Characterization of *Pseudomonas putida* genes responsive to nutrient limitation

Chris K. C. Syn,¹† Jon K. Magnuson,² Mark T. Kingsley² and Sanjay Swarup¹

¹Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore 117 543
²Pacific Northwest National Laboratory, Richland, WA, USA

The GenBank accession numbers for the *P. putida* PNL-MK25 *gdhA*, *nql* and *cyoD* genes reported in this paper are AF321093, AF321092, and AF321090, respectively.

INTRODUCTION

There is considerable ecological, agronomic and financial interest in the use of genetically modified micro-organisms for bioremediation of xenobiotics in soils or biocontrol of soilborne pathogens (Dowling & O’Gara, 1994; Timmis & Pieper, 1999). Success of these applications is largely dependent on the establishment of a metabolically active population of the bacterial inoculant, which is capable of sustained expression of the desired traits. The performance of bioremediation or biocontrol strains in the soil environment is often orders of magnitude lower than that observed under optimal laboratory conditions. Among the several limiting factors that have been described in the literature, the general lack of nutrients such as carbon, nitrate and phosphate, and of oxygen, in the soil environment is of primary concern (Leahy & Colwell, 1990; Atlas, 1991; Cases & de Lorenzo, 1998; Ferenci, 1999).

Bioavailability of organic nutrients is inherently low in both bulk and vegetated soils (Williams, 1985; van Overbeek & van Elsas, 1997). In bulk soils, polymeric organic nutrients are readily adsorbed to negatively charged clay particles or humic material, rendering them unavailable (Greenland, 1971; Danish & Stotzky, 1986), while other studies have shown that bacterial populations in the rhizosphere are actually starved (Meikle et al., 1995; Marschner & Crowley, 1996a, b; Normander et al., 1999). Hence, bacteria introduced into either soil type are likely to face conditions of carbon/energy shortage.

Poor conditions in the soil environment extend beyond organic nutrients to include inorganic nutrients and oxygen limitations as well. Instances of phosphate and nitrate limitation have been widely reported (Kragelund et al., 1997; Jensen & Nybroe, 1999). Due to the low diffusion coefficients of most gases, the static nature of soil, and respiration by plant roots and soil microfauna and microflora, oxygen in the soil environment becomes depleted while carbon dioxide accumulates (Kozlowski et al., 1991; Vande Broek et al., 1993; Hojberg et al., 1999). Additionally, waterlogging leads to oxygen deficiency. Consequently, many genes that
are normally active under aerobic laboratory conditions become downregulated (Lin & Iuchi, 1991; Spiro & Guest, 1991).

Traditional use of indigenous micro-organisms in environmental bioremediation involved expression of the biodegradation genes under the regulation of their native promoters, which may not be most suitable in certain field applications. Aromatic catabolic operons are generally controlled by transcriptional activators that interact with the aromatic substrates or with pathway intermediates, which serve as inducer molecules and provide regulatory specificity (Diaz & Prieto, 2000). Many such regulators have a narrow inducer specificity and/or have a minimum threshold concentration for their activation (Timmis & Pieper, 1999). Several studies have also demonstrated that the catabolic pathways of Pseudomonas spp. involved in the biodegradation of aromatic xenobiotics are regulated by carbon catabolite repression (CCR) (Hartline & Gunsalus, 1971; O’Connor et al., 1996; Duetz et al., 1997).

The above observations suggested a need to perform a broad-spectrum screening for Pseudomonas spp. gene promoters that are highly active under low-nutrient and low-oxygen conditions typical of soil environments. De novo identification of Pseudomonas genes and subsequent isolation of their promoters may be necessary since regulatory mechanisms in the well-studied Escherichia coli are not always transferable to soil pseudomonads. Hence, genes and promoters from heterologous species are not likely to express suitably in Pseudomonas spp. Promoters from pseudomonads would allow the expression of biodegradation and biocontrol genes to be uncoupled from the signals that ordinarily control or limit their expression and would thus offer greater flexibility in their application. We describe here three genes, gdhA, nqj and cyoD, from a plant-growth-promoting Pseudomonas putida strain, which show strong expression under conditions of low nutrient availability, anoxia, or both. Genes with desired expression characteristics were identified using Tn5-gus transcriptional fusions and screening the mutant strains for β-glucuronidase activity under different nutritional and environmental conditions using reporter gene assays. Selected strains were used for further studies and gene isolation.

**METHODS**

**Bacterial strains and culture conditions.** Pseudomonas putida PNL-MK25 (spontaneous Cm’ and Rf’ mutant of ATCC 39169; M. T. Kingsley, unpublished results) was used as the parental wild-type strain. Escherichia coli IM109 and pBluescriptII-SK+ were used as the host strain and plasmid vector for recombinant DNA experiments, respectively. LB medium was used for routine growth of the strains. All Pseudomonas cultures were grown at 30 °C. Antibiotic concentrations for P. putida during growth in LB were as follows: 15 μg chloramphenicol (Cm) ml⁻¹; 20 μg rifampicin (Rf) ml⁻¹; 15 μg tetracycline (Tc) ml⁻¹; 15 μg kanamycin (Km) ml⁻¹. Ampicillin (Amp) at 100 μg ml⁻¹ is ineffective on P. putida PML1 and its derived strains used in this study. Antibiotic concentrations were halved during growth in the low-nutrient conditions detailed below.

The following five classes of culture conditions were used for the detailed expression studies.

1. **Minimal-nutrient studies:** half-strength Stanier’s medium (Stanier et al., 1966) supplemented with 0.5% (v/v) glycerol (SG), 0.5% (w/v) glucose (GLC) or 0.5% (w/v) monosodium glutamate (MSG).

2. **Low-light studies:** light content was reduced by either 20- or 50-fold fold by supplementing half-strength Stanier’s medium with 0.025% (w/v) glucose (LGLC), 0.025% (w/v) monosodium glutamate (LMSG) or 0.01% (v/v) glycerol (LGL).

3. **Phosphate- and nitrate-limitation studies:** were performed in a similar way to CCR studies of aromatic catabolic operons (O’Connor et al., 1996; Duetz et al., 1997). The phosphate and nitrate concentrations in SG and GLC media were reduced from the original 20 mM to 2 mM (LP, LPGLC) and 15-12 mM to 1-21 mM (LN, LNLGC), respectively. Ionic strength was maintained by addition of an equimolar concentration of NaCl.

4. **Anoxic studies:** cells were cultured in (capped) tubes filled to the brim with SG medium (SGA), supplemented with 10 mM KNO₃, as previously described (Sawers, 1991; Zimmerman et al., 1991).

5. **Root exudate studies:** were done with half-strength Stanier’s medium supplemented with 0 p.p.m. total organic carbon of wheat root exudates (RE).

**Preliminary screening for nutrient-responsive genes.** Tn5 with a promoterless β-glucuronidase reporter gene (gus) (Sharma & Signer, 1990) was used to generate insertion mutants in PNL-MK25 and these were screened for enhanced or consistently strong β-glucuronidase (GUS) expression in low-nutrient media (SG-based) as compared to rich medium (LB). Twelve NRM (nutrient-responsive mutant) strains were selected for GUS expression studies, from which three were selected for molecular characterization.

**Preparation of wheat root exudates.** Wheat seeds (Carolina Biological Supply) were surface-sterilized and germinated for 4 days as previously described (van Overbeek & van Elsas, 1995). Seedlings were then grown for 6 weeks (16 h light: 8 h dark) either in solid substrate or hydroponically.

**Solid substrate growth.** Fifteen seedlings were planted per Phytacon (Sigma-Aldrich Chemicals) containing 350 ml sand/vermiculite (1:1) mix. The containers were opened weekly to allow exchange of gases and for addition of 0·1 × Stanier’s medium supplemented with 0·25 mg CaCl₂ ml⁻¹ (SC).

**Hydroponic growth.** Three hundred seedlings were transferred onto a support mesh placed in a plastic tank filled with 3·5 l SC. An airstone (of the type used in fish tanks) was placed under the mesh and connected to an air-pump through a 0·22 μm syringe filter. The tank was sealed. SC medium was added by injecting through the 0·22 μm syringe filter. All sterilization, transfer, germination and growth procedures were performed aseptically.

Root exudates were collected from the solid substrate by washing the sand/vermiculite mix twice with three volumes of water. For hydroponic growth, the growth medium in the tank was collected and filtered sequentially through 30 μm, 20 μm, 8 μm and 2-7 μm filter paper to remove particulate matter. Root exudates were concentrated by rotary evaporation, filtered through a 0·22 μm filter, and stored at -20 °C. Total organic carbon content in the root exudates was
determined with an O.I. Analytical model 1020A Combustion TOC Analyser at the Wastewater Biotreatment Group, Department of Civil Engineering, National University of Singapore.

**Analysis of gus-tagged gene expression.** Tn5-gus-tagged NRM strains were cultured in different test media for pre-conditioning, inoculated at a dilution of 1:1000 (v/v) in fresh media, and grown to mid-exponential phase (OD$_{600}$ 0-2). Bacterial cultures were harvested, washed once with 0-1× Stanier’s medium, and resuspended in GEB (50 mM sodium phosphate, pH 7-0; 10 mM EDTA; 0-3 % SDS; 0-1 % Triton X-100; 1 mM DTT; 1 mM PMSF). Aliquots were used for both protein and GUS activity determination. Protein quantity was determined using the Bio-Rad Protein Assay Dye Reagent Concentrate with BSA as standard.

GUS activity was determined fluorometrically based on previously described methods (Gallagher, 1992), using a Perkin Elmer LS50B Luminescence Spectrometer. Enzyme activity was expressed as nmol 4-methylumbelliferone formed h$^{-1}$ (µg total protein)$^{-1}$. Mean and standard error values were determined from at least two independent assays with five replicates each. One-way ANOVA followed by Tukey’s test were performed to determine significance of difference in gus reporter gene expression between strains.

**DNA gel blot analysis of NRM strains.** Genomic DNA from the 12 NRM strains was isolated using a previously described method (Sym & Swarup, 2000). EcoRI- and Sall-digested DNA fragments were separated by electrophoresis and transferred onto Hybond-N (Amersham Pharmacia Biotech) nylon membranes using standard protocols (Sambrook et al., 1989). aph2 (GenBank accession. no. AF061930) and tetA (GenBank accession. no. J01830) gene fragments were generated with PCR using NRM17 genomic DNA as the template. The gel-purified PCR products were used as templates for $^{32}$P-labelled DNA probe synthesis using the Amersham Rediprime II Random Prime Labelling System.

**Cloning and sequencing of chromosomal DNA flanking transposon-insertion sites.** Genomic DNAs of NRM5, NRM7 and NRM17 were digested with EcoRI for cloning of the fragment upstream of the transposon; digestion with Sall was used for cloning the downstream fragment. These enzymes were chosen since they cut only once within the transposon (Fig. 1), such that they preserve either an intact gusA-aph2 (conferring Km resistance in the upstream region) or a tetA-IS50R (conferring Tc resistance in the downstream region). Digested genomic DNA was ligated to pBlueScriptII-SK$^+$ and used to transform E. coli JM109 cells. LB agar supplemented with either Km and Amp or Tc and Amp was used to select for clones containing sequences upstream or downstream of the Tn5-gusA transposon, respectively. Plasmids from selected positive clones were isolated and digested with either EcoRI or Sall.

DNA was sequenced using the ABI PRISM BigDye Terminator Kit in accordance with the manufacturer’s instructions. DNA sequences were compared against all known sequences in the GenBank DNA Database using the BLASTn homology search program (Altschul et al., 1997). DNA sequences were translated and amino acid sequence pairwise alignments were performed using CLUSTALW 1.80 (Thompson et al., 1994) and output produced using BoxShade v3.33c (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html).

**RESULTS**

A set of 12 NRM strains of *P. putida* PNL-MK25 with gus-tagged genes was identified from a previous screening based on their visibly high GUS expression both on minimal medium plates and on wheat roots. After preliminary studies, GUS fluorometric analyses were performed in detail to study expression levels of three gus-tagged genes under rich-nutrient (LB), low-nutrient (SG) and wheat rhizosphere (RE) conditions (Table 1). DNA blot analysis of NRM strains with transposon-specific probes indicated that Tn5-gusA had inserted into single, independent sites in the chromosomes of the mutant strains (Fig. 1).

**The *P. putida* gdhA, nql and cyoD genes**

A comparison was made to identify the genes with consistently strong expression under most of the conditions studied by gus reporter analysis. In mineral medium supplemented with 0-5 % (v/v) glycerol (SG), nql showed the highest expression, followed by cyoD (Table 1). The

---

**Fig. 1.** Southern analyses of NRM strains. (a) Tn5-gus transposon (Sharma & Signer, 1990). SL, synthetic DNA consisting of the minimal 19 bp from IS50L required for transposition together with stop codons in all three reading frames fused with a promoterless gus reporter gene. Insertion of the transposon results in transcriptional fusion. The aph2 and tetA genes confer resistance to kanamycin and tetracycline, respectively. Restriction sites in the transposon are indicated. E, EcoRI; P, PstI; S, SaI; H, HindIII, X, XhoI; Xb, XbaI; N, NotI. (b, c) Genomic DNA of NRM strains digested with either EcoRI (b) or Sall (c) was separated by electrophoresis through a 0-7 % agarose gel, transferred to a Hybond-N nylon membrane and hybridized against the Tn5-gus-specific aph2 or tetA probes, respectively. Numbers above the lanes indicate the respective NRM strains.
The further analysis.

Table 1. Expression of Tn5-gus-tagged gdhA, nql, and cyoD genes under various conditions

<table>
<thead>
<tr>
<th>Nutrient conditions</th>
<th>gdhA (NRM5)</th>
<th>nql (NRM7)</th>
<th>cyoD (NRM17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rich medium (LB)</td>
<td>0.45±0.01</td>
<td>5.07±0.09</td>
<td>48.4±1.9</td>
</tr>
<tr>
<td>Minimal medium (SG)</td>
<td>6.57±0.13</td>
<td>23.0±0.7</td>
<td>13.5±0.0</td>
</tr>
<tr>
<td>SG/LB ratio (fold increase/decrease)</td>
<td>14.6</td>
<td>4.5</td>
<td>0.28</td>
</tr>
<tr>
<td>Glucose (GLC)</td>
<td>9.01±0.20</td>
<td>18.2±0.9</td>
<td>32.1±0.8</td>
</tr>
<tr>
<td>Glutamate (MSG)</td>
<td>11.7±0.8</td>
<td>14.2±0.7</td>
<td>74.4±2.8</td>
</tr>
<tr>
<td>Low glycine (LGL)</td>
<td>6.68±0.23</td>
<td>33.2±2.3</td>
<td>13.4±1.8</td>
</tr>
<tr>
<td>Low glucose (LGLC)</td>
<td>11.2±0.3</td>
<td>17.0±0.1</td>
<td>10.9±0.2</td>
</tr>
<tr>
<td>Low glutamate (LMSG)</td>
<td>7.45±0.80</td>
<td>27.8±1.0</td>
<td>25.7±0.6</td>
</tr>
<tr>
<td>Low phosphate (LP)</td>
<td>4.53±0.09</td>
<td>48.4±1.4</td>
<td>9.62±0.31</td>
</tr>
<tr>
<td>Low phosphate, glucose (LPGLC)</td>
<td>11.9±0.2</td>
<td>15.6±0.3</td>
<td>37.5±0.6</td>
</tr>
<tr>
<td>Low nitrate (LN)</td>
<td>6.24±0.04</td>
<td>13.1±0.1</td>
<td>7.01±0.19</td>
</tr>
<tr>
<td>Low nitrate, glucose (LNGLC)</td>
<td>2.21±0.07</td>
<td>1.16±0.02</td>
<td>20.2±1.0</td>
</tr>
<tr>
<td>Low oxygen (SGA)</td>
<td>4.78±0.13</td>
<td>77.9±2.8</td>
<td>15.8±0.4</td>
</tr>
<tr>
<td>Wheat root exudates (RE)</td>
<td>7.19±0.07</td>
<td>24.6±0.3</td>
<td>13.5±0.6</td>
</tr>
</tbody>
</table>

absolute expression of gdhA was lower than that of many of the other genes studied, but its increased expression during growth in low-carbon conditions was particularly striking (14.6-fold). Thus, on the criteria of strong absolute expression, or highly amplified expression under limiting nutrient conditions, nql, cyoD and gdhA were chosen for further analysis.

The P. putida gdhA gene

Sequencing of cloned chromosomal DNA flanking the Tn5-gus showed that P. putida NRM5 carried the insertion in the 3’ region (position 1285) of the gdhA (glutamate dehydrogenase) gene (Fig. 2a). The partial nucleotide sequence (1095 nt) and deduced amino acid sequence showed 84 % and 82 % identity, respectively, to the Pseudomonas aeruginosa PA01 gdhA gene. CLUSTALW pairwise alignment analysis (Fig. 2a) showed that the P. putida Gdh was well conserved, and showed >63 % sequence identity with GdhA of enterobacteria (E. coli, Klebsiella pneumoniae and Salmonella typhimurium); similarity with GdhA from other species was lower (non-enterobacteria, 62 %; fungi, 57 %; archaea, 26 %; mammals, 28 %). A rho-dependent terminator was identified 70 bp downstream of the gdhA stop codon (Fig. 2a).

Fig. 2. The P. putida PNL-MK25 gdhA, nql and cyoD genes. (a) The P. putida gdhA gene (GenBank accession no. AF321093) showing the Tn5-gus insertion site and the 9 bp target site duplication (figure is not drawn to scale). R, rho-dependent transcription terminator. (ii) Nucleotide sequence of the region flanking the transposon insertion site and the corresponding amino acid translation. (iii) CLUSTALW v1.8 comparison of amino acid sequences of P. putida gdhA with two Family I (P. aeruginosa and E. coli) and two Family II (human and Thermococcus litoralis) glutamate dehydrogenases. (b) The P. putida nql gene (GenBank accession no. AF321092) showing the insertion of Tn5-gusA. The nql gene is separated by 277 bp from the tgtS gene (figure not drawn to scale). BLASTP analysis of the putative OrfX located 672 nt downstream of nql showed no significant similarity to any known sequences in the GenBank database. L, LysR-type transcriptional regulator binding site (CATGGAAATTTTGCCCATG); A, ANR binding site (TTGATGCCGAATCAA); C, core promoter region (ATGACA-18 bp-ATTAAC) similar to the E. coli σ70 –35 and –10 consensus sequence. (ii) Nucleotide sequence of the region flanking the transposon insertion site and the amino acid translation. (iii) CLUSTALW comparison of amino acid sequences of P. putida Nq1 (aa 231–263) with bacterial (P. aeruginosa and E. coli) QORs and mammalian (human, cow and guinea pig) 1-crystallins. A zinc-ADH motif, which is absent in all other QORs, is found in Nq1. The QOR/1-crystallin signature motif has been modified in Nq1 with the conserved central glycine pair replaced with histidine-cysteine (indicated by arrows). (c) The P. putida cytochrome c ubiquinol oxidase (cyo) operon consisting of five genes. Tn5-gusA inserted into the 5’ region of the cyoD gene (GenBank accession no. AF321090). The 9 bp target site duplication is shown. (ii) Nucleotide sequence of the region flanking the transposon insertion site and the corresponding amino acid sequence translation. (iii) CLUSTALW comparison of amino acid sequences of PNNL-MK25 CyoD (aa 21–70) with the corresponding proteins of P. putida IH2000, P. aeruginosa PA01 and E. coli revealed strong sequence similarities (68 %, 51 % and 45 %, respectively).
(a) *P. putida* **gdhA** gene

(i) $\text{gdhA}$

\[\text{EcoRI} \quad \text{CGCTACTGC} \quad \text{CGCTACTGC} \quad \text{TAA} \quad \text{R} \quad \text{Sall}\]

\[
\text{9bp direct repeats}
\]

(ii) $\text{GAT GCC GGA GCC CGG AGC CTA CTG CGC AAT GGC TGC GTC TGT}$

\[
\text{DAGAARTTLRNLGNCVCV}
\]

(iii) *P. putida* DIALPACATQVNEVDAGAARTTLRNLGCVCV

*Paeruginosa* DIALPACATQNELDAEDARRILNRNLGCVCV

*Ecoli* DIALPACATQNELDLGRILNRNLGCVCV

*Human* DILIPACATQNELDLQKSLRAVRVAK

*Tritoralis* DVLAPSAIEEVITKKNANIKK

(b) *P. putida* **nql** gene

(i) $\text{tgS}$

\[\text{EcoRI} \quad \text{ATG nql} \quad \text{LAC} \quad \text{GTCTGGTGC} \quad \text{TGTGCTGGTGC} \quad \text{TAA} \quad \text{orfX} \quad \text{672 bp}\]

\[
\text{277 bp}
\]

\[
\text{9bp direct repeats}
\]

(ii) $\text{GTC TGG TGC TGT AGC TGC AGA GTC GTA ACC}$

\[
\text{QATPQVWCTACRVT}
\]

(iii) *P. putida* GHESCAGVVTAVGADV

*Paeruginosa* QSEGAGVVEAAGVESV

*Ecoli* GTGAAAVSVSGLSV

*Human* GSDVAGVIEAVGDNA

*Cow* GFDVGAVIAGRSV

*Guinea pig* GVDVAGVIESIGV

* zinc-ADH motif*

*QOR/ζ-crystallin motif*

(c) *P. putida* **cyoD** gene

(i) $\text{cyoA}$

\[\text{cyoB} \quad 676 \text{ aa} \quad \text{cyoC} \quad 210 \text{ aa} \quad \text{ATG cyoD} \quad \text{CGTAATCC} \quad \text{CGTAATCC} \quad \text{TGA cyoE} \quad 295 \text{ aa}\]

\[
\text{9bp direct repeats}
\]

(ii) $\text{TTC AGC CTG TCG GTA ATC CTG ACC GTC ATT CCG TTC GGT CTG GTG ATG}$

\[
\text{PSLSLVITLVIVIPFLGLVM}
\]

(iii) *PpPNL-MK25* VKSYAIGFLSLSVILTVIPFLGLVM

*PpIN2000* VSKSYAGFILSILTAIPFFLMSPKPLNLTVILIVAMAVIQVVLHIVYLHMDREKRN

*PaPA01* LGSYAGIFILSILTAIPFFLMSPKPLNLTVILIVAMAVIQVVLHIVYLHMDREKRN

*Ecoli* VTYMTPGFLSILTVIPFFLMVMTAGASDIVLTILAMAVIQVVLHIVYLHMDREKRN

: * * * * * * * * * * * * * * * * * * * * * * * * * * *
The *P. putida nql* gene

Based on DNA sequencing, the Tn5-gus was inserted at position 727 of a 906 nt ORF in the mutant strain NRM7 (Fig. 2b). This 906 bp ORF was predicted to encode a protein of 301 aa with a molecular mass of 32.4 kDa. BLASTP and CLUSTALW analysis of the predicted amino acid sequence indicated that the protein showed low-level similarity (<20%) to NADPH:quinone oxidoreductases (QORs), ζ-crystallins and zinc-type alcohol dehydrogenases (ADH), which are members of a medium-chain dehydrogenase/reductase superfamily (Edwards et al., 1996).

Analysis of the amino acid sequence indicated that the protein possessed a QOR/ζ-crystallin motif (Gonzalez et al., 1993) in the central region of the protein, with a key difference: the pair of glycine residues conserved in all QOR/ζ-crystallins is replaced by histidine-cysteine in Nql (Fig. 2b). Interestingly, the protein also possessed a zinc-ADH signature motif (Jornvall et al., 1987) in the N-terminus region, which was absent in other QOR/ζ-crystallins. Phylogenetic relationship analysis suggested that the protein was not closely related to QOR/ζ-crystallins (data not shown). This ORF was, therefore, named nql (NADPH:quinone oxidoreductase like).

The promoter of the nql gene was predicted to be localized to within a 140 nt region upstream of the initiation codon. A 14 bp partially dyadic sequence (TG-N10-CA) was found centred at the −65 position (Fig. 2b), which closely matched the consensus LysR-type transcriptional regulator binding motif (TG-N9-CA; Schell, 1993). An ANR box (TTGAT-N5-ATCAA) identical to the ANR box-crystallin motif (Gonzalez et al., 1987) in the N-terminus region was also observed. The promoter region upstream of the nql promoter was sequenced and found to contain the first 336 nt of an ORF transcribed divergently from nql (Fig. 2b). The deduced amino acid sequence of this ORF showed strong similarity to two efflux pump regulatory proteins, *P. putida* DOT-T1E Ttg5 (93%) and *P. aeruginosa* PAO1 MexT (91%). An ORF with no matches to GenBank database entries was located downstream of nql.

The *P. putida cyoD* gene

In the mutant strain NRM17, Tn5-gus was inserted at position 80 of the 336 nt cyoD (subunit IV of the cytochrome o ubiquinol oxidase complex) gene (Fig. 2c). Sequencing of the region flanking cyoD identified the other members of the five-subunit cytochrome o ubiquinol oxidase complex. The predicted amino acid sequences of all five subunits showed strong similarity to corresponding subunits in *P. putida* IH-2000, *P. aeruginosa* PAO1 and *E. coli* cytochrome ubiquinol oxidases. A Kyte–Doolittle hydropathy plot (Kyte & Doolittle, 1982) of CyoD indicated three putative transmembrane regions (data not shown). These transmembrane regions are similar to those of *E. coli* CyoA determined experimentally using phoA-fusion protein studies (Chepuri & Gennis, 1990; Chepuri et al., 1990).

Gene expression under various nutrient-limiting conditions

Detailed analyses of the expression profiles of the three genes under various nutrient conditions (Table 1) were performed. The presence of two other carbon sources, viz. 0.5% glucose (GLC) or 0.5% glutamate (MSG), resulted in increased gdhA and cyoD expression compared to that observed in SG. This increased gene expression by a readily metabolized carbon source coupled with previous observations that growth of the other NRM strains in mineral medium was fastest when the culture was supplemented with glutamate (data not shown) suggested that these two genes disrupted by the transposon were associated with growth and metabolism. In contrast, expression of nql was reduced 1.3–1.6-fold, which suggested possible regulation by a carbon catabolite repression (CCR) mechanism (Table 1). Under carbon-limiting conditions (0.01% glycerol, 0.025% glucose, 0.025% glutamate, or wheat root exudates), expression of these three genes was comparable to that observed during growth in SG. These data further supported the potential use of the three gene promoters in driving expression of foreign genes in the nutrient-limited soil environment.

The possible effects of CCR were also examined under low phosphate and low nitrate levels. In the presence of glucose or glutamate (LPGLC), expression of gdhA and cyoD was increased 2.6- and 3.9-fold, respectively, while that of nql was reduced 3.1-fold when compared with LP medium (Table 1). When the glycerol in low-nitrate medium (LN) was replaced with glucose (LNGLC), expression of gdhA and nql was reduced by 2.8- and 11.3-fold, respectively, whereas that of cyoD was increased by 2.9-fold. The marked reduction in nql gene expression in the presence of glucose, a preferred carbon source, further supports the hypothesis that this gene is regulated by a CCR mechanism.

Low-oxygen-responsive gene expression

During growth under low-oxygen conditions the expression of nql was highest amongst the genes screened (Table 1), and was upregulated 3.4-fold (Table 1, SGA compared to SG). In contrast, only slight changes in the expression of gdhA and cyoD were observed – expression of gdhA decreased 1.4-fold while expression of cyoD increased by 1.2-fold.

Root-exudate-responsive gene expression

Bacteria were grown in half-strength Stanier’s medium supplemented with wheat root exudates to simulate growth in the rhizosphere. The quantity of total organic carbon in eight different root exudate preparations ranged from 0.15 to 0.49 mg per plant. Root exudate was added to the medium at 40 p.p.m. total organic carbon concentration, similar to levels detected in the sand/vermiculite substrate after the seedlings had been grown for 6 weeks. No qualitative differences were observed between the different batches of root exudates with respect to induction of reporter gene expression in the three NRM strains (data not shown). Expression of nql was the highest, followed by cyoD.
DISCUSSION

Twelve nutrient-responsive mutants (NRM) were studied. The expression driven by the promoters of three of these gus-tagged genes was studied in detail under conditions of low nutrient availability. The expression levels of the different genes in the Stanier’s synthetic medium (SG) were similar to those observed in the presence of root exudates (Table 1). Their ranking in order of gene expression levels was also similar: nql and cyoD showed the highest expression of the twelve NRM mutants (data not shown). The corroboration of the two findings supported the hypothesis that these genes, selected based on their expression in Stanier’s medium, were likely to be active in the rhizosphere – an important property in view of their potential use for driving foreign gene expression in the soil environment.

**gdh expression differs in various bacteria**

The partial sequence of the *P. putida* PNL-MK25 gdhA gene showed strong identity to other bacterial glutamate dehydrogenase genes. The expression of the *P. putida* gdhA gene was carbon quality- and quantity-dependent but was nitrate-independent; expression of gdhA was highest in glutamate-containing, followed by glucose- and glycerol-containing mineral media. Such regulation is similar to that observed in *S. typhimurium* and *Corynebacterium callunae* (Brenchley *et al.*, 1975; Ertan, 1992). The absolute expression of gdhA was lower than that of many of the other genes studied, but it was increased 5–26-fold, relative to rich medium (LB), under all low-nutrient conditions studied. The increased gdh expression observed during carbon-limiting conditions suggested that *P. putida*, like *E. coli*, may also use Gdh for glutamate synthesis when the cell is limited for carbon (Helling, 1994). The Gdh pathway may also be the preferred pathway in energy-limiting conditions since the GOGAT (glutamine synthetase–glutamate synthase) system requires 20% higher ATP levels than the Gdh pathway. However, in the case of *E. coli*, utilization of Gdh occurred when ammonium and phosphate were not limiting. In contrast, the *P. putida* gdh promoter-probe construct showed strong increase in expression even when the carbon-limited cells were further deprived of phosphate or nitrate. These results suggest that the *P. putida* gdhA promoter might be suitable for regulating expression of foreign genes in oligotrophic bulk soil.

**Regulation of nql gene expression**

The observation that nql expression was repressed in the presence of glucose and glutamate coupled with the further decline in expression when glycerol in the low-phosphate (LP) or low-nitrate (LN) media was replaced with glucose (LPGLC or LNLGC) suggested a CCR-like regulation of gene expression. However, it should be noted that this result was based on studies using synthetic media designed to evoke a CCR-type response. nql expression during growth under low-nutrient conditions was comparable to that observed in the presence of root exudates. This suggested that nql was unlikely to be affected by CCR-linked repression during expression in the soil environment. This is unlike several aromatic xenobiotic degradation pathways that are CCR-regulated (Hartline & Gunsalus, 1971; O’Connor *et al.*, 1996; Duetz *et al.*, 1997).

Bacteria respond to low oxygen availability by down-regulating the expression of ‘aerobic’ genes and up-regulating ‘anaerobic’ genes (Lin & Iuchi, 1991; Spiro & Guest, 1991) as well as slowing or stopping growth (Stretton & Goodman, 1998). nql was, however, found to be synergistically upregulated by low-carbon and low-oxygen conditions: expression of nql was increased 4–5-fold in SG (compared to LB) and further increased 3–4-fold under anoxic conditions (SGA compared to SG; Table 1), yielding a total induction of 15–3-fold in the presence of both conditions relative to rich media (LB). As in the case of Gdh, Nql may represent a potential parallel pathway for balancing growth efficiency during nutrient-limited growth as suggested by Helling (1994).

The 4–5-fold increase in nql gene expression under minimal conditions is similar to that observed for the *E. coli* qor gene (Tao *et al.*, 1999). The further increase of nql during anoxic growth suggested a synergistic interaction between the two conditions. An ANR box, similar to the *E. coli* FNR and *P. aeruginosa* ANR binding motifs (Ye *et al.*, 1995), was located immediately preceding the −35 promoter sequence, which suggested that the anaerobic induction of nql was mediated by ANR (anaerobic transcriptional regulator). The region upstream of the nql gene showed strong sequence similarity to *P. aeruginosa* MexT, which is an efflux pump regulatory protein. MexT is a member of the LTTR family (LysR-type transcriptional regulator; Kohler *et al.*, 1999) and genes controlled by LTTRs are often located adjacent to their activators, which are transcribed in the opposite direction (Schell, 1993). Given these observations, it is possible that this upstream regulatory protein also regulates the nql promoter. This hypothesis is supported by the presence of the consensus LTTR binding sequence centred at the −65 position of the nql gene.

**cyo expression is nutrient-responsive and oxygen-independent**

The cytochrome o oxidase complex has been extensively studied in *E. coli* (Chepuri *et al.*, 1990) but similar studies in other bacteria have been very limited. Among the pseudomonads, the cyo operon has only recently been cloned from another strain of *P. putida*, IH−2000 (Hirayama *et al.*, 1998). Our cyoD–gus transcriptional fusion studies indicated that expression of the cyo operon in *P. putida* was carbon-quality-dependent: expression was highest when the cells were grown in glutamate, followed by LB broth, glucose and glycerol, consistent with a CCR-like regulation. This contrasts with previous reports that cytochrome o levels in *E. coli* are regulated by cAMP, which is indicative of a global transcriptional regulation mechanism (Minagawa...
et al., 1990; Tao et al., 1999). Interestingly, cyo expression in P. putida was oxygen-independent (only 1.2-fold greater for SGA versus SG; Table 1), which again contrasts with E. coli, where expression of the cyo operon is derepressed under high oxygen (Kranz & Gennis, 1983; Minagawa et al., 1990). Our results suggest that expression of cyo in P. putida may be influenced more by nutrient conditions than by oxygen tension, somewhat like regulation in Bacillus subtilis, where expression of the qoxABCD operon is highest during growth in rich medium (Liu & Taber, 1998).

In the present study, the nql and cyo genes were consistently amongst the most highly expressed under the variety of low-nutrient conditions tested. Their promoters are thus of potential use in driving the expression of foreign genes in various soil conditions where carbon, nitrogen, phosphorus or a combination of these nutrients is typically scarce. Additionally, the strong inducibility of the nql promoter under anoxic conditions makes it particularly suited for driving the expression of foreign genes in water-saturated soils and other conditions where oxygen is scarce.

ACKNOWLEDGEMENTS

This work was supported by a National University of Singapore research grant RP982329 to S.S. and a research scholarship to C. K. C. S.

REFERENCES


