Microbiology (1997), **143**, 127–134 Printed in Great Britain

High affinity iron acquisition in *Rhizobium leguminosarum* requires the *cycHJKL* operon and the *feuPQ* gene products, which belong to the family of two-component transcriptional regulators

Kay H. Yeoman,[1]† Maria-Jesus Delgado,[2]‡ Margaret Wexler,[1] J. Allan Downie[2] and Andrew W. B. Johnston[1]

Author for correspondence: Andrew W. B. Johnston. Tel: +44 1603 592264. Fax: +44 1603 542250. e-mail: a.johnston@uea.ac.uk

[1] School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK

[2] John Innes Centre, Colney Lane, Norwich NR4 7UH, UK

The *cycHJKL* operon of *Rhizobium leguminosarum* has previously been shown to be involved in the maturation of cytochrome *c*, possibly by its involvement in the covalent attachment of haem to the apoprotein. Mutations in the *cycHJKL* genes abolish symbiotic nitrogen fixation. Here, we show that *cyc* mutants are pleiotropically defective. They have lost a high affinity iron acquisition system due to their failure to make or to export siderophores. They also accumulate protoporphyrin IX, the immediate precursor of haem. A model to account for these phenotypes is presented. Immediately upstream of *cycH* is a gene, *lipA*, which is predicted to encode an outer-membrane lipoprotein. Further upstream of *lipA*, there are two other genes, whose products are similar in sequence to the widespread family of two-component transcriptional regulators. These two genes, *feuP* and *feuQ*, did not affect the transcription of *lipA*, or of the *cycHJKL* operon. However, a mutation in *feuQ* also led to the loss of the high affinity iron uptake system, although siderophores were still produced.

Keywords: cytochrome *c*, iron acquisition, nitrogen fixation, *Rhizobium*, siderophore

INTRODUCTION

Iron is essential as a trace element for the growth of nearly all micro-organisms. Despite being the fourth most abundant element in the earth's crust, its extreme insolubility at normal biological pH severely decreases its bioavailability. To obtain sufficient iron for growth, most micro-organisms have developed high affinity iron acquisition systems. These usually involve the synthesis and export of siderophores, which are low-molecular-mass ferric-ion-specific ligands. The iron–siderophore complex is recognized by an outer-membrane protein that is involved in the transport of the complex (Crosa, 1989; Guerinot, 1991; Reigh & O'Connell, 1993). The majority of siderophores can be classified as either hydroxamates or catecholates, according to the main chelating group. One notable exception is rhizobactin produced by *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*) DM4, which possesses ethylenediamine as a ligand (Smith *et al.*, 1985).

Iron is of particular importance in the legume root nodules that are induced and occupied by species of the bacterial genera *Rhizobium*, *Azorhizobium*, *Sinorhizobium* and *Bradyrhizobium*. The iron-containing enzymes and proteins involved in nitrogen fixation include nitrogenase, ferredoxin, cytochromes and leghaemoglobin, the last being the single most abundant protein in the nodule. Iron also plays a regulatory role since it is involved in the oxygen-sensitive expression of the nitrogen fixation regulatory protein NifA (Fischer, 1994). The transcription of *nifA* in *S. meliloti* is regulated by the genes *fixL* and *fixJ* in response to a low-oxygen

†**Present address:** Institute of Food Research, Colney Lane, Norwich NR4 7UA, UK.

‡**Present address:** Estacion experimental del Zaidin, c/o Professor Albareda, Granada, Spain.

Abbreviation: PPIX, protoporphyrin IX.

The EMBL accession number for the sequence reported in this paper is X89983.

signal (Gilles-Gonzales *et al*., 1991) and FixL is known to be one of a group of haem-based sensors (Monson *et al*., 1995).

Nadler *et al*. (1990) isolated a mutant of *Rhizobium leguminosarum* biovar *viciae* (which nodulates peas and vetches) with a complex phenotype that included a defect in high affinity iron acquisition. This mutant, termed Pop116, was initially isolated because it fluoresced under UV light due to accumulated protoporphyrin IX (PPIX), the immediate precursor of haem that lacks iron. The mutant had at least two other defects; it had lower levels of *b* and *c*-type cytochromes than the wild-type and it was completely unable to fix nitrogen in pea nodules although it formed bacteroids of essentially normal morphology. These pleiotropic effects were interpreted as being due to a defect in high affinity iron acquisition; thus, it was argued, cells that were iron-deficient might be expected to accumulate PPIX, have low cytochrome content and be Fix^- due to a lack of functional nitrogenase or other iron-containing proteins needed for nitrogen fixation.

In *R. leguminosarum*, *S. meliloti* and *Bradyrhizobium japonicum*, the *cycHJKL* genes are in a single operon (Delgado *et al*., 1995; Kereszt *et al*., 1995; Ritz *et al*., 1995). This operon is essential for the biogenesis of *c*-type haem proteins, and in rhizobia, mutations in *cycHJKL* completely abolish symbiotic nitrogen fixation. It has been speculated that the *cycHJKL* gene products are involved in the covalent attachment of haem to the apocytochrome *c*.

Homologues of *cycHJKL* have been found in a variety of other bacteria, including *Escherichia coli* (Thöny-Meyer *et al*., 1995) and *Paracoccus denitrificans* (Page & Ferguson, 1995); two of them, *cycK* and *cycL*, occur in *Rhodobacter capsulatus*, where they are referred to as *ccl1* and *ccl2*, respectively (Beckman *et al*., 1992). Homologues have also been identified in chloroplasts (Ohyama *et al*., 1988) and in mitochondria (Pritchard *et al*., 1990). By a combination of functional and genetic comparisons, we show here that the pleiotropic phenotype found in the *R. leguminosarum* mutant Pop116 is, in fact, due to a mutation in the *cycHJKL* operon.

METHODS

Bacterial strains, plasmids and growth conditions. The strains used are shown in Table 1. General growth conditions for *Rhizobium* and *E. coli* were as described by Beringer (1974).

Growth in iron-deficient media. Iron was removed from Y minimal medium by adding 5 g Chelex resin l^{-1} (Bio-Rad) to Y medium lacking salts, and stirred at room temperature for 4 h. The resin was removed by filtration, and the minimal medium salts added. Where necessary, uracil and tryptophan were added to final concentrations of 30 μg ml^{-1}. Aliquots of medium (100 ml) were dispensed into 250 ml polypropylene Erlenmeyer flasks and autoclaved. Starter cultures were grown for 48 h in Y medium with no added iron, then 5 ml volumes were inoculated into the Chelex-treated medium. Cultures were grown at 28 °C on a rotary shaker until late-exponential phase. Cells were harvested by centrifugation, washed in iron-depleted medium, and subsequently used for siderophore production analysis and ^{55}Fe-uptake studies.

Analysis of ^{55}Fe uptake. This was performed according to Nadler *et al*. (1990), with the following changes. Iron-starved cells of *R. leguminosarum* were resuspended to an OD_{600} of 0·7. $^{55}FeCl_3$ was added in a solution of sodium nitrilotriacetate (NTA). The latter was made in a microcentrifuge tube by adding 1 μl $^{55}FeCl_3$ (0·242 MBq in 0·1 M HCl) to 200 μl 2 mM NTA. After 30 min equilibration at 30 °C, 100 μl of the ^{55}Fe-NTA was added to 10 ml of the cell suspension. Iron uptake was expressed as ng ^{55}Fe (mg protein)$^{-1}$.

Analytical methods. Protein determinations were carried out using the Bio-Rad protein assay. β-Galactosidase assays were performed according to Rossen *et al*. (1985), and the enzyme activity expressed in Miller units.

Siderophore analysis. Siderophore production was analysed using chrome azurol sulphonate (CAS) agar plates described by Schwyn & Neilands (1987). Iron-starved cells (10 μl at OD_{600} of 0·7) were spotted onto the centre of the CAS-agar plates and incubated at 28 °C for 3 d. Siderophore production was seen as a bright orange halo around the bacterial growth.

Haem staining. Fractionation of *Rhizobium* cells into membrane and soluble fractions, followed by SDS-PAGE, blotting of the proteins and subsequent staining for haem was performed as described by Vargas *et al*. (1993).

Protoporphyrin IX detection. PPIX accumulation was measured by spectrofluorimetry (Miyamoto *et al*., 1992). *R. leguminosarum* strains were grown to stationary phase in 100 ml complete TY medium. Cells were recovered by centrifugation, resuspended in acetone/0·1 M NH_4OH (9:1, v/v) and vortexed. Cell extracts were recentrifuged and the supernatants collected. Fluorescent emission spectra were recorded on a Howe Dr-15 spectrofluorimeter using an excitation wavelength of 405 nm.

***In vivo* genetic manipulations.** Plasmids were transferred into *R. leguminosarum* by conjugation as described by Buchanan-Wollaston *et al*. (1980) using the plasmid pRK2013 as a helper in tri-parental matings (Figurski & Helinski, 1979). Tn*5lacZ* mutagenesis of plasmid pKN16 was performed as follows. pKN16 was introduced by transformation to *E. coli* strain A118, which has a chromosomally located copy of Tn*5lacZ* (Simon *et al*., 1989). This strain was used in a tri-parental mating (with pRK2013) in which *R. leguminosarum* biovar *viciae* strain 8401pRL1JI was the recipient. Selection was made on TY medium containing streptomycin (8401pRL1JI), plus kanamycin (Tn*5lacZ*) plus tetracycline (pKN16). Transconjugant colonies arose at a frequency of about 10^{-6} per recipient, these being due to the transfer of derivatives of pKN16 into which Tn*5lacZ* had transposed. Plasmid DNA was isolated from these transconjugants and, using appropriate restriction enzymes, the sites of insertion were roughly established, and the orientations of the *lacZ* were determined.

For precise mapping of the *feuQ*::Tn*5lacZ* insertion, an internal *Bam*HI restriction site in Tn*5lacZ* and a *Hin*dIII site in the *feuQ* gene were used to generate a subclone in the sequencing vector pUC18. The junction between the end of IS*50L* and the pKN16 DNA was sequenced. This was done using a fluorescently labelled 25-mer oligonucleotide primer with the sequence 5′ GAAGTCAGATCCTGGAAAACGGGAA 3′, designed to bind to the end of Tn*5*.

The *feuQ*::Tn*5lacZ* mutation in pKN16 was introduced into the *R. leguminosarum* genome by marker exchange (Ruvkun

Table 1. Strains and plasmids

Strain or plasmid	Relevant properties	Reference
Strain		
8401pRL1JI	*R. leguminosarum* bv. *viciae* carrying the symbiotic plasmid pRL1JI; Str^r	Downie *et al.* (1983)
A266	Rifampicin-resistant derivative of 8401pRL1JI carrying *cycK67*::Tn*5*	Delgado *et al.* (1995)
A267	Rifampicin-resistant derivative of 8401pRL1JI carrying *cycH271*::Tn*5*	Delgado *et al.* (1995)
1062	*R. leguminosarum* bv. *viciae ura-14 trp-16* Str^r	Beringer *et al.* (1978)
Pop116	Derivative of 1062; *pop-1*	Nadler *et al.* (1990)
J100	Derivative of 8401 pRL1JI carrying *feuQ*::Tn*5lacZ*	This work
Plasmids		
pIJ1942	Cosmid carrying 30 kb of *R. leguminosarum* DNA, including *feuPQ*, *lipA*, *cycHJKL*	Delgado *et al.* (1995)
pRK2013	Helper plasmid	Figurski & Helinski (1979)
pKN16	Similar to pIJ1942	Nadler *et al.* (1990)
pMP220	Broad-host-range cloning vector; Tet^r	Spaink *et al.* (1985)
pLAFR3	Broad-host-range cloning vector; Tet^r	Staskawicz *et al.* (1987)
pRK415	Broad-host-range cloning vector; Tet^r	Keen *et al.* (1988)
pIJ7345	Derivative of pMP220 containing *cycHJKL* promoter region	This work
pIJ7346	Derivative of pMP220 containing *lipA* promoter region	This work
pBIO260	Derivative of pLAFR3 containing *feuPQ*	This work
pBIO275	Derivative of pKN16 containing *cycK*::Tn*5lacZ*	This work
pBIO279	Derivative of pKN16 containing *feuQ*::Tn*5lacZ*	This work
pBIO280	Derivative of pKN16 containing *feuP*::Tn*5lacZ*	This work
pCHO8	Cloned *cycHJK* genes of *S. meliloti* strain 41 cloned in pRK290	Kereszt *et al.* (1995)
pCHO8::Tn*5*(87)	Derivative of pCHO8 with Tn*5* in *cycK*	Kereszt *et al.* (1995)

& Ausubel, 1981) using the P1 incompatibility plasmid pPH1JI to eliminate the pKN16 as described by Downie *et al.* (1983).

Pea seedlings (cv. Wisconsin Perfection) were inoculated, grown and assayed for nitrogen fixation as described by Beynon *et al.* (1980).

DNA manipulations. Restriction enzymes and DNA ligase were used according to the manufacturer's instructions. Transformation of *E. coli* was done according to Maniatis *et al.* (1982). DNA sequencing was done with the aid of an ALF (Pharmacia) semi-automated sequencer according to the manufacturer's instructions. The data were analysed using programs in the DNA-Star package. DNA searches of the EMBL database were carried out using BLAST in the GCG package.

The genes *feuP* and *feuQ* were amplified by PCR using the primers 5′ CTCCTGCAGTCGATTTGAAAGCGGGACG 3′ and 5′ CTCCTGCAGCGAGGCACTCTTCGTCGTGC 3′, designed to bind 30 bp upstream of the 5′ end of *feuP* and 187 bp downstream of the 3′ end of *feuQ*, respectively. A *Pst*I site was engineered into the 5′ end of each primer to facilitate direct cloning of the PCR product into the sequencing vector pUC18. The fidelity of the PCR product was checked by sequencing; this product was then recloned into the broad-host-range plasmid pLAFR3 to form pBIO260.

RESULTS AND DISCUSSION

Phenotypic similarity of Pop116 and Cyc mutants

The previously described phenotypes of the Pop116 mutant of *R. leguminosarum* had some similarities to those of strains with mutations in *cycH* (strain A267) or *cycK* (strain A266). All three strains induced pea root nodules that contained bacteroids, but which failed to fix nitrogen. They were also defective in *c*-type cytochrome synthesis. Further, we noted that the *pop116* mutation of *R. leguminosarum* (Nadler *et al.*, 1990) and the *cycHJKL* genes of *S. meliloti* (Kereszt *et al.*, 1995) were in corresponding positions of the highly conserved (Kondorosi *et al.*, 1980) chromosomes of the two species. Given these similarities, strains Pop116, A266 and A267 were examined further to ascertain if they shared all their previously described phenotypes. Delgado *et al.* (1995) showed that mutants A266 and A267 did not catalyse the synthesis of indophenol blue from *N*,*N*-dimethyl-*p*-phenylenediamine and α-naphthol (NADI test), indicating their loss of cytochrome *c* function (Jones & Poole, 1985). In an NADI test, colonies of the wild-type *R. leguminosarum* strain 1062 stained blue ($NADI^+$), but colonies of the Pop116 mutant derivative were white ($NADI^-$). Therefore, Pop116 was also unable to respire via the cytochrome aa_3 pathway.

Delgado *et al.* (1995) also showed that mutations in *cycH* and *cycK* blocked the formation of *c*-type haem proteins in *R. leguminosarum*. Membrane and soluble fractions from cells of strains 1062 and Pop116 were analysed for such proteins. As found previously (Delgado *et al.*, 1995), the wild-type had two *c*-type haem proteins, of 23 and 31 kDa, in the membrane fraction and one *c*-type haem protein of 14 kDa in the soluble fraction. In contrast, the mutant Pop116 had no detectable *c*-type protein in either fraction (results not shown).

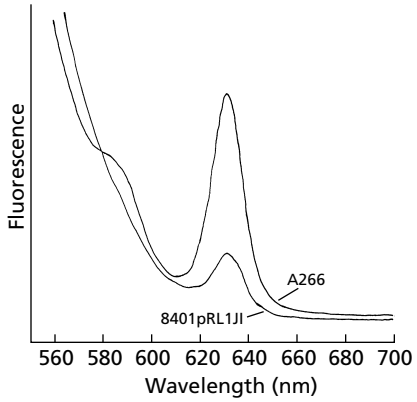

Fig. 1. Fluorescence spectra of extracts of *R. leguminosarum* strains 8401pRL1JI and A266. The extracts were excited at 405 nm, and emission measured at 633 nm.

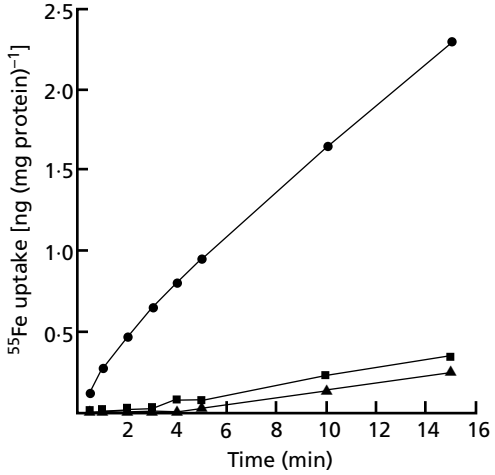

Fig. 2. ^{55}Fe uptake by *R. leguminosarum* strains grown in low-iron minimal medium broth. ●, 8401pRL1JI; ■, A266; ▲, J100.

The mutant Pop116 had two further characteristics, namely an accumulation of PPIX and a defect in iron uptake. As shown in Fig. 1, strain A266 was also found to accumulate PPIX (by approximately fivefold) relative to the wild-type strain 8401pRL1JI. In iron-uptake experiments, A266 was also found to be defective in this property (Fig. 2). The *cycH* strain A267 was identical to A266 in these two properties (not shown).

Some iron-uptake mutants are due to a defect in siderophore biosynthesis (Schwyn & Neilands, 1987). The mutants A266, A267 and Pop116 were each tested for siderophore production, using CAS-agar plates. Siderophores remove the iron from the CAS–ferric dye complex, turning its colour from blue to orange. As expected, colonies of wild-type strains 8401pRL1JI and 1062 had a bright halo. There was no such halo surrounding colonies of A266, A267 or Pop116, indicating that these mutants were unable to synthesize or export siderophores.

Taken together, these observations showed that the known phenotypic defects of Pop116 were very similar to those in the *cycH* and *cycK* mutants, and vice versa.

The mutation in Pop116 is in *cycK*

The *cycHJKL* operon that complements the A266 and A267 *cyc* mutants had been cloned on a pLAFR1-based plasmid, pIJ1942 (Delgado *et al.*, 1995). Similarly, Nadler *et al.* (1990) isolated a pLAFR1-based plasmid, pKN16, which complemented the defects of the mutant Pop116. Digestion of pIJ1942 and pKN16 with *Eco*RI revealed almost identical restriction fragment patterns for the two plasmids, indicating that they contained cloned DNA in common. When mobilized into the mutants A266 and A267, pKN16 was able to restore the NADI, ^{55}Fe acquisition, nitrogen fixation and siderophore defects to normal. Likewise, when pIJ1942 was mobilized into Pop116, it corrected all its mutant phenotypes.

To determine which gene was mutated in Pop116, pKN16 was mutagenized with Tn*5lacZ* and mutant plasmids were screened for those that no longer corrected Pop116, as determined by the NADI test. One mutant plasmid, pBIO275, was unable to correct Pop116 for any of its defects. By restriction mapping, the Tn*5lacZ* was shown to be located in *cycK*, approximately 500 bp upstream of *cycL*. Delgado *et al.* (1995) proposed that *cycK* and *cycL* were translationally coupled; thus, in pBIO275, *cycL* may also be defective. However, we found that a plasmid, pCHO8 (Kereszt *et al.*, 1995), which contained the cloned *cycHJ* and *K* (but lacked *cycL*) genes of *S. meliloti*, also restored Pop116 to NADI$^+$ but that pCHO8::Tn*5*(87), a derivative with Tn*5* in *cycK*, did not. These observations show that the mutation in Pop116 is in *cycK*, and that the *cycK* gene products of *Rhizobium* and *Sinorhizobium* are functionally interchangeable. [Johnston *et al.* (1994) reported tha the Pop$^-$ phenotype was corrected by a fragment of DNA containing the *feuPQ* genes, previously termed *popPQ*. That observation was erroneous.]

Genes upstream of *cycHJKL* are predicted to encode an outer-membrane lipoprotein and a two-component transcriptional regulator

During the course of sequencing the *cycHJKL* operon, three genes upstream of *cycH* were identified (Fig. 3).

The gene immediately upstream of *cycHJKL* was termed *lipA*; this gene is in the same orientation as *cycH* and encodes a protein of predicted molecular mass 13·5 kDa. The LipA protein appears to be a lipoprotein on the basis of its sequence, the best match being to the 17 kDa antigenic outer-membrane protein described from several *Rickettsia* species. There is also a distinctive SerGlyCys (SGC) motif located at amino acid residues 13–15, which is a potential peptidase cleavage sequence, typical of outer-membrane lipoprotein cleavage sites (Wu & Tokunga, 1986). Database searches also revealed

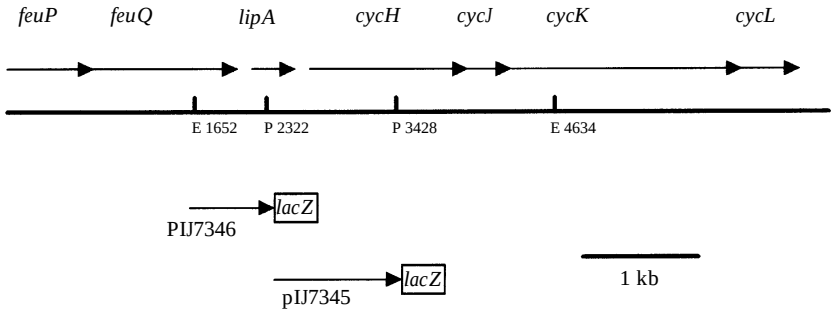

Fig. 3. Locations of the *feuPQ* and *lipA* genes in relation to the *cycHJKL* operon. The arrows indicate the orientation of these genes and the numbers refer to the base pair positions in the DNA sequence. The fragments cloned to form the two *lacZ* fusion plasmids pIJ7345 and pIJ7346 and the positions of restriction sites for *Eco*RI (E) and *Pst*I (P) are shown.

```
MRIKSLTARVLLLTTVWSTVALVVIGLLISTLYRKSAERGFQDLLRAQLY FeuQ 50

NVINSVTIGDQGALSGSPQLGDLRFAQPKTGWYWVVEPLGTYTTAPLVSP FeuQ 100

SLGSALIPVPSVVEAPFDKNYERYYQVTDASGNRVQVAETEVVLDTDGRA FeuQ 150

ARVRVTGNVDVVEDDVRTFSHSLYLALAGFGVGSLIVNALAILYGLKPLD FeuQ 200


                                    *           *
KARAAWE..RIRAGESEQLKGDFPREILPLANEVNALIDSNRRIVERARM FeuQ 248
IEALAKEVRELEEHNRELLNPATTRELTSLVRNLNRLLKSERERYDKYRT PhoQ 270
HAALQVG....KGIIPPPLREYGASEVRSVTRAFNH.MAAGVKQLADDRT EnvZ 235
RAAERLS....MDLNTQPVPIDGPSVVQEAARAMNR.MQQRIQDLIRNRT QrsA 218

        * * **
Q...VGNLAHSLKTPIAVLLNEARVLEK.....SHGELVR.SQAEAMQGQ FeuQ 289
T...LTDLTHSLKTPLAVLQSTLRSLRSEKMSVSDAEPVMLEQISRISQQ PhoQ 317
..LLMAGVSHDLRTPLTRIRLATEMMSEQDGYLAESINKDIEECNAII.. EnvZ 282
QML..AAISHDLRTPITRMKLRAQFL..DNSTTRNALVKDLNEMEVMINE QrsA 264


VQSYLNRARIAA......QRESVLARTDAEPALERLVRVMRRLNVDTEFD FeuQ 333
IGYYLHRASMRGGTLLSRELHPV..APLLDNLTSALNKVYQRKGVNISLD PhoQ 365
EQFI........DYLRTGQEMPMEMA.DLNAVLGEVIAAESGYEREIETA EnvZ 322
TLSF........ARDDFADNAKVN..LDLVSLVCSLVESMQDMGYNIQFH QrsA 304

                    *   **
LVVSPPHLAVAMEQQDLEETVGNLLENAARFAKSRVRLSAVEAAEDVKGV FeuQ 383
I...SPEISFVGEQNDFVEVMGNVLDNACKYCLEFVE.............I PhoQ 400
LYPGSIEVKMHPLSIKRA..VANMVVNAARYG...NGWI.........KV EnvZ 358
SHPQRIKILGRASALKRAFT..NLLNNAIRYAKNVNV...........RI QrsA 341

             ******              *           * * **
EASARRHWVELAVEDDGPGLEPDQIREALKRGRRSNESK....PGTGLGL FeuQ 429
SARQTDEHLYIVVEDDGPGIPLSKREVIFDRGQRVDTLR...PGQGVGL PhoQ 446
SSGTEPNRAWFQVEDDGPGIAPEQRKHLFQPFVRGDSARTI..SGTGLGL EnvZ 406
QW..RQNRVKVLIEDDGPGIAEKELEQVFEPFYRGEHSRSRDTGGVGLGL QrsA 389

     *                  **
SIVTEISNEYQGRLELSRGE..WGGLKAKLILP...GVTKDVA...     FeuQ 467
AVAREITEQYEGFTKIVAGESMLGGARMEVIFGRQHSAPKDE....     PhoQ 488
AIVQRIVDN..HNGMLELGTSERGGLSIRAWLPVPVTRAQGTTKEG     EnvZ 450
AVTRDIISDFTHNGKVILTNRPNGGLCATVELLSEVH.........     QsrA 426
```

Fig. 4. Sequence comparisons of the deduced amino acid sequence of *R. leguminosarum* FeuQ with other sensor proteins: *E. coli* PhoQ; *E. coli* EnvZ; *Coxiella burnetii* QsrA. The residue number of each protein is provided at the end of each line. Amino acid residues that are common to all sequences are indicated by asterisks. Two hydrophobic domains of FeuQ from *R. leguminosarum* (residues 10–32 and 173–197) are underlined. The position of the Tn*5lacZ* insertion is marked with a triangle.

that the 3′ region of a homologue of *lipA* is present in *S. meliloti*, also upstream of *cycH* (Kereszt *et al*., 1995).

Upstream of *lipA* in *R. leguminosarum*, we identified two other genes; it was clear from the sequences of their predicted products that they belonged to the large family of two-component transcriptional regulators. These regulators tend to comprise two protein components. The 'sensor' protein is often located in the cytoplasmic membrane, and monitors the external cell environment. In response to particular environmental signals, the sensor protein usually phosphorylates or otherwise modifies a cytoplasmic 'response regulator' protein. The response regulator is usually a DNA-binding protein that, depending on its state of phosphorylation, can act as a transcriptional activator at one or more 'target' promoters (Parkinson & Kofoid, 1992). The most similar gene products to those of the genes identified here were PhoP and PhoQ of *E. coli*. Given this similarity and their role in iron uptake (see below), the genes were termed *feuP* and *feuQ* (*f*erric *u*ptake).

The FeuQ protein has a predicted molecular mass of 51·2 kDa, and has significant homology to sensor proteins belonging to the family of histidine protein kinases. In database searches, 40 such sensors with similarity to FeuQ were found. The conserved domain of these proteins extends over approximately 250 amino acids and is usually located at the C-terminus (Albright *et al*., 1989). FeuQ has four highly conserved regions when compared to other sensor proteins. Region 1 extends between amino acid positions 252–262 (Fig. 4) and contains a conserved histidine at position 255, this being the putative site of autophosphorylation. Region 2 extends between positions 347–361 and has conserved asparagine and alanine residues at positions 356, 360 and 361. Regions 3 and 4, between positions 396–405 and 423–432, respectively, are glycine-rich and resemble nucleotide-binding motifs (Parkinson & Kofoid, 1992). These blocks of conserved amino acid residues probably form the catalytic centre involved in phosphorylation (Stock *et al*., 1989). The N-termini of sensor proteins tend to be variable and this region may contain the motifs needed to identify the specific environmental signals (Albright *et al*., 1989). Residues 10–32 and 173–197 of FeuQ are hydrophobic (Fig. 4), consistent with their being transmembrane domains. This would be consistent with residues 33–173 being exposed to the periplasm, with the C-terminal end of the protein exposed to the cytoplasm. A database search of residues 33–172, which probably form part of the sensor domain, showed no significant homology to any other sensor proteins.

The predicted *feuP* gene overlaps the predicted translational start of *feuQ* by 11 nucleotides, suggesting translational coupling. The *feuP* gene encodes a protein of predicted molecular mass 24·9 kDa, with similarity to many response regulator proteins. These include the PhoP of *E. coli* and *Salmonella typhimurium* (67% identify) and the *basR* gene product of *Haemophilus*

```
            * *  **                    ▼**    *
FeuP         MRILVVED DVNLNRQLAD TLKEAGYVVD QAFDGEEGHF LGDTEPYDAI 48
               +++++ +  +++++       ++++++  + +++++++ +++++++++
Bm  PhoP     ..VLVVED DKDLNRQLSE AMIAAGYVVD SAYDGEEGHY LGDTEPYDAV
Ec  PhoP     MRVLVVED NALLRHHLKV QIQDAGHQVD DAEDAKEADY YLNEHIPDIA
St  PhoP     MMRVLVVED NALLRHHLKV QLQDSGHQVD AAEDAREADY YLNEHLPDIA
BasR         MRILLIED DNLIGNGLQI GLTKLGFAVD WFTDGKTGMA ALTSAPYDAV

            *  **  * *      **       * * * ***      *      *****
FeuP      ILDIGLPEMD GVTVLEKWRG AGRGVPVLIL TARDRWSDKV AGIDAGADDY 98
           ++++++ ++ +  + + ++   ++  +++ + ++++++++++ ++++++++++
Bm  PhoP  VLDIGLPQMD GISVVERWRR SGRTIPVLML TARDRWSDKV AGIDAGADDY
Ec  PhoP  IVDLGLPDED GLSLIRRWRS NDVSLPILVL TARESWQDKV EVLSAGADDY
St  PhoP  IVDLGLPDED GLSLIRRWRS SDVSLPVLVL TAREGWQDKV EVLSSGADDY
BasR      VLDLTLPKLD GLEVLQQWRS NHQDVPVLIL TARDTLDERV KGLQSGADDY

             **   **  **  ** **   *     *                    *
FeuP      VTKPFHVEEV LARIRALIRR AAGHSSSEII CGPVRLDTKS SKATVNGTTL 148
           +
Bm  PhoP  IT
Ec  PhoP  VTKPFHIEEV MARMQALMRR NSGLASQVIS LPPFQVDLSR RELSINDEVI
St  PhoP  VTKPFHIEEV MARMQALMRR NSGLASQVIN IPPFQVDLSR RELSVNEEVI
BasR      LCKPFALAEV AARLQALIRR RYGYHHSVIE QAGVKLDQNQ RSVWLNNQPI

            *   **             *  *                        *     *
FeuP      KLTSHEYRLL AYLMHHMGEV VSRTELVEHM YDQDFDRDSN TIEVFVGRLR 198
Ec  PhoP  KLTAFEYTIM ETLIRNNGKV VSKDSLMLQL YPDAELRESH TIDVLMGRLR
St  PhoP  KLTAFEYTIM ETLIRNNGKV VSKDSLMLQL YPDAELRESH TIDVLMGRLR
BasR      SLTSREYKLL ELFMLNKDRV LSRSSIEEKL SSWDEEISSG ALDVHIYNLR

            *           *    * * *
FeuP      KKMGV....D L.IETVRGLG YRIQAPKHAN .                     223
Ec  PhoP  KKIQAQYPQE V.ITTVRGQG YLFELR.... .
St  PhoP  KKIQAQYPHD V.ITTVRGQG YLFELR.... .
BasR      QKL....GKQ F.IRTVHGVG YALGQVEK.. .
```

Fig. 5. Sequence comparisons of the deduced amino acid sequence of *R. leguminosarum* FeuP with those of other proteins: *B. melitensis* (Bm) PhoP; *E. coli* (Ec) PhoP; *S. typhimurium* (St) PhoP and *H. influenzae* BasR. The residue number of FeuP is provided at the end of each line. Amino acid residues identical in both *R. leguminosarum* FeuP and *B. melitensis* PhoP are indicated by crosses. Amino acids common to all sequences are indicated by asterisks. The position of the Tn*5lacZ* insertion is marked with a triangle.

influenzae (61% identity). These DNA-binding regulatory proteins tend to be similar to each other throughout their length; an alignment of FeuP with the response regulators mentioned above is shown in Fig. 5. An acid pocket is a feature of these regulatory proteins; thus, these residues are conserved among all sequences (Parkinson & Kofoid, 1992). The residues in FeuP that correspond to Glu-7, Asp-8, Asp-9 and Asp-51 probably form part of the acid pocket into which the side chain of Lys-101 protrudes. The Asp residue at position 51 could be the site of phosphorylation by FeuQ (Parkinson & Kofoid, 1992).

In addition to these similarities, there is a much stronger identity between FeuP and the deduced product of a partially sequenced region of DNA in the pathogen *Brucella melitensis* (Fig. 5), a close relative of *Rhizobium* (Woese, 1987). The identity (92% at the amino acid level) is so high as to suggest that the *B. melitensis* gene may encode a protein that is functionally equivalent to FeuP (B. W. Wren, personal communication).

A mutation in FeuQ affects iron uptake

To determine the phenotype of a *feu* mutant strain, plasmid pKN16 was mutagenized with Tn*5lacZ*. Transposon insertions in the *feuPQ* region were identified initially by restriction mapping and were located precisely by DNA sequencing. One Tn*5lacZ* insertion in *feuQ* was isolated; precise mapping showed that *lacZ* was transcribed in the opposite orientation to *feuQ* and so was not under the control of the *feuPQ* promoter. The *feuQ*::Tn*5lacZ* insertion in this mutant plasmid (termed pBIO279) was marker-exchanged into the corresponding region of the genome of the wild-type *R. leguminosarum* strain 8401pRL1JI. DNA hybridization of genomic DNA using a probe that spanned *feuPQ* confirmed that a recombinant strain, termed J100, had undergone homologous recombination. This mutant was prototrophic, produced wild-type levels of siderophore, was NADI$^+$ and formed nitrogen-fixing nodules on the roots of peas. J100 did not accumulate PPIX at more than normal levels. However, J100 did have one striking characteristic in common with strains A266, A267 and Pop116 in that it also had a significantly reduced rate of ^{55}Fe uptake (Fig. 2). This phenotype was corrected by plasmid pBIO260, which contains a PCR product comprising *feuPQ* cloned in the broad-host-range plasmid pLAFR3.

We also wished to determine the effects of a *feuP* mutation. A Tn*5lacZ* insertion into the *feuP* gene in pKN16 was isolated, the orientation of the insertion being such that *lacZ* would be transcribed from the *feuPQ* promoter. Repeated attempts to marker-exchange this mutation into the corresponding position in the chromosome of *R. leguminosarum* strain 8401pRL1JI failed. It may be that under the conditions used here, *feuP* genomic mutants are non-viable or are slow-growing and thus difficult to isolate. Such an observation is not unprecedented; Osteras *et al.* (1995) reported an essential two-component regulatory system in *Rhizobium* spp.

Effect of a feuQ mutation on the transcription of *lipA* and *cycHJKL*

Two-component regulators often control the transcription of nearby genes. Given the close linkage of *feuPQ* and *cycHJKL*, plus the similarity of the effects of mutations in these genes with respect to iron uptake, the effects of the *feuQ* mutation on the transcription of the *cycHJKL* operon and also of *lipA* were tested.

Fragments spanning the 177 bp intergenic region between *lipA* and *cycH* and the 118 bp region between *feuQ* and *lipA* (Fig. 3) were each cloned into the wide-host-range promoter probe plasmid pMP220 to form pIJ7345 and pIJ7346, respectively. In these plasmid fusions, the levels of transcription of *lacZ* in pMP220 depend on the native promoter activity in the two intergenic sequences. Each plasmid was mobilized into the wild-type strain 8401pRL1JI and into the *feuQ* mutant strain J100. As shown in Table 2, the *lipA–lacZ* fusion (pIJ7346) had considerably higher levels of activity than the *cycH–lacZ* fusion. Comparison of the levels of β-galactosidase activities of both fusions in the wild-type and in the *feuQ* mutant strain J100 shows that the *feuQ* mutation has no effect on the expression of either the *lipA* or *cycH* fusion (Table 2).

Since the *cycHJKL* and *feuQ* genes play a role in iron uptake, measurements of the *cycH–lacZ* and the *lipA–lacZ* activities were also made in cells of

Table 2. *β*-Galactosidase activities of wild-type (8401pRL1JI) and *feuQ* mutant (J100) carrying a *cycHJKL* (pIJ7345) or *lipA* (pIJ7346) fusion plasmid

Strain	*β*-Galactosidase (Miller units)	
	Succinate + Fe	Succinate − Fe
8401pRL1JI(pMP220)	12 ± 8	14 ± 5
8401pRL1JI(pIJ7345)	405 ± 90	234 ± 30
8401pRL1JI(pIJ7346)	1626 ± 59	1080 ± 35
J100(pMP220)	63 ± 10	25 ± 8
J100(pIJ1345)	391 ± 27	347 ± 30
J100(pIJ7346)	1708 ± 163	1270 ± 83

8401pRL1JI and J100 grown in iron-depleted medium. As shown in Table 2, the expression of both fusions was reduced by growth in the low-iron conditions. However, this was not affected by the *feuQ* genotype and may just be a reflection of the poor cell growth in the iron-deficient medium.

Why are *cycHJKL* mutants defective in iron uptake?

The *cycHJKL* genes have been identified in a number of bacterial genera, in mitochondria and chloroplasts. These genes, which appear to form a single operon in *Rhizobium*, *Sinorhizobium* and *Bradyrhizobium*, have been shown by several groups (Delgado *et al*., 1995; Kereszt *et al*., 1995; Ritz *et al*., 1995) to have a possible role in the attachment of haem to apocytochrome *c*. Here, we have shown that mutations in the *cycHJKL* operon of *R. leguminosarum* lead to a number of other, unexpected, phenotypic defects, previously identified in the Pop116 mutant. These are the accumulation of PPIX and a lack of siderophore production leading, presumably, to a defect in high affinity iron uptake.

Interestingly, the upstream gene *feuQ*, which with *feuP*, appears to encode a two-component regulator, is also involved in iron acquisition. This suggested a role for *feuPQ* in the regulation of iron uptake. However, a mutation in *feuQ* does not affect PPIX accumulation, siderophore production, cytochrome *c* function or expression of the *cycHJKL* operon. Thus, *cyc* and *feuQ* mutants do not confer identical phenotypes. We considered the possibility that the loss of high affinity iron acquisition in both *feuQ* and *cyc* mutants might be because functional *cycHJKL* genes are required for FeuQ to function (for example, for incorporation of haem into the FeuQ protein). However, if this were the case, one would have to argue that siderophore production is not regulated by FeuQ and that the absence of siderophore production in *cyc* mutants is independent of the FeuQ-mediated regulator.

At present, we are unable to explain the precise basis of the role of the *cycHJKL* genes in siderophore synthesis or excretion. One possibility is their direct involvement in the transport of siderophores from the cytoplasm into the periplasm. Since the functions of the *cycHJKL* gene products are thought to be in the attachment of haem to apocytochrome *c*, this explanation seems unlikely, given the lack of any chemical similarity between haem (or any of its precursors) and any known *Rhizobium* siderophore molecule.

A more plausible explanation is that the biosynthesis of siderophores may require an electron transfer step involving a *c*-type cytochrome. Since *c*-type cytochromes are located mostly on the periplasmic side of the membrane, such a reduction could, in principle, occur in the periplasm simply by diverting electrons from the electron transport pathway into an enzyme or substrate required for siderophore biosynthesis.

Another, somewhat speculative model to explain why *cyc* mutants do not synthesize (or export) siderophores relates to the mechanisms by which bacteria sense the concentration of available iron. This model suggests that the primary signal for the activation of genes involved in siderophore biosynthesis may be the concentration of haem in the bacterial periplasm. The *cycHJKL* mutants would accumulate haem in that compartment since it would not be sequestered by *c*-type cytochromes or other haem-containing proteins in the membranes or periplasm. Thus, such mutants might sense that the concentration of available iron is higher than is, in fact, the case; in consequence, genes involved in siderophore production would not be expressed. The accumulation of PPIX in the *cyc* mutants could, in turn, be due to the stripping of iron from the excess haem, adding the metal to the inorganic iron pool.

Given the widespread occurrence of the *cycHJKL* genes in bacteria and organelles, it will be of interest to establish if the various phenotypic defects caused by mutations in the *cycHJKL* genes of *R. leguminosarum* are relevant to these other systems.

ACKNOWLEDGEMENTS

This work was funded largely by the BBSRC and by an EU-HCM grant (930497) to M. J. D. We thank A. Kereszt for supplying strains and plasmids and B. W. Wren for communication of results prior to publication.

REFERENCES

Albright, L. M., Huala, E. & Ausubel, F. M. (1989). Prokaryotic signal transduction mediated by sensor and regulator pairs. *Annu Rev Genet* **23**, 311–336.

Beckman, D. L., Trawick, D. R. & Kranz, R. G. (1992). Bacterial cytochrome *c* biogenesis. *Genes Dev* **6**, 268–283.

Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *J Gen Microbiol* **84**, 188–198.

Beringer, J. E., Hoggan, S. A. & Johnston, A. W. B. (1978). Linkage mapping in *Rhizobium leguminosarum* by means of R plasmid-mediated recombination. *J Gen Microbiol* **104**, 201–207.

Beynon, J. L., Beringer, J. E. & Johnston, A. W. B. (1980). Plasmids and host-range in *Rhizobium leguminosarum* and *Rhizobium phaseoli*. *J Gen Microbiol* **120**, 421–429.

Buchanan-Wollaston, A. V., Beringer, J. E., Brewin, N. J., Hirsch, P. R. & Johnston, A. W. B. (1980). Isolation of symbiotically

defective mutations in *Rhizobium leguminosarum* by insertion of the transposon Tn5 into a transmissible plasmid. *Mol Gen Genet* **178**, 185–190.

Crosa, J. H. (1989). Genetics and microbiology of siderophore-mediated iron transport in bacteria. *Microbiol Rev* **53**, 517–530.

Delgado, M. J., Yeoman, K. H., Wu, G., Vargas, C., Davies, A. E., Poole, R. K., Johnston, A. W. B. & Downie, J. A. (1995). Characterisation of the *cycHJKL* genes involved in cytochrome *c* biogenesis and symbiotic nitrogen fixation in *Rhizobium leguminosarum*. *J Bacteriol* **177**, 4927–4934.

Downie, J. A., Hombrecher, G., Ma, Q. S., Knight, C. D., Wells, B. & Johnston, A. W. B. (1983). Cloned nodulation genes of *Rhizobium leguminosarum* determine host-range specificity. *Mol Gen Genet* **190**, 359–365.

Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. *Proc Natl Acad Sci USA* **76**, 1648–1652.

Fischer, H. M. (1994). Genetic regulation of nitrogen fixation in rhizobia. *Microbiol Rev* **58**, 352–386.

Gilles-Gonzalez, M. A., Ditta, G. S. & Helinski, D. R. (1991). A haemoprotein with kinase activity encoded by the oxygen sensor of *Rhizobium meliloti*. *Nature* **350**, 170–172.

Guerinot, M. L. (1991). Iron uptake and metabolism in the rhizobia/legume symbiosis. *Plant Soil* **130**, 199–209.

Johnston, A. W. B., Brightwell, G., Tibertius, A. & Yeoman, K. H. (1994). Exopolysaccharide synthesis and haem production in *Agrobacterium* and *Rhizobium* – an unexpected link. In *Proceedings of the First European Nitrogen Fixation Conference*, pp. 91–94. Edited by G. B. Kiss & G. Endre. Szeged, Hungary: Officiana Press.

Jones, C. W. & Poole, R. K. (1985). Analysis of cytochromes. *Methods Microbiol* **18**, 285–328.

Keen, N. T., Tamaki, S., Kobayashi, D. & Trollinger, D. (1988). Improved broad-host range plasmids for DNA cloning in Gram-negative bacteria. *Gene* **70**, 191–197.

Kereszt, A., Slaska-Kiss, K., Putnoky, P., Banfalvi, Z. & Kondorosi, A. (1995). The *cycHJKL* genes of *Rhizobium meliloti* involved in cytochrome *c* biogenesis are required for 'respiratory' nitrate reduction *ex planta* and for nitrogen fixation during symbiosis. *Mol Gen Genet* **247**, 39–47.

Kondorosi, A., Vincz, E., Johnston, A. W. B. & Beringer, J. E. (1980). A comparison of three *Rhizobium* linkage groups. *Mol Gen Genet* **178**, 403–408.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Miyamoto, K., Nishimura, K., Musuda, T., Tsuji, H. & Inokuchioo, H. (1992). Accumulation of protoporphyrin IX in light-sensitive mutants of *Escherichia coli*. *FEBS Lett* **310**, 246–248.

Monson, E. K., Ditta, G. S. & Helinski, D. R. (1995). The oxygen sensor protein, FixL, of *Rhizobium meliloti*. *J Biol Chem* **270**, 5243–5250.

Nadler, K. D., Johnston, A. W. B., Chen, J. W. & John, T. R. (1990). A *Rhizobium leguminosarum* mutant defective in symbiotic iron acquisition. *J Bacteriol* **172**, 670–677.

Ohyama, K., Fukuzawa, T., Kohchi, T., Sano, T., Sano, S. & Shirai, H. (1988). Structure and organisation of *Marchantia polymorpha* chloroplast genome. *J Mol Biol* **203**, 281–298.

Osteras, M., Stanley, J. & Finan, T. M. (1995). Identification of *Rhizobium*-specific intergenic mosaic elements within an essential two-component regulatory system of *Rhizobium* species. *J Bacteriol* **177**, 5485–5494.

Page, M. D. & Ferguson, S. J. (1995). Cloning and sequence analysis of the *cycH* gene from *Paracoccus denitrificans*; the *cycH* gene product is required for assembly of all *c*-type cytochromes, including cytochrome c_1. *Mol Microbiol* **15**, 307–318.

Parkinson, J. S. & Kofoid, E. C. (1992). Communication modules in bacterial signalling proteins. *Annu Rev Genet* **26**, 71–112.

Pritchard, A. E., Seilhamer, J. J., Mahalingam, R., Sable, C. L., Venuti, S. E. & Cummings, D. J. (1990). Nucleotide sequence of the mitochondrial genome of *Paramecium*. *Nucleic Acids Res* **18**, 173–180.

Reigh, G. & O'Connell, M. (1993). Siderophore-mediated iron transport correlates with the presence of specific iron-regulated proteins in the outer membrane of *Rhizobium meliloti*. *J Bacteriol* **175**, 94–102.

Ritz, D., Thöny-Meyer, L. & Hennecke, H. (1995). The *cycHJKL* gene cluster plays an essential role in the biogenesis of *c*-type cytochromes in *Bradyrhizobium japonicum*. *Mol Gen Genet* **247**, 27–38.

Rossen, L., Shearman, C. A., Johnston, A. W. B. & Downie, J. A. (1985). The *nodD* gene of *Rhizobium leguminosarum* is autoregulatory and in the presence of plant root exudate induces the *nodABC* genes. *EMBO J* **4**, 3369–3374.

Ruvkun, G. B. & Ausubel, F. M. (1981). A general method for site-directed mutagenesis in prokaryotes. *Nature* **289**, 85–88.

Schwyn, B. & Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* **160**, 47–56.

Simon, R., Quamlt, J. & Klipp, W. (1989). New derivatives of transposon Tn5 suitable for mobilisation of replicons, generation of operon fusions and induction of genes in Gram-negative bacteria. *Gene* **80**, 161–169.

Smith, M. J., Shoolery, J. N., Schwyn, B., Holden, I. & Neilands, J. B. (1985). Rhizobactin, a structurally novel siderophore from *Rhizobium meliloti*. *J Am Chem Soc* **107**, 1739–1743.

Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Pees, E. & Lugtenberg, B. J. J. (1985). Promoters in the nodulation region of the *Rhizobium leguminosarum* symbiotic plasmid pRL1JI. *Plant Mol Biol* **9**, 27–39.

Staskawicz, B., Dahlbeck, D., Keen, N. T. & Napoli, C. A. (1987). Molecular characterisation of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv *glycinae*. *J Bacteriol* **169**, 5789–5794.

Stock, J. B., Ninfa, A. J. & Stock, A. M. (1989). Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol Rev* **53**, 450–490.

Thöny-Meyer, L., Fischer, F., Kunzler, P., Ritz, D. & Hennecke, H. (1995). *Escherichia coli* genes required for cytochrome *c* maturation. *J Bacteriol* **177**, 4321–4326.

Vargas, C., McEwan, A. G. & Downie, J. A. (1993). Detection of *c*-type cytochromes using enhanced chemiluminescence. *Anal Biochem* **209**, 323–326.

Woese, C. R. (1987). Bacterial evolution. *Microbiol Rev* **51**, 221–271.

Wu, H. C. & Tokunaga, M. (1986). Biogenesis of lipoproteins in bacteria. *Curr Top Microbiol Immunol* **125**, 127–157.

Received 13 June 1996; revised 5 August 1996; accepted 8 September 1996.