifH* promoter.

Pretorius *et al.* (1987) sequenced the *nifH* gene of *T. ferrooxidans* and reported that it had a σ^{54} -dependent promoter which in common with NtrC-PO₄ activated promoters, also contained a T at position -15. In this paper we describe how a *T. ferrooxidans nifH-lacZ* fusion was used to isolate a clone containing the *T. ferrooxidans ntrBC* genes. We demonstrate through sequencing and Southern hybridization that the *T. ferrooxidans ntrBC* genes are not linked to the *glnA* gene. In addition we show that *T. ferrooxidans ntrC* expression is regulated by nitrogen in an *E. coli* background.

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 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i>		
YMC10	$\Delta lacU169$	Backman <i>et al.</i> (1981)
ET8556	<i>ntrC1488 lacZ::IS1 gyrA rbs hutC_K^c</i>	Merrick (1983)
ET8051	$\Delta(rba-glnA) gyrA rbs hutCKc$	Pahel & Tyler (1979)
Plasmids		
pEcoR252	Ap ^r EcoRI	P. Janssen, Ghent
pBluescript SK ⁺	Ap ^r	Stratagene, San Diego
pACYC184	Cm ^r Tc ^r	
pHC79	Ap ^r Tc ^r cosmid vector	Hohn & Collins (1980)
pT101	Ap ^r <i>T. ferrooxidans</i> genomic fragment in vector pHC79	This work
pHlac	Ap ^r <i>T. ferrooxidans nifH-lacZ</i> in vector pMC1403	Berger <i>et al.</i> (1990)
pHlac10	Cm ^r <i>T. ferrooxidans nifH-lacZ</i> in Tc ^r gene of pACYC184	This work
pHlac20	Cm ^r pHlac10 but Tc ^r promoter deleted	This work
pK70	Ap ^r <i>K. pneumoniae glnA⁺ ntrB⁺ ntrC⁺</i> , HindIII fragment of pFB514 cloned into HindIII site of pHC79	This work
pFB514	Cm ^r <i>K. pneumoniae glnA⁺ ntrB⁺ ntrC⁺</i>	De Bruijn & Ausubel (1981)
p804	Ap ^r <i>E. coli ntrB⁺ ntrC⁺</i>	Tuli <i>et al.</i> (1982)
pMEB100	Ap ^r <i>T. ferrooxidans glnA⁺</i>	Barros <i>et al.</i> (1985)

Table 2. Expression of the *T. ferrooxidans* *nifH-lacZ* fusion in *E. coli* in the presence and absence of *ntrC* genes

Strain and relevant genotype*	β -Galactosidase activity (Miller units)	
YMC10 <i>ntrBC</i> ⁺ (pHlac) <i>Tf nifH-lacZ</i>	32024† (4220)	
ET8556 <i>ntrC</i> (pHlac) <i>Tf nifH-lacZ</i>	214† (38)	
ET8556 <i>ntrC</i> (pFB514, pHlac) <i>Kp ntrBC</i> ⁺ , <i>Tf nifH-lacZ</i>	13326† (1952)	
	Low N‡	High N‡
ET8556 <i>ntrC</i> (pHlac20, pHc79) <i>Tf nifH-lacZ</i>	312 (26)	266 (42)
ET8556 <i>ntrC</i> (pT101, pHlac20) <i>Tf ntrBC</i> ⁺ , <i>Tf nifH-lacZ</i>	1048 (125)	553 (74)
ET8556 <i>ntrC</i> (pT130, pHlac20) <i>Tf ntrBC</i> ⁺ , <i>Tf nifH-lacZ</i>	2035 (420)	483 (65)
ET8556 <i>ntrC</i> (pHlac20) <i>Tf nifH-lacZ</i>	654 (77)	413 (58)
ET8556 <i>ntrC</i> (pK70, pHlac20) <i>Kp ntrBC</i> ⁺ , <i>Tf nifH-lacZ</i>	2185 (415)	452 (61)
YMC10 <i>ntrBC</i> ⁺ (pHC79, pHlac20) <i>Tf nifH-lacZ</i>	7634 (1015)	464 (62)
ET8051 <i>ntrBC</i> (pHlac20) <i>Tf nifH-lacZ</i>	341 (49)	418 (55)
ET8051 <i>ntrBC</i> (pT130, pHlac20) <i>Tf ntrBC</i> ⁺ , <i>Tf nifH-lacZ</i>	1252 (325)	980 (157)
ET8051 <i>ntrBC</i> (p804, pHlac20) <i>Ec ntrBC</i> ⁺ , <i>Tf nifH-lacZ</i>	2816 (633)	1577 (139)

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

† Strains were grown aerobically under nitrogen-limiting conditions.

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

Fig. 2. Restriction endonuclease map of the 13.2 kb *HindIII* *T. ferrooxidans* chromosomal DNA insert of construct pT110 and derivatives. The *ntrB* gene, *ntrC* gene and ORF of unknown function as well as the direction of transcription are indicated by the open arrows. pEcoR252 and SK are the cloning vectors pEcoR252 and pBluescript SK⁺ respectively. B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sc, *Sac*II; Sl, *Sma*I; St, *Stu*I; X, *Xho*I.

scribed. When required, antibiotics were added at the following concentrations: ampicillin, 100 μ g ml⁻¹; chloramphenicol, 25 μ g ml⁻¹; tetracycline, 20 μ g ml⁻¹.

DNA techniques, sequencing and analysis. The alkaline lysis method was used for both small- and large-scale DNA preparation. Standard methods (Sambrook *et al.*, 1989) were used for restriction digests, gel electrophoresis, ligations and Southern blot hybridizations. Probes used in hybridization experiments were labelled by nick-translation using the DIG system (Boehringer Mannheim). DNA sequencing was carried out by the dideoxy-chain-termination method with overlapping

templates generated by exonuclease III shortening. Where templates failed to overlap, custom-made single-stranded sequencing primers were used. Sequencing reactions were carried out by using the Sequenase kit (version 2.0) from US Biochemical. The entire sequence reported in Fig. 3 was determined from both strands. The DNA sequences were analyzed with the Genepro (version 4.1) program and the Genetics Computer Group Inc. software package (version 7.1).

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 *Eco*RI-*Sal*I fragment from pHlac (containing the *T. ferrooxidans* *nifH-lacZ* fusion and the *lacY A* genes) was ligated to pACYC184 that had been digested with *Bam*HI and *Sal*I. After ligation of the *Sal*I ends, the remaining *Eco*RI and *Bam*HI ends were filled-in with the Klenow fragment of DNA polymerase and these blunt ends ligated. This gave plasmid pHlac10, containing a pBR322-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 *Xba*I and *Eco*RV (both unique sites upstream and downstream of the *tet* promoter), the *Xba*I 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

CTGCAGGCTGTTGGCGCGGTAAATGAGCAGTTCCACCGGAGACCTACGGTGACATTGCTGCGCATGGTGAATTCATGGAGACCAGCGCACAGCGTGCCG 100
CAGCCTCCAGCGAGAGGTGGGCGCTGACTACGCGGTCCAGGATCGGTTTGGCGTACTTGATTTCGCCGATTTCAGAGAAAGGATGGTCCGACGATTTCATG 200
GATATAGTTGCCCTTGTGGATAGATCATATAGGTGGCTGGCATCTCTGCCCGCATCTGCCACCGAGTATGAAGGTGCGCTCGAAATTGGTGTGGCGGTA 300
TCGCGGTTGGCTTGGTGGCGTGACCTCGGCATTGATGAGGCGGACATAATCCGCGCGCTCCGCCATGCTGCCAGATTGAGCAGATGGATTTCCGCGT 400
CCTGGTCGATATCTTGGTGCATCCGCTTGACCAACGCCCTGGGTGGTGGCGAGATTGCCCGCTGAAAGCAGGAGAGAAAACGATCCCGGGCCAGCAGAA 500
GTGATGCATTTTCGAGTAGATGCTGACATTGTGCGGTGCCCGCGTTGGTCCGCGAGTCTGAGCAGAAAACAGACCCCGGTGACGTGGGTGGTGAAGCAA 600
TAGGTCATGGAGTATCTGTGAGCCCGACGACATGGATGGATGATAGTCGTCCGAAAATACCGTCTTGGGAAGCCCGACCAAGAGGTGCGTCCGCAAT 700
GTGCGCTAGGCGCCTCGTCCATAATGCACTATTATGGTGCATCATATGCGTCTGCACCGGCTGCCGCACGATAAGGGCATGCTGGTGTGCTGCGTGGGG 800
ATGCGGAAGCATGTCCATGGTATGCCGGGTGACGGGGGTGTGTTCTGACGGTCTGGTGGCGCTTTTGGCTTTACTGAGCACGGAGCCTGTTTGGTGAAT 900
CATAAGATCGAGCTTGATGGCGCGCCGCAAAAACATAACCACTGCCCGCGGAGATATGGTGTGACGCGCTGGAATCAGCGGTACTGTTCTG 1000
M A R R K N I T T L P A E N M V L D A L E S A V L V L G 28
GAGCGGATTACCATATCCGATATATGAATCCGCGCGCGGAGAAATCTGCTGCGCTCAGCCAGCACAGGCAATGGGGCAATATTTGCCGCGCTGTGCAGC 1100
A D Y H I R Y M N P A A E N L L R S A S T R H W G N I C R R C A A 61
GTCGTGGCGAAGAGCGATTTGCCACACTTTTCTGGATACCATCGCGCGAACACGCGCAACGCTGCGTAGAGCCCTGCCCTGGCGCTGAGTGAGGGC 1200
S L A K S D L P H F S W I P S R R T R Q R C V E P L P L A L S E G 94
GGCGCGCGCTGGTGGATTGGTGGTGACACGGCACCAACCGACGGCCTCATTATTGAAATGCGGCCATGGAATGCCGCGCGGACGCGGAGGAGG 1300
G G A V V D L V V T P A P T D G L I I E M R P M E L P A R H A E E E 128
AAATGAGGACCGCATTTACGGGGCAGCGCAGGAGATGCTGCGCGCCTCGCCACGAAATCAAAAATCCCTCGCGGCTTTCGCGGGGCTGCGCAACT 1400
M Q D R I Y G A A Q E M L R G L A H E I K N P L G G L R G A A Q L 161
CCTGGATCGCGAGTTGCAACGCCGGAATGAAAGATTACCCCGCTCATTCTCCATGAGGTGGATCGTCTGCATCATCTGGTGGATCGCTCCAGGGA 1500
L D R E L Q R P E L K D Y T R V I L H E V D R L H H L V D R L Q G 194
CCCCGTAGCGCGCGGTTTTCGCCAGGTGAATATCCATCAGGTGCTGGAGCAGTGCCTGCTGCTGAATGCGGAATGCCACCGGTATTGCCGTGC 1600
P R S R P V F A E V N I H Q V L E H V R R L L N A E L P T G I A V R 228
GTTTCGACTACGACCCCTCCATTCCCGACCTTATGGCGGACAGGAGCAACTGGTGCAAGTTTTCTGAATCTGATGCGCAATGCCAACAGGCGCTGGA 1700
F D Y D P S I P D L M A D Q E Q L V Q V F L N L M R N A Q Q A L D 361
CCGCCACGGTGAATTTCTGATCCGTACCCGGGTGCAACGCTACGTTACCTGCTGCACCAAGTTCGCCCTGGCGCTGCGTGTGGATGTAGTCGACAA 1800
R H G A I L I R T R V E R Y V T L L H H K F R L A L R V D V V D N 394
GGCCCCGGAATCTCCCGGATCTGCTGCCACGGATCTTTTGGCCCTGGTCAACAGTCTGCGCGGAGGATGGGAATGGGGCTCGCCATCTACAGGGAC 1900
G P G I S P D L L P R I F L P L V T S R A E G M G M G L A I V Q G L 428
TGGTGGCGGGCATGGCGGCTGGTCCACTGCCATTCAACCGGGAGAAACGGTATTTTCCGTTTCTGCTGCCCTGTACCGCACCCCGGAGGATCCGC 2000
V R G H G G V V H C H S Q P G E T V F S V S L P L S P H R E A S A 461
ATGACACAAGTCTGGATTATCGATGACGACCTTCCATTGCTGGGTTTGGAGAAGGCGTTGACTCAGGCCGATATCTGGTGGCGAGCTTTGACAGCG 2100
*
M T Q V W I I D D D P S I R W V L E K A L T Q A D I L V R S F A D A 34
CCGACAGCGCGCTGCGGGCCTTGGGGCTGAGCAGCCAGGGGCGCTTGTACCCGATCTGCGTATGCCCGGCTGGACGGATTGGCGTTTCTGCGGAAGT 2200
D S A L R A L G R E Q P G A V V T D L R M P G L D G L A F L R E V 67
TCAGTCGCGCTGGCCAAAATGCCGGTATCGTGATGACGGCGCACTCTGATCTCGACAATGCCCTGCGCGCTTTTCAAAGTGGGGCTTTGAGTATCTG 2300
Q S R W P K L P V I V M T A H S D L D N A V A A F Q S G A F E Y L 100
CCCCAGCGGTTGACATGGACGAGGCGGTTACGCTGGTGACGCGCGCTTCCGCGAGCGGGCTGGACGGGCGCGGGGCTGGAGGAGAGCGATCCGG 2400
P K P F D M D E A V T L V Q R A L G S R A G P G A A G V E E S D P A 134
CGGAAATGATCGGTGAGGCTCCGGCCATGCAAGAGGTGTTTCTGCCATCGGGCTCTGTCGCGCTCACAGATCAATGTGCTGATTACCGCGAATCGGG 2500
E M I G E A P A M Q E V F R A I G R L S R S Q I N V L I T G E S G 167
ATCCGGCAAGGAATGGTGGCCAGTGCATGCAACCGGACAGCCCGCTGCGCGGACCTTTCATTGCCATCAACAGCGCGGCATTCCCGCCGAGTTG 2600
S G K E L V A S A L H R H S P R A R G P F I A I N T A A I P A E L 200
CTGGAATCGGAACCTTTTCGCCCATGAAAGGGCGCTTTTACCGGGGCGGTGCGAGCTCGGAGGGGCGCTTCGAGCAGGCTCCGGGGGAACCTCTTTT 2700
L E S E L F G H E K G A F T G A V Q T R Q G R F E Q A S G G T L F L 234
TGGATGAAATCGCGATATGCCGGCGCGCTGCAACCGCGCTGCTGCGGGTGTCTTCCGACGGCACTTTCTATCGGGTGGTGGCCACGCGCCCGAGCG 2800
D E I G D M P A A L Q T R L L R V L S D G T F Y R V G G H A P Q R 267
TGGGAGCTCCGCTCTCGCGCGGACCCACGAACTCGAAGCCAAGGTGCGCGATGGCAGCTTCCGGGAAGATCTTACCACCGTCTCAACGTCATC 2900
A D V R V L A A T H Q N L E A K V R D G S F R E D L Y H R L N V I 300
CATATCCATCTTCCGCCATACGCGAGCGGCGTGAGGACATTCGCGCTGTCGCCCGCCATTTTCTCCGTGCTAGCGCGGAGCAATGGATGTGAGCGGC 3000
H I H L P P I R E R R E D I P R L A R H F L R R S A E Q L D V E R R 334
GAATATTGAGCCCGGAGCGAGTCGCTTCATGAATGCGATTGGCCCGCAATGTGCGCAATGGAAAACGTCTGCTGCTGGATTACGGTGTGAGCACC 3100
I L S P E R V A F M N W H W P G N V R Q L E N V C R W I T V M A P 367

Fig. 3. For legend see facing page.

CACCCGCGAGGTCCAGGTGACGGATTGCGCGCGGAAATGGCGCATCGCCGATGTCGCCATGATGCCGGATCCGAACACCCAGGATTGGCGCTGCTG 3200
 T R E V Q V T D L P P E M A A S P M S A M M P D P N T Q D W R L L 400
 CTGGGGGGGGTGGTGCAGGACGCTCTCGCGCGCTGAAGAAGCCTTGCTGGACAAGATTACGCCGCTGTTTGAACGCTGTCTGCTGGAAGCGGCCCTGCAAC 3300
 L G A V V R H V S R R E E A L L D K I Q P L F E R C L L E A A L Q H 434
 ATACCCGTGGCCATAAGCAGAGCGCGCGCACAACTCGGGTGGGGGCGCAATACGCTACCCGCAAGCTGAAAGAGTTGGGCATGGCGGAGGATACGTA 3400
 T R G H K Q S A A H K L G W G R N T L T R K L K E L G M A E D T * 466
 AGGCGTCGGTTCTGGCGAATCCCGGCTTCGACTAAAACCCCTTGTATGTGGTTATAAAGGTATGCTTGGTCGCGCTTGGGTGTCTATGCAAGGTG 3500
 M Q G V 4
 TAATGTTACTGTTGGAATCGTTGTCTGCTCTGCTACTGTTGGAGGTTTTCATTCTGCTGAGGGCACGGCGCCGCTTTACAGAACAAAAGCAGCGC 3600
 M L L L E I V V V L L L L V E V F I R L R A R R R R R L Q N K S S A 37
 GCCAGTCTCTCAGCAGCGGTTGGCACCCGCCACAGCGTCTGCAGATTGACCCGAAGTGTCCGGCGTAAATGCTGTGTCCACCCGTAACCACT 3700
 P V S S A A R S A P A T D V L Q I R P E V S G V N A V V P P V T T 70
 ACCGGTGGCGCGAGCAGCAAGCGCTCAGCATTGACGACCTCCTCAATGAAGCGACCATTTACTGGAGTATGGCCACTATGCGCAGGCGCGACCGTGT 3800
 T G G A E Q Q A L S I D D L L N E A T I Y L E Y G H Y A Q A A T V L 104
 TGGCTGTGTATGTGGATATCAACCCGCATGTGACCAAAACCCATCAACATGCTCCTGGATGCCTATCTTGGCCATGGCCGATATCGACAGCTATGCCGACCT 3900
 R W Y V D I N P H V T K P I N M L L D A Y L A M A D I D S Y A D L 137
 GCTCGAAAGTCTGGGAGAAAAGCGGGGCTGCCCCATGGACGAGTCTGTGGCGGGGAACGGGTAAATACCGGTCTGAGTCAGGATCCCGGCAATCTT 4000
 L E S L G E K P G A A P M D E S W W R E R V N T G L S Q D P G N L 170
 GAACTCCTCGTCTTGGCGAGAAAGTGGGCATGGCCATCCCTCTCCGCAAGTGCGAACCTCCGATGCCGTATGACCGCGGAGATGGCGCTGGCCCTGG 4100
 E L L V L A E K V G M A I P L P Q V R T P D A V M T A E M A L A L V 204
 TGTCCCGCAACCATGATCCGGCTACGGCATGGCGATCTGTGGCGGGTATCGCCCATGAACCGCGGCGACTGCCGTTGTATGCCGAATCTTGGCGCAT 4200
 S R N H D P A Y G M A I L W R A I A H E P R R L P L Y A E L L R I 237
 AACCTACCAGCAACCGCGTATCGAGGATTATATCAATACCTGATTCTGCTCTTCTGGCGGTGGGCGAGTGGTGGCAAAACCTTGGCTGAACCGCATGTTG 4300
 T Y Q Q R R I E D Y I N T L I L L F L A V G S G G K T L R E R M L 270
 CGGGCAGGTGAAGACCTCGGCCCGCACCTCCTGTGGGATACCTTGCACAATGGAATGGCGACCCGGAAGTATTGAGACGATTGGCCAGATCAGCCCAAC 4400
 R A G E D L G P H L L W D T L A Q W N G D P E V L R R L A R S R Q L 304
 TGGAGATTCCGGTCCGGTTGTCCGGCGCTGCGGCACAAGCGGATGACCGCGCTTGACAAGCTTATCGAT 4470
 E I P V G L S G A A A Q G G * 328

Fig. 3. Nucleotide sequence of the 4.47 kb *EcoRI*–*HindIII* fragment containing *T. ferrooxidans* *ntrB*, *ntrC* and the unidentified ORF. Predicted amino acid sequences are given below in single-letter code. Putative ribosome-binding sites are indicated in bold, the putative σ^{54} promoter is underlined and potential NtrC-binding sites are overlined.

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). *E. coli* YMC10(pHlac10 or 20) and *E. coli* ET8556(pHlac10, pK70) colonies which contained either the *E. coli* or *K. pneumoniae* *ntrC* gene produced bright yellow colonies within 5–15 min. A problem with colony wash-off on flooding with ONPG was experienced initially. Incubation at 4 °C to 'harden' the colonies solved the problem of 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 colonies turned blue. MacConkey/lactose medium contained too high a concentration of undefined nitrogen sources to be workable.

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 plasmid. 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 GNM 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-

(a)	Tf	MARRKNIT.TLP..AENMVLDALSAVLVLGADYHIRMNPAENLLRSASTRHWNICRRCAASLAKSDLPHFWSIPSRRT	100
	Ec	MATGTPQPDAGQ..ILNSLINSILLDONLAIHYANPAQAQLL.AQSSRLKFTPLPELLSYF.S.LNIELMQESLEAG	
	Kp	MATGTPDPAGQ..ILNSLINSILVDODLAVHYANPAQAQLL.AQSSRLKFTPLPELLSYF.S.LNIELMQESLEAG	
	Va	MVTATLILDDGLAIRYANPAEELF.SQSAKRIVQSLSLQIQA.S.LDLALLTQPLQSG	
	Pv	METGNLP..DNLTILDSLIHSLV/INQEFICYANHAALQVL.AQSSRLKFTPTPTDLSYH.S.FDAELMQETLANG	
	Bp	MTSAMEFRPVPPTDGEA.IILNALPNPVL.VAPDGRVIDANIAAESFF.EISTQFLRRQSLKELVPFG.SPLLLALIEQVTS	
	Rc	MNLPP..PGIWNLSLPLALDVLNDVRVIEINPAEELF.NLSARALKQAGLBERLAI..SAPLEIIPARVRKN	
	Ab	MARASAAAPLPRRPARPRAPSSSRVPLPCIDPSVMLNALPDVPLV.DGSDIRYVNLAEQEFF.GLSAAMMEGPLAEELPPN.S.PVSQLEIQVQQR	
	con	L N AA S S	
	Tf	QRCVEPLPLALSEGGA..VVDLVVTPAPTDLGLIEMRPMELPARHAE..EMQDRIYGAAQE...MLRGLAHEIKNPLGGLRGAQQLDRELQ..RP	200
	Ec	QGTPTNEVLTVIDGRSH...ILSVTAQRMMPDGMILEMAPMDNQRHLSQE..QLQHAQQVAARD...LVRGLAHEIKNPLGGLRGAQQLSKALP..DP	
	Kp	QGTPTNEVLTVIDGRSH...ILSVTAQRLPEGYILEMAPMDNQRHLSQE..QLQHAQQIAARD...LVRGLAHEIKNPLGGLRGAQQLSKALP..DP	
	Va	QSTIDSDEVTVFVVDGRPLMLEVTVSPITWQKQLMLLVEMRKIDQQRHLSQE..LNQHAQQAAKL...LVRGLAHEIKNPLGGLRGAQQLSKALP..DP	
	Pv	QSTFNDNEVLVIHMQSH...TMSLSAQPISEQHILLELAPMDSQRHLSQE..QIQQAQQIAARE...LVRGLAHEIKNPLGGLRGAQQLSKALP..DP	
	Bp	SPV.NEYKVDLGTPTRI..GGDRQVDLHVAPLTERPGHIVVMLEHETIADKMDROLTHRSAAARS.VIALAAMLAHEIKNPLSGIRGAQQLLEQASSED	
	Rc	RSALFVNVDL.TTGE..RAPVCNLQAAPITADDETIVLLISPIEADRLGRASVMKSAARSAGIGMAEMLAHEIKNPLAGIAGAQLLQSGLED	
	Ab	HRASQEG.VVID.TTAH..RAPACHRAGTPRWASPTMCLTVNEITLARKIDNSLTHRNAARS.VTAMASMLGHEVKNPLSGIRGAQQLLENCESEDR	
	con	I AA LAHEIKNPL G RGAQQL	
	Tf	ELKDYTRVILHEVDRLHHLVDRL..QGPRSRPVFAEVNIHQVLEHVRLLNLAELPTGIARVFDYDPSIPDLMDQEQVLQVFLNLMRNAQALDRHGA..	300
	Ec	SLLEYTKVIEQADRLRLNLDRL..LGPQLPGTRVETSIHKVAERVVLVSMELPDNVRILRDYDPSLPELAHPDQIEQVLLNVRNALQALGPPEGGE	
	Kp	ALMEYTKVIEQADRLRLNLDRL..LGPQHPGMHVETSIHKVAERVVLVSMELPDNVRILRDYDPSLPELPHDPDQIEQVLLNVRNALQALGPPEGGE	
	Va	SLTEYTHVIEQADRLRLNLDRL..LGPQKPKKTKQENLHQILEKVRQLEVESQRSIVIERDYDPSLPEILMDAQIEQVLLNVRNALQALGPPEGGE	
	Pv	ALTEYTVQVIEQADRLRLNLDRL..LGPQHPGKTKQSIHHVVERVAQLISLECPENVTLLKDYDPSLPELSHYPDQIEQVLLNVRNALQALGPPEGGE	
	Bp	LLTR...LICDEADRIVTVLDRMEVFGDPRVARGPVNIHVSVDLHRAKQSGFARNVRFIEDYDPSLPPVLANQAGVAEDKGTDA	
	Rc	ELTD...LIVDETRRIVKLEQVEQFGNVRPPEMKPVNIHVDLDRHAKSAGVGFAGHMLIVEDYDPSVPTLGDADQILQVFLNLRNALQALGPPEGGE	
	Ab	VLTR..RLICDEADRIVTVLDRMEVFGDPRVARGPVNIHVSVDLHRAKQSGFARNVRFIEDYDPSLPPVLANQAGVAEDKGTDA	
	con	L R I DR LV R G P IH V V DYDPS P DQ Q LN NA G	
	Tf	LIRTRTVERVYTL.....LHHKFLRLALRVDVVDNGPGISPDLLPRILPLVTSRAEGMCMGLAIVQGLVRHGCVVHCHTFTFVSFVSLPSPHRE	400
	Ec	LIRTRTAFQTL.....HGERVRLAARIDVEDNGPGIPSHLQDILFYPMVSGREGGTGLGLSIARNLDQHSKIEFTSWPGHTEFVSFVSLPIRK	
	Kp	LIRTRTAFQTL.....HGVYRLAARIDVEDNGPGIPSHLQDILFYPMVSGREGGTGLGLSIARNLDQHSKIEFTSWPGHTEFVSFVSLPIRK	
	Va	LIRTRTAFQTL.....HGKRCCKLAARVETDNGPGIPPELQDILFYPMVSGREGGTGLGLSIARNLDQHSKIEFTSWPGHTEFVSFVSLPIRK	
	Pv	LIRTRTAFQTL.....HGERHRLVARIDVITDGTSGIPPHLQDILFYPMVSGREGGTGLGLSIARNLDQHSKIEFTSWPGHTEFVSFVSLPIRK	
	Bp	EQLTARFPGV...RLSVGPKKSRVSLPLECFKVDNGSGVPELDPVLDPFVTTKQYSGGLGALVAKIVGDHGGIICESQPRKTTFRVLVDADVQRRO	
	Rc	..LRITFYDYALRLRRPDGGERCAAPLQVEVIDDGPPIADIASIIFEPVSGRENGTGLGLALVSKIISEHNGWISVESAPGRVPLRIISLPVVRKN	
	Ab	IISTSYQHGV...RMALPGDTRHLPLLVSVQDNGDIPDDLRSVLDPFITTVMGTGLGLALVAKIVGDHGGIIFEDSQPRRTVFRVSLPMPDEAQ	
	con	L D G GIP L P F V G GLGL H G I S P T F	
	Tf	ASA	
	Bp	ATRPQKPR	
	Ab	MSGDPAPARGIRGAIG	
(b)	Tf	MTQVWIIDDDPSIRWLEKALQADILVRSFADADSALRALGREPGAVVTDLRMPGLDGLAFLEVRQSRWPKLPVIMTAHSDLDNAVAAPQSGAPE	100
	Ec	MQRGIWVVDVDDSSIRWVLERALAGAGLTCTTFENGAEVLEALASKPDVLLSDIRMPMDGLLALKQIKQRHMLPVIIMTAHSDLDNAVAAPQSGAPE	
	Kp	MQRGIWVVDVDDSSIRWVLERALAGAGLTCTTFENGAEVLEALASKPDVLLSDIRMPMDGLLALKQIKQRHMLPVIIMTAHSDLDNAVAAPQSGAPE	
	Pv	MQRGIWVVDVDDSSIRWVLERALAGAGLTCTTFENGAEVLEALASKPDVLLSDIRMPMDGLLALKQIKQRHMLPVIIMTAHSDLDNAVAAPQSGAPE	
	Bp	MPAGSLVADDSDAIRTVLQALSRAGYEVRLTGNAAATLWRWVSGQGLDVLITDVMVMDENAFDLPRIKKRPPDLVLMVAQNTFMTAIRPSERGAYE	
	Rm	MTGATILVADDSDAIRTVLQALSRAGYEVRLTGNAAATLWRWVSGQGLDVLITDVMVMDENAFDLPRIKKRPPDLVLMVAQNTFMTAIRPSERGAYE	
	Rc	MDGTVLVADDSDAIRTVLQALSRAGYEVRLTGNAAATLWRWVSGQGLDVLITDVMVMDENAFDLPRIKKRPPDLVLMVAQNTFMTAIRPSERGAYE	
	At	MTATILVADDSDAIRTVLQALSRAGYEVRLTGNAAATLWRWVSGQGLDVLITDVMVMDENAFDLPRIKKRPPDLVLMVAQNTFMTAIRPSERGAYE	
	Ac	MSAATILVADDSDAIRTVLQALSRAGYEVRLTGNAAATLWRWVSGQGLDVLITDVMVMDENAFDLPRIKKRPPDLVLMVAQNTFMTAIRPSERGAYE	
	con	DDD IR VL AL G D D MP L I P LPV M A A GA	
	Tf	YLPKPFDMDEAVTLVQALSRAGYEVRLTGNAAATLWRWVSGQGLDVLITDVMVMDENAFDLPRIKKRPPDLVLMVAQNTFMTAIRPSERGAYE	200
	Ec	YLPKPFDMDEAVTLVQALSRAGYEVRLTGNAAATLWRWVSGQGLDVLITDVMVMDENAFDLPRIKKRPPDLVLMVAQNTFMTAIRPSERGAYE	
	Kp	YLPKPFDMDEAVTLVQALSRAGYEVRLTGNAAATLWRWVSGQGLDVLITDVMVMDENAFDLPRIKKRPPDLVLMVAQNTFMTAIRPSERGAYE	
	Pv	YLPKPFDMDEAVTLVQALSRAGYEVRLTGNAAATLWRWVSGQGLDVLITDVMVMDENAFDLPRIKKRPPDLVLMVAQNTFMTAIRPSERGAYE	
	Bp	YLPKPFDMDEAVTLVQALSRAGYEVRLTGNAAATLWRWVSGQGLDVLITDVMVMDENAFDLPRIKKRPPDLVLMVAQNTFMTAIRPSERGAYE	
	Rm	YLPKPFDMDEAVTLVQALSRAGYEVRLTGNAAATLWRWVSGQGLDVLITDVMVMDENAFDLPRIKKRPPDLVLMVAQNTFMTAIRPSERGAYE	
	Rc	YLPKPFDMDEAVTLVQALSRAGYEVRLTGNAAATLWRWVSGQGLDVLITDVMVMDENAFDLPRIKKRPPDLVLMVAQNTFMTAIRPSERGAYE	
	At	YLPKPFDMDEAVTLVQALSRAGYEVRLTGNAAATLWRWVSGQGLDVLITDVMVMDENAFDLPRIKKRPPDLVLMVAQNTFMTAIRPSERGAYE	
	Ac	YLPKPFDMDEAVTLVQALSRAGYEVRLTGNAAATLWRWVSGQGLDVLITDVMVMDENAFDLPRIKKRPPDLVLMVAQNTFMTAIRPSERGAYE	
	con	YLPKPF D RA AMQ R RL I GESGTGKELVA ALH R PF A	
	Tf	INTAAIPAELESELFGEHKGAFGTGAVTQGRFEQASGGTFLDEIDMPALQATRLRLVLSDGTFFYRVGGHAPQADVRVLAATHQNLAKVRDGS.F	300
	Ec	INTAAIPAELESELFGEHKGAFGTGANTIRQGRFEQADGGTFLDEIDMPALQATRLRLVLSDGTFFYRVGGHAPQADVRVLAATHQNLAKVRDGS.F	
	Kp	INTAAIPAELESELFGEHKGAFGTGANTIRQGRFEQADGGTFLDEIDMPALQATRLRLVLSDGTFFYRVGGHAPQADVRVLAATHQNLAKVRDGS.F	
	Pv	INTAAIPAELESELFGEHKGAFGTGASVQRGRFEQAGGSLFLDEIDMPALQATRLRLVLSDGTFFYRVGGHAPQADVRVLAATHQNLAKVRDGS.F	
	Bp	INTAAIPAELESELFGEHKGAFGTGANTRASGRFEQAGGSLFLDEIDMPALQATRLRLVLSDGTFFYRVGGHAPQADVRVLAATHQNLAKVRDGS.F	
	Rm	INTAAIPAELESELFGEHKGAFGTGANTRASGRFEQAGGSLFLDEIDMPALQATRLRLVLSDGTFFYRVGGHAPQADVRVLAATHQNLAKVRDGS.F	
	Rc	INTAAIPAELESELFGEHKGAFGTGANTRASGRFEQAGGSLFLDEIDMPALQATRLRLVLSDGTFFYRVGGHAPQADVRVLAATHQNLAKVRDGS.F	
	At	INTAAIPAELESELFGEHKGAFGTGANTRASGRFEQAGGSLFLDEIDMPALQATRLRLVLSDGTFFYRVGGHAPQADVRVLAATHQNLAKVRDGS.F	
	Ac	INTAAIPAELESELFGEHKGAFGTGANTRASGRFEQAGGSLFLDEIDMPALQATRLRLVLSDGTFFYRVGGHAPQADVRVLAATHQNLAKVRDGS.F	
	con	N AAIP L ESELFGEHKGAFGTGA GRFEQA GG LDET:DMP QTRLLRLV G VGG P DVRI AAT L G F	
	Tf	REDLYHRLNVIHILPPIRERREDIPRLARHFLRRSAEQDLVERILLSPERVAFNMW.HWPGNVRLQENVCRWITVMAPTREVQVTDLPPEMAASPMAS	400
	Ec	REDLYHRLNVIHILPPIRERREDIPRLARHFLRRSAEQDLVERILLSPERVAFNMW.HWPGNVRLQENVCRWITVMAPTREVQVTDLPPEMAASPMAS	
	Kp	REDLYHRLNVIHILPPIRERREDIPRLARHFLRRSAEQDLVERILLSPERVAFNMW.HWPGNVRLQENVCRWITVMAPTREVQVTDLPPEMAASPMAS	
	Pv	REDLYHRLNVIHILPPIRERREDIPRLARHFLRRSAEQDLVERILLSPERVAFNMW.HWPGNVRLQENVCRWITVMAPTREVQVTDLPPEMAASPMAS	
	Bp	REDLYHRLNVIHILPPIRERREDIPRLARHFLRRSAEQDLVERILLSPERVAFNMW.HWPGNVRLQENVCRWITVMAPTREVQVTDLPPEMAASPMAS	
	Rm	REDLYHRLNVIHILPPIRERREDIPRLARHFLRRSAEQDLVERILLSPERVAFNMW.HWPGNVRLQENVCRWITVMAPTREVQVTDLPPEMAASPMAS	
	Rc	REDLYHRLNVIHILPPIRERREDIPRLARHFLRRSAEQDLVERILLSPERVAFNMW.HWPGNVRLQENVCRWITVMAPTREVQVTDLPPEMAASPMAS	
	At	REDLYHRLNVIHILPPIRERREDIPRLARHFLRRSAEQDLVERILLSPERVAFNMW.HWPGNVRLQENVCRWITVMAPTREVQVTDLPPEMAASPMAS	
	Ac	REDLYHRLNVIHILPPIRERREDIPRLARHFLRRSAEQDLVERILLSPERVAFNMW.HWPGNVRLQENVCRWITVMAPTREVQVTDLPPEMAASPMAS	
	con	REDL RL V PPIR R ED P L RH F G WPGNVRLQENVCRWITVMAPTREVQVTDLPPEMAASPMAS	
	Tf	MPDPNTQD.WRLLGAVVRHVSRRREA.LLDKIQP.....LFEKCLLEAALQHTRGHKQSAHKLWGWRNTLTKRLKELGMAEDT	
	Ec	TSQMPQDS.WATLLAQWADRALRSQHNLSEAQP.....LEFETLLTALRHTQGHKQEAARLLGWGRNTLTKRLKELGME	
	Kp	PTQMLPDS.WATLLAQWADRALRSQHNLSEAQP.....EMERTLLTALRHTQGHKQEAARLLGWGRNTLTKRLKELGME	
	Pv	NINRLTSQHSQSLSLWADEALGEGKENILNDALP.....QFERTLLLSALAYTQGHKQDAARLLGWGRNTLTKRLKELGIEDY	
	Bp	GSTATVGDV...LGGAVEAYLSSHPSFGPPNGVPPGLYHRLKEIEIPLLTAAALATRGNQIRAADLLGLNRRNLRKKIRDLIDQVYRSGG	
	Rm	PIEKAARVSGSLSIQAVERNMRQYFASFGDALPPSGLYDRVLAEMEYPLILAAALTATRGNQIKAADLLGLNRRNLRKKIRDLIDQVYRSGG	
	Rc	LGAGGEGEK...LSASLARHLRRYFDLHGGNLPPLPGLYDRVLAEMEYPLILAAALTATRGNQIKAADLLGLNRRNLRKKIRDLIDQVYRSGG	
	At	PLDKMVRVTSGLTIQAVERNMRQYFASFGDALPPSGLYDRVLAEMEYPLILAAALTATRGNQIKAADLLGLNRRNLRKKIRDLIDQVYRSGG	
	Ac	REDLYHRLNVIHILPPIRERREDIPRLARHFLRRSAEQDLVERILLSPERVAFNMW.HWPGNVRLQENVCRWITVMAPTREVQVTDLPPEMAASPMAS	
	con	P E L AL T G AA LLG RNTL K L	

Fig. 4 (a, b). For legend see facing page.

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 several restriction fragments in common and appeared to 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 *Hind*III 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 *Hind*III-*Kpn*I and 4.47 kb *Eco*RI-*Hind*III fragments (pT120 and pT130 respectively) were subcloned into the vector pBluescript 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 M_r values of 40278 and 51894 respectively. The *ntrBC* genes overlapped slightly, with the TGA stop codon of *ntrB* providing part of the ATG start codon of *ntrC*. Using the *TF*FASTA 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 γ 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, -CTGGTGCCTTTT-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-TTGCA) of σ^{54} -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 σ^{54} (Buck & Cannon, 1992). At least two potential NtrC-binding sites were located upstream of the proposed *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-N₄-TGGTGCA-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 M_r 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)

Fig. 4. Comparison of the amino acid sequences of (a) the *T. ferrooxidans* (Tf) NtrB protein and (b) the NtrC protein with those of other organisms. Ec, *E. coli* (Miranda-Rios *et al.*, 1987); Kp, *K. pneumoniae* (MacFarlane & Merrick, 1985; Buikema *et al.*, 1985); Va, *Vibrio alginolyticus* (Maharaj *et al.*, 1989); Pv, *Proteus vulgaris* (Steglitz-Mörsdorf *et al.*, 1993); Bp, *Bradyrhizobium parasponia* (Nixon *et al.*, 1986); Rc, *Rhodobacter capsulatus* (Jones & Haselkorn, 1989); Ab, *Azospirillum brasilense* (Liang *et al.*, 1993); At, *Agrobacterium tumefaciens* (Wardhan *et al.*, 1989); Rm, *Rhizobium meliloti* (Szeto *et al.*, 1987). Consensus amino acids are listed below the sequence alignments. Only amino acids which were identically conserved in seven out of eight NtrB proteins and eight out of nine NtrC sequences compared have been included in the consensus.

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 cosmid 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.

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 M_r 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 M_r 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

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 pT120. (b) Autoradiograph of panel (a).

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

low levels of nitrogen (Table 2). ET8556(pHlac20, pT101) cells, which contained the *T. ferrooxidans ntrBC* genes as a cosmid insert in the vector pH79, 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 ntrBC* 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, pH79). 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 *nifLA* 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 nifH* 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. Reitzer *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 closed 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 *glnALG* or *glnA/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 *nifR1*) 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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