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A chromosomal locus required for copper resistance, competitive fitness, and cytochrome *c* biogenesis in *Pseudomonas fluorescens*

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ABSTRACT **A chromosomal locus required for copper resistance and competitive fitness was cloned from a strain of *Pseudomonas fluorescens* isolated from copper-contaminated agricultural soil. Sequence analysis of this locus revealed six open reading frames with homology to genes involved in cytochrome *c* biogenesis in other bacteria, *helC*, *cycJ*, *cycK*, *tipB*, *cycL*, and *cycH*, with the closest similarity being to the *aeg-46.5(yej)* region of the *Escherichia coli* chromosome. The proposed functions of these genes in other bacteria include the binding, transport, and coupling of heme to apocytochrome *c* in the periplasm of these Gram-negative bacteria. Putative heme-binding motifs were present in the predicted products of *cycK* and *cycL*, and TipB contained a putative disulfide oxidoreductase active site proposed to maintain the heme-binding site of the apocytochrome in a reduced state for ligation of heme. Tn*3-gus* mutagenesis showed that expression of the genes was constitutive but enhanced by copper, and confirmed that the genes function both in copper resistance and production of active cytochrome *c*. However, two mutants in *cycH* were copper-sensitive and oxidase-positive, suggesting that the functions of these genes, rather than cytochrome *c* oxidase itself, were required for resistance to copper.**

Biological processes that regulate the transport and homeostasis of toxic but essential copper ions are not well understood. However, significant progress has been made recently with the discovery of copper-transporting P-type ATPases in mammals and in several bacterial species (1–4). These studies suggest that separate P-type ATPases may be generally present in bacteria and higher organisms for transporting copper into and out of cells. In addition, investigations of bacteria that have adapted to high levels of copper exposure have revealed other mechanisms of handling copper, such as the copper-sequestering activity of proteins produced by the *cop* operon of *Pseudomonas syringae* (5–7) and related copper-resistance systems in *Xanthomonas campestris* (8, 9) and *Escherichia coli* (10). Further analysis of copper-resistant strains of *E. coli* has shown that in addition to the plasmid-borne copper-resistance operon, several chromosomal loci, designated *cutA, cutB, cutC, cutD, cutE,* and *cutF*, are required for expression of resistance and for normal copper metabolism (11). Several have now been cloned and sequenced (12–14), but their exact functions in copper metabolism are not yet known. One of the *E. coli* genes, *cutA2,* identified from copper-sensitive mutants (13), was independently identified from mutants defective in the production of *c*-type cytochromes under anaerobic growth conditions and called *dipZ* (15). The amino acid sequence of DipZ contained a protein disulfide isomerase-like motif and was suggested to function in the reduction of cysteine pairs of cytochrome *c* (Cyt *c*) apoproteins for covalent attachment of heme groups (15). The specific function of CutA2/DipZ in copper transport is not clear, but this work established a linkage between general copper metabolism and Cyt *c* biogenesis.

This report describes a linkage between a separate set of chromosomal genes required for Cyt *c* biogenesis and copper metabolism and their additional role in competitive fitness. In previous work (16), we demonstrated that copper resistance was associated with competitive fitness in a strain of *Pseudomonas fluorescens* from copper-contaminated agricultural soil. Copper-sensitive mutants of this strain survived well in sterile soil but not in nonsterile soil, suggesting that genes required for copper resistance were also necessary for competition with other soil organisms. Here we describe the cloning and characterization of chromosomal genes required for copper resistance and competitive survival and their additional role in the biogenesis of an important branch of the bacterial electron transfer chain.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media. The wild-type *P. fluorescens* 09906 was isolated from citrus rhizosphere soil and had a minimum inhibitory concentration of 1.6 mM cupric sulfate (16, 17). Three copper-sensitive mutants of 09906 were generated by Tn*5* mutagenesis, and the minimum inhibitory concentrations of $CuSO_4$ for these mutants 09906.2, 09906.3, and 09906.4 were 0.16, 0.16, and 1.0 mM (16). For routine work, *P. fluorescens* was grown on mannitol-glutamate (MG) medium (18) or MG supplemented with yeast extract at 0.25 g/liter (MGY). *E. coli* cultures were grown on Luria–Bertani agar (19). Antibiotic concentrations used were as follows: 100 μg of kanamycin and 30 μg of tetracycline per ml for *P. fluorescens* or 50 μg of ampicillin, 35 μg of chloramphenicol, 50 μg of kanamycin, and 12.5 μg of tetracycline per ml for *E. coli* cultures.

Construction and Screening of a Genomic Library. The wide-host range cosmid pLAFR3 (20) was used to construct a genomic library of wild-type *P. fluorescens* 09906. Total DNA was isolated as described (21). The strategy used for cosmid cloning was a modification of the method of Ish-Horowicz and Burke (22). The DNA was partially digested with enzyme *Sau*3A and treated with calf intestinal alkaline phosphatase. The DNA was then ligated with the individual arms of pLAFR3, and packaged *in vitro* as described (20). *E. coli* HB101 was transduced with this mixture, and colonies were selected on Luria–Bertani agar plates containing tetracycline.

Cosmid clones were mobilized from *E. coli* to the copper-sensitive Tn*5* mutants 09906.2, 09906.3, and 09906.4 by triparental mating using the helper plasmid of pRK2013 as described (16). Mating mixtures were suspended in sterile distilled water and plated on MG agar plates containing kanamycin, tetracycline, and 0.8 mM of $CuSO_4$. Several transconjugants from each mating were grown in MG broth

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Abbreviations: Cyt *c*, cytochrome *c*; MG, mannitol glutamate; MGY, MG supplemented with yeast extract; ORF, open reading frame.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U44827).

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containing antibiotics and $CuSO_4$, and cosmids were isolated. These cosmids were again mobilized into the copper-sensitive transposon mutants of *P. fluorescens* to confirm their complementation to copper resistance. One of the cosmid clones that complemented the copper-sensitive Tn*5* mutants 09906.2 and 09906.3 was designated as pPF1. Subclones from pPF1 were made and tested for copper resistance.

Detection of Homology to Cosmid pPF1. The cosmid pPF1 was labeled by random primed labeling with digoxigenin-11-dUTP and used as a probe as outlined in the Genius DNA Labeling and Detection Kit (both from Boehringer Mannheim). Southern blotting of complete digests of total DNA of 09906, 09906.2, 09906.3, and 09906.4 with *Eco*RI, *Bam*HI, and *Hind*III; hybridization; wash conditions; and detection of labeled DNA were done essentially as described by the manufacturer of the detection kit.

Transposon Mutagenesis and Marker Exchange. Plasmid pPF2 was a subclone of pPF1 with a deletion of a 15.2-kb *Xho*I fragment (see Fig. 1). pPF3 was a subclone of pPF2 with a further deletion of a 5.2-kb *Xba*I–*Hin*dIII fragment. Plasmids pPF2 and pPF3 in *E. coli* HB101 were subjected to random insertion mutagenesis by using Tn*3–gus* as described by Bonas *et al.* (23), and the insertions were mapped by restriction enzyme analysis. Wild-type *P. fluorescens* 09906 cells were transformed with the mutated plasmids by electroporation and selection on MGY supplemented with tetracycline and kanamycin. To obtain mutants of *P. fluorescens* in which the wild-type chromosomal region was replaced by homologous recombination with the Tn*3–gus*-containing mutant DNA, cultures were subcultured at least 10 times in MGY broth with shaking for 12 h without selection and then once with kanamycin added. Cells were then plated onto MGY supplemented with kanamycin and replica-plated onto MGY with tetracycline. Potential marker-exchange mutants that lost tetracycline resistance and retained kanamycin resistance were further characterized by Southern blot analysis. Total DNA of the recombinants was digested with *Bam*HI, separated by agarose gel electrophoresis, blotted, and hybridized with a 1.7-kb *Eco*RI–*Eco*RI fragment internal to Tn*3–gus* to confirm the homologous recombination and the orientation of the Tn*3–gus* insertion. These recombinants were then spotted on MGY plates supplemented with 0.8 mM $CuSO_4$ for testing copper resistance. To determine the precise insertion sites of some mutants, a primer complementary to an internal sequence of Tn*3–gus* (5′-AAGAGGCGTCAGAGGCAGAA-3′) was used to sequence across the junction of the transposon and target DNA.

Assays for β-Glucuronidase and Oxidase Activities. Strains of *P. fluorescens* containing Tn*3–gus* insertions after marker exchange were grown overnight in MGY broth, centrifuged, and washed once with sterile distilled water. The bacterial suspensions were then spotted onto MGY plates containing 25 μg of 5-bromo-4-chloro-3-indolyl β-D-glucuronide per ml (X-Gluc; Sigma) in the absence or presence of $CuSO_4$ (0.005 mM). Colonies that displayed a blue color after 6 days of incubation in 28C were considered to have β-glucuronidase activity. The oxidase activity of *P. fluorescens* strains was determined by streaking colonies from MGY plates onto a filter paper saturated with 1% of tetramethyl-*p*-phenylenediamine solution (24).

DNA Sequencing and Analysis. The 3.6-kb (pPF5) and 3.0-kb (pPF6) *Eco*RI fragments of pPF1 (see Fig. 1) were subcloned into the vector pBluescriptKS+ (Strategene). The 3.6-kb insert from pPF5 was subcloned into the broad-host-range cosmid pLAFR3 to obtain pPF4.1 for complementation analysis in *P. fluorescens*, and pPF5 was also subcloned into two parts in pBluescriptKS+ to obtain pPF7 and pPF8 (see Fig. 1) for DNA sequencing. The high G+C content of *P. fluorescens* DNA made it necessary to use deaza deoxynucleotides from the Sequenase sequencing kit (United States Biochemical) and terminal deoxynucleotidyl transferase with excess dNTPs to eliminate the artifact banding associated with double-stranded DNA templates. Computer-assisted sequence analysis and alignments were made by using programs of the Wisconsin Package of the Genetics Computer Group (Madison, WI) and the BLAST programs provided by the National Center for Biotechnology Information, World Wide Web.

RESULTS

Cloning and Complementation of Copper-Sensitive Mutants. A single cosmid, pPF1, was identified that complemented copper-sensitive Tn*5* mutants 09906.2 and 09906.3, but not 09906.4, by allowing growth on MGY agar plates containing 0.8 mM of $CuSO_4$. Several subclones from the cosmid pPF1 were electroporated into 09906.2 and 09906.3 (Fig. 1). Plasmids pPF2 and pPF3, but not pPF4.1, were able to complement the copper resistance of 09906.2 and 09906.3. The third Tn*5* mutation causing a similar copper-sensitive phenotype was apparently not within this chromosomal region and has not yet been characterized.

When pPF1 was labeled and probed to *Eco*RI-digested chromosomal DNA from wild-type *P. fluorescens* 09906 and copper-sensitive Tn*5* mutants 09906.2 and 09906.3, one band from the mutants had shifted up about 6 kb (data not shown), corresponding to the size of the 5.8-kb Tn*5* (25). However, in the copper-sensitive mutant 09906.4, there was no difference in the band pattern from the wild-type when the chromosomal DNA was probed with pPF1. The insertions causing copper sensitivity in 09906.2 and 09906.3 were therefore inside the chromosomal region shown to be homologous to the cosmid pPF1, but the insertion in 09906.4 was outside of this region. From the physical map of the cosmid clone pPF1 and Southern blot analysis of the chromosomal DNA of 09906.2 and 09906.3 digested with different enzymes, the Tn*5* insertion sites of these two mutants were determined (Fig. 1). These insertions were in the same 3.6-kb *Eco*RI fragment of pPF1 about 1.9 kb apart from each other in 09906.2 and 09906.3.

Transcriptional Organization of the Chromosomal Locus. Chromosomal Tn*3–gus* insertions in *P. fluorescens* were obtained through mutagenesis of pPF2 and pPF3, followed by marker exchange (Fig. 2). With added $CuSO_4$, β-glucuronidase activity was induced for insertions within the chromosomal region required for copper resistance. With copper induction, blue colonies were observed within 6 days. Without copper induction, 12 days were required for colonies of these mutants to show a blue color. Southern blot analysis of *Bam*HI-digested DNA of Tn*3–gus* mutants indicated that each mutant had a single transposon insertion (data not shown). The positions of the Tn*3–gus* insertions suggested that at least 3.4 kb of DNA were essential for copper resistance in pPF1. The oxidase activity of each mutant was also determined (Fig. 2). Tn*3–gus* mutants G1–G7 were copper-sensitive and oxidase-negative. However, copper-sensitive mutants G8 and G9 were oxidase positive.

DNA Sequence Analysis. The nucleotide sequence was determined for the first 4.8 kb of the pPF3 clone, which spanned all of the insertions causing copper sensitivity in this region (Fig. 3). Six open reading frames were found; they were designated *helC, cycJ, cycK, tipB, cycL,* and *cycH* by homology to other characterized genes. The *helC* homolog, at the 5′ end of our subclone, was not complete but contained the last 234 bp of the gene that would encode an amino acid sequence with 36% identity to the corresponding C-terminal region of HelC of *Rhodobacter capsulatus* (26). The predicted protein product of *cycJ* of *P. fluorescens* was homologous to CycJ of *Bradyrhizobium japonicum* (46% amino acid sequence identity) and to YejS of *E. coli* (48% identity) (ref. 27; GenBank accession no. U00008). Homology was found between CycK of *P. fluorescens* and several other conserved bacterial and mitochondrial pro-

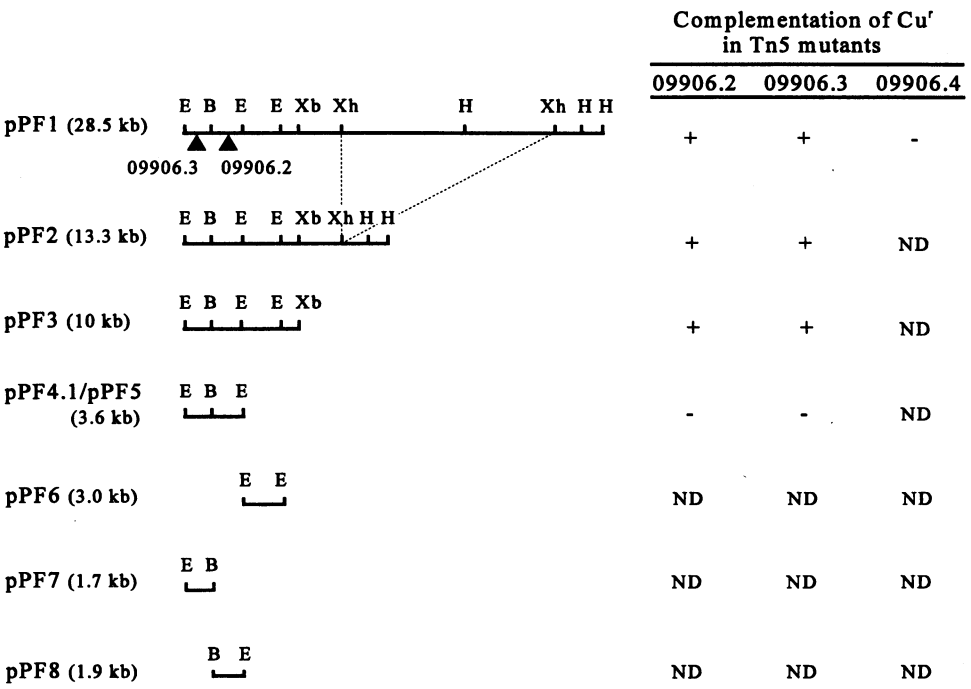

FIG. 1. Complementation of the copper-resistance phenotype by conjugation of the cosmid clone pPF1 or subclones into three copper-sensitive Tn*5* mutants of *P. fluorescens* 09906. Arrowheads indicate the positions of Tn*5* insertions in mutants 09906.2 and 09906.3. E, *Eco*RI; B, *Bam*HI; Xb, *Xba*I; Xh, *Xho*I; H, *Hin*dIII; ND, not determined.

teins encoded by *cycK* of *B. japonicum* (47% identity), *ccl1* of *R. capsulatus* (50% identity), *yejR* of *E. coli* (55% identity), *nrfE* of *E. coli* (45% identity), mitochondrial open reading frame (ORF) 509 of *Marchantia polymorpha* (36% identity), and several related ORFs in other plant mitochondria (refs. 26–31; GenBank accession no. U00008). Similarity was also found between TipB of *P. fluorescens,* TipB of *B. japonicum* (37% identity), HelX of *R. capsulatus* (40% identity), and YejQ of *E. coli* (47% identity) (refs. 32 and 33; GenBank accession no. U00008). CycL of *P. fluorescens* was similar to CycL of *B. japonicum* (38% identity) and YejP of *E. coli* (43% identity) (ref. 27; GenBank accession no. U00008). Following *cycL*, a sixth ORF would encode a product with homology to CycH of *B. japonicum* (22% identity) and NrfG of *E. coli* (25% identity) (28, 34).

Putative heme-binding motifs proposed for CycK and CycL of *R. capsulatus* and *B. japonicum* (35) were also present in the predicted products of *cycK* and *cycL* of *P. fluorescens*, as well as their homologs in *E. coli* and other organisms (Fig. 4). In addition, a consensus disulfide oxidoreductase active site, present in the periplasmic thioredoxin-like proteins HelX of *R. capsulatus* (33), TipB (from ORF132) of *B. japonicum* (32), and YejQof *E. coli* (GenBank accession no. U00008), as well as the periplasmic disulfide oxidoreductase DsbA of *E. coli* (36) and disulfide isomerase-like protein DipZ(CutA2) of *E. coli* (13, 15), was also found in the predicted product TipB of *P. fluorescens* (Fig. 4).

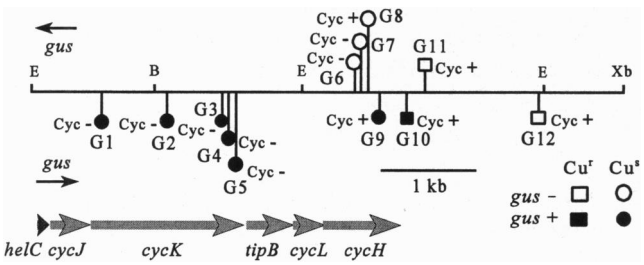

FIG. 2. Transcriptional orientation, copper resistance, and oxidase phenotypes determined by Tn*3*–*gus* insertions in pPF3. The direction of arrows indicates the orientation of the promoterless *gus* gene in Tn*3*–*gus* for each insertion. Insertions resulting in a copper-sensitive phenotype are indicated by circles; insertions that did not inactivate resistance are indicated by squares. Filled symbols indicate that *gus* expression was detected. +, Oxidase positive; −, oxidase negative.

Hydrophobicity profiles of the predicted gene products were consistent with descriptions of probable membrane and periplasmic locations of homologs of these products in other bacteria (35). Although our clone did not contain the intact *helC* gene, the predicted C-terminal region contained one of the hydrophobic stretches predicted to be a membrane-spanning domain of this protein (Fig. 3). CycJ, CycL, and CycH each contained two hydrophobic domains, one at the N terminus and one further in the sequence, which was consistent with predictions of these proteins being anchored in the membrane (35). The putative heme-binding site of CycL was located between the two hydrophobic domains, consistent with the location of this site in the periplasm. CycK contained several hydrophobic stretches that may be membrane-spanning domains. TipB contained only one hydrophobic domain at its N terminus, which is consistent with its predicted periplasmic location (33).

DISCUSSION

Copper resistance in *P. fluorescens* required chromosomal genes that were not directly related to the *cop* operon (5), to copper-transporting P-type ATPases (1–4), or to the known *cut* genes required for normal copper uptake and transport in *E. coli*. In *E. coli*, *cutA, cutC, cutE*, and *cutF* have been cloned and sequenced (12–14). These separate chromosomal loci encode a cytoplasmic copper-binding protein (CutC), an apolipoprotein *N*-acyltransferase (CutE), and an outer membrane lipoprotein (CutF), but their specific roles in copper metabolism are not completely clear. In addition, the *cutA* locus consists of three genes, one of which (*cutA2*) encodes a protein disulfide isomerase required for the biogenesis of *c*-type cytochromes under anaerobic conditions (13, 15). The chro-

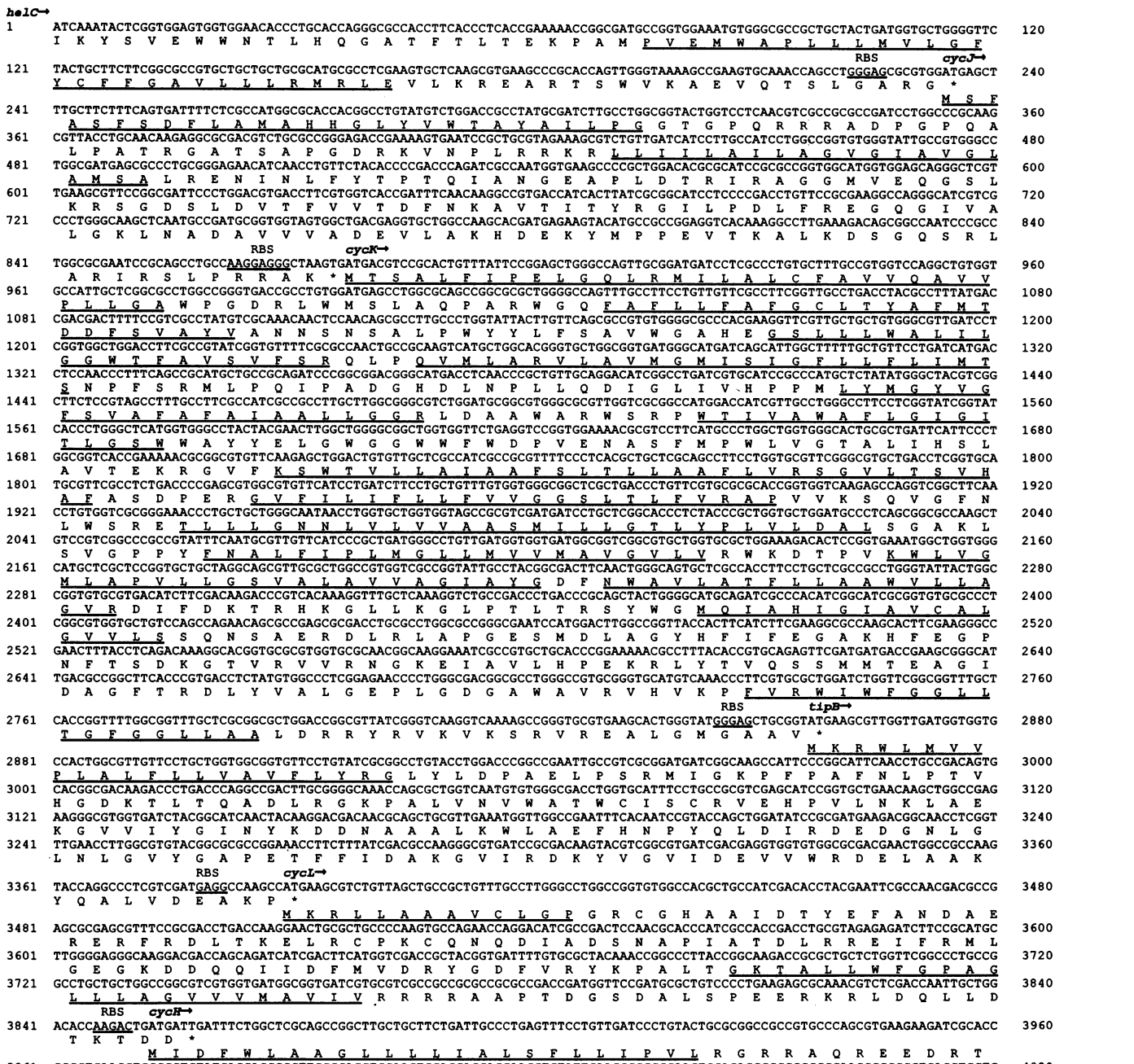

FIG. 3. DNA sequence and predicted translation products of *helC*, *cycJ*, *cycK*, *tipB*, *cycL*, and *cycH* of *P. fluorescens*. Putative ribosome binding sites in the nucleic acid sequence are underlined and labeled RBS, and regions of hydrophobicity in the amino acid sequence are underlined.

mosomal locus required for copper resistance in *P. fluorescens* was also involved in the biogenesis of Cyt *c*, but other than the presence of similarities between the active site of CutA2(DipZ) and a similar putative disulfide oxidoreductase active site in TipB, the genes were not obviously related to the *E. coli cut* genes. Instead, the locus in *P. fluorescens* was related to another region of the *E. coli* chromosome, the *aeg-46.5* region, as discussed below.

The six ORFs in the chromosomal region cloned from *P. fluorescens*, *helC*, *cycJ*, *cycK*, *tipB*, *cycL*, and *cycH*, were similar to genes involved in Cyt *c* biogenesis, as first described from *B. japonicum* and *R. capsulatus*, but the genes were organized

FIG. 4. (*A*) Conservation of a putative disulfide oxidoreductase active site in TipB of *P. fluorescens* (Pf), YejQ of *E. coli* (Ec), TipB of *B. japonicum* (Bj), HelX of *R. capsulatus* (Rc), DipZ(CutA2) of *E. coli*, and DsbA of *E. coli*. (*B* and *C*) Conservation of putative heme-binding motifs in CycK and CycL of *P. fluorescens*, CycK and CycL of *B. japonicum*, Ccl1 and Ccl2 of *R. capsulatus*, YejR and YejP of *E. coli*, mitochondrial ORF509 of *Marchantia polymorpha* (Mp), and CCHL of *Neurospora crassa* (Nc).

differently in *P. fluorescens* than in those bacterial species. In *B. japonicum*, *cycH–cycJ–cycK–cycL* are organized as a cluster but another region contains *cycV–cycW*–ORF263(*helC*)–*cycX–tipB* (32, 34, 35). *R. capsulatus* had a similar arrangement as *B. japonicum*, with one region containing *helA(cycV)–helB(cycW)–helC–helD(cycX)–tipB*, but the other region containing *ccl1(cycK)–ccl2(cycL)* was preceded by a divergently transcribed *argD*, rather than *cycH–cycJ* (26, 33).

The gene order in *P. fluorescens* was more similar to a homologous region of the *E. coli* chromosome, identified first through operon-fusion mutagenesis (37) as an anerobically expressed gene region (*aeg-46.5*), and later through sequencing as *yejW(helA)–yejV(helB)–yejU(helC)–yejS(cycJ)–yejR(cycK)–yejQ(TipB)–yejP(cycL)* (GenBank accession no. U00008). This similarity is consistent with the closer phylogenetic relationship between *P. fluorescens* and *E. coli* than between *P. fluorescens* and *B. japonicum* or *R. capsulatus* (38).

Cyt *c* oxidase activity and copper resistance were disrupted in Tn*3–gus* mutations in this gene cluster of *P. fluorescens*, except that two mutations (G8 and G9) localized to the 3′ end of *cycH* that caused copper sensitivity did not disrupt Cyt *c* oxidase activity. In a recent study, Page and Ferguson (39) also found that a deletion of the 3′ end of *cycH* from *Paracoccus denitrificans* did not abolish Cyt *c* biogenesis. Our G8 and G9 mutants effectively uncoupled the resistance phenotype from Cyt *c* function and suggest that the end product (Cyt *c*) is not what is required for resistance. Apparently, a function of the CycH product, and possibly other products of this gene cluster, are required for resistance. *c*-Type cytochromes are electron carriers with the heme group covalently attached to the apocytochrome. The mature holoprotein is localized on the periplasmic side of the cytoplasmic membrane, either membrane bound or present in the periplasmic space of Gram-negative bacteria (26). Heme is thought to be transported to the periplasm and coupled to the apocytochrome in the periplasm (39). This process is thought to involve several membrane-bound and periplasmic proteins, including homologs to each of the genes described in this study. HelC may be a subunit of an ABC-transporter of heme, which includes additional proteins HelA and HelB in other bacteria (35). TipB is thought to function as a disulfide oxidoreductase that may function in reduction of the cysteines of the apocytochrome *c* heme-binding site, and a number of membrane-associated proteins are thought to function in heme ligation to this site, including CycJ, CycK, CycL, and CycH (35). It is not known how these proposed functions could affect copper sensitivity. None of the putative copper-binding, repeated sequences (MXXMDHXXM) present in Cop proteins of *P. syringae* (5) and *Enterococcus hirae* (2), or the related MXXM repeats in the CTR1 protein of yeast (40), were found in the predicted products from the *P. fluorescens* genes.

However, the cysteine-containing motifs CXXC conserved in the putative heme-binding site of CycL and in the active site of the thioredoxin-like protein TipB could potentially interact with copper, as proposed for CXXC motifs in the mammalian and bacterial copper ATPases (1–4). Cyt *c* oxidase itself contains copper atoms (41), but the mechanism of copper insertion into the complex does not appear to be well characterized. A role for some of these genes in the handling of copper for Cyt *c*, and in the management of toxic levels of copper, might therefore be considered. Alternatively, the effects of these genes may be less direct, by contributing to other cellular processes that are also required for copper metabolism. It will therefore be important to explore possible secondary effects on the functions of *cop* gene homologs (5, 21), the locus that was mutagenized in *P. fluorescens* 09906.4 (16), copper-transporting ATPases (1–4), and other genes that may be analogous to the *cut* genes of *E. coli* (12–14).

Greater β-glucuronidase activity was observed with Tn*3–gus* mutants grown with supplemental copper than without copper, suggesting that, in addition to the basal level of expression, transcription of these genes was induced to higher levels by copper. Copper-responsive expression of Cyt *c*6 and an enzyme in the heme biosynthetic pathway has been reported in a green alga, and many other examples of regulation of gene expression by metal ions associated with catalytic metalloproteins exist (42). This pattern of expression is consistent with a bifunctional role of these genes in copper resistance and Cyt *c* biogenesis, but whether enhanced expression is an adaptation of copper-resistant bacteria, or a normal characteristic of oxidase-positive bacterial species is not yet known. No copper-responsive regulatory sites such as the CopR-binding site (*cop* box) of *P. syringae* (43, 44) were detected in the DNA sequence from *P. fluorescens,* but the probable operon structure of this gene cluster suggests that any such regulatory site would be upstream from the incomplete *helC* gene in our clone.

Many bacterial species have multiple respiratory oxidases (41). The different respiratory oxidases may allow the cells to customize their respiratory system to meet the demands of a variety of environmental growth conditions, including changes in the availability of metal ion cofactors. Mutations affecting a single cytochrome assembly pathway would therefore not necessarily be fatal, but may reduce their ability to meet all the demands to grow in certain microenvironments. The oxidase-negative, copper-sensitive Tn*5* mutants of *P. fluorescens* identified in our previous study (16) survived well in sterile soil, but they were less competitive for survival when other microbes were present in the soil. The importance of Cyt *c* oxidase itself in competitive fitness, versus more general effects from the loss of proteins involved in transport and handling of heme or copper, will be evaluated in further studies.

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