# Global Regulation of Expression of Antifungal Factors by a *Pseudomonas fluorescens* Biological Control Strain

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**The root-colonizing bacterium *Pseudomonas fluorescens* BL915 protects a variety of seedlings from damping-off disease caused by the fungal pathogen *Rhizoctonia solani*. Spontaneous pleiotropic mutants of *P. fluorescens* strain BL915 which fail to synthesize antifungal factors such as chitinase, cyanide, and pyrrolnitrin and exhibit altered colony morphology were isolated. Such mutants fail to inhibit the growth of *R. solani in vitro*, and their biological control capability is sharply reduced. We characterized a genomic DNA fragment from strain BL915 which, when introduced into these pleiotropic mutants, restored the lost functions, the wild-type colony morphology, and biocontrol activity. DNA sequence analysis of the genomic fragment revealed the presence of genes homologous to those of numerous bacterial global regulatory systems and identified a cluster of genes identical in organization to the *Escherichia coli* gene cluster consisting of *uvrY, uvrC, pgsA,* and *glyW*. Coordinate biosynthesis of multiple antifungal products in some heterologous *Pseudomonas* strains in response to the introduction of the strain BL915 genomic fragment confirmed the regulatory nature of sequences contained on this fragment. Further genetic analysis indicated a gene homologous to response regulators of bacterial two-component systems was sufficient to complement the pleiotropic mutants and to activate antifungal genes in heterologous strains. Marker exchange of a truncated version of this gene into the *P. fluorescens* BL915 chromosome generated pleiotropic mutants indistinguishable from the original spontaneous mutants. Cloning and sequencing of the response regulator gene from several spontaneous mutants allowed identification of various nucleotide changes associated with the gene in such mutants.**

*Additional keyword:* transcriptional activator.

The ability of rhizosphere-associated fluorescent pseudomonads to inhibit the growth of plant-pathogenic fungi has generated increased interest in their use as crop protectants (Gutterson 1990; Lam and Gaffney 1993; Weller 1988). Effective bacterial biological control agents with activity against phytopathogenic fungi often synthesize a variety of antifungal metabolites and enzymes (Gutterson 1990; Haas *et al.* 1991; Lam and Gaffney 1993; Laville *et al.* 1992; Loper and Buyer 1991; Thomashow and Pierson 1991; Weller 1988). For example, chitinase, pyrrolnitrin, and cyanide, which are among the products synthesized by the biological control bacterium *Pseudomonas fluorescens* strain BL915, are all capable of negatively impacting fungal growth (Jones *et al.* 1986; van Pée *et al.* 1983; Voisard *et al.* 1989). It is anticipated that the study of how biocontrol bacteria regulate the expression of diverse genes involved in pathogen inhibition will prove critical for determining environmental conditions under which such strains function optimally and may identify previously unrecognized aspects of bacterial regulatory circuits.

We have determined that certain mutant derivatives of the biocontrol bacterium *P. fluorescens* BL915 are deficient or altered in a variety of functions, with one consequence being loss of the ability to inhibit fungal growth. An 11-kb genomic DNA fragment from strain BL915, originally identified on the basis of its ability to restore the production of an agar-diffusible antibiotic to an antibiotic-deficient mutant (Hill *et al.*, in press), restored all functions to these pleiotropic mutants. We discuss the genetic organization of this genomic fragment, the identification of genes homologous to those of bacterial two-component regulatory systems, the similarity of a portion of this fragment to an *E. coli* gene cluster and to a region recently described in the biocontrol strain *P. fluorescens* CHA0 (Laville *et al.* 1992), and evidence that expression of antifungal factors by *P. fluorescens* BL915 is governed by a global regulatory circuit.

## RESULTS

### Isolation and features of spontaneous pleiotropic mutants.

Colonies of *P. fluorescens* BL915 appear circular, opaque, and convex on Luria-Bertani (LB) agar. After storage of such colonies on LB agar plates at room temperature for approximately 1 wk, we noted the occasional presence of undulate, translucent, flat sectors growing outward beyond the edges of the wild-type colonies. Analysis of clonal populations derived from such sectors revealed that they consisted of pleiotropic mutant derivatives of strain BL915 which had lost the ability

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to inhibit growth of the fungal phytopathogen *Rhizoctonia solani*. The characteristics of the pleiotropic mutants are listed in Table 1.

**Complementation of pleiotropic mutants.**

An 11-kb *Eco*RI restriction fragment (Fig. 1) was originally identified in a cosmid library of *P. fluorescens* strain BL915 genomic DNA on the basis of its ability to restore an antibiotic activity against *R. solani* to a strain BL915 mutant lacking this activity (Hill *et al.*, in press). Introduction of pPRN-E11 (a derivative of the broad-host-range plasmid pRK290 containing this 11-kb *Eco*RI fragment) into strain BL915 pleiotropic mutants such as BL915-1, BL915-2, and BL915-3, with features described in Table 1, fully restored all lost or altered functions to such mutants (data not shown).

**Activation of latent genes in heterologous *Pseudomonas* strains.**

*P. fluorescens* soil isolates designated strains BL914 and BL922 fail to produce detectable levels of chitinase or cyanide. Upon prolonged incubation (more than 48 hr) at 28° C in LB medium, strain BL914 produced a barely detectable level of pyrrolnitrin (less than 0.1 μg/L), while pyrrolnitrin production was not observed in strain BL922. Both strains BL914 and BL922 formed large, circular, flat, translucent colonies with undulate edges. In contrast, strain BL914 transconjugants carrying pPRN-E11 produced chitinase, cyanide, and significantly higher amounts of pyrrolnitrin (approximately 2.0 μg/L). These transconjugants formed small, circular, convex, opaque colonies with entire edges, which were virtually indistinguishable from those of *P. fluorescens* BL915. It should be noted that genomic restriction endonuclease profiles of *P. fluorescens* strains BL914 and BL915 distinguished the two strains, indicating that the BL914 soil isolate is not simply a naturally occurring mutant derivative of strain BL915 (data not shown). Strain BL922 transconjugants carrying pPRN-E11 also produced chitinase, cyanide, and pyrrolnitrin (approximately 2.5 μg/L), but did not undergo an obvious change in colony morphology.

**DNA sequence analysis of the 11-kb *Eco*RI fragment.**

A restriction map of the 11-kb *Eco*RI fragment from strain BL915, cloned in pPRN-E11, is included in Figure 1. Analysis of the DNA sequence obtained from this 11-kb fragment revealed the presence of five major open reading frames and a tRNA gene. The positions of these loci are indicated in Figure 1. Two of these open reading frames, ORF2 and ORF5, bear striking homology to sensor and activator components, respectively, of bacterial two-component global regulatory systems (Albright *et al.* 1989; Bourret *et al.* 1991; Stock *et al.* 1989). The DNA sequence of ORF2 is presented in Figure 2, and that of ORF5 is given in Figure 3.

ORF1, predicted to encode a 492–amino acid protein, shares homology with the *cheR* gene (Mutoh and Simon 1986) of *E. coli* (27% identity at the level of predicted amino acid sequence in a 317–amino acid overlap) and the *frzF* gene (McCleary *et al.* 1990) of *Myxococcus xanthus* (27% identity at the level of predicted amino acid sequence in a 373–amino acid overlap). CheR has a methyltransferase activity which is involved in chemotactic responses (Springer and Koshland 1977), while FrzF appears to have a similar function in *M. xanthus* (McCleary *et al.* 1990). Both the putative ORF1 product and FrzF contain an additional C-terminal domain absent in CheR, but these additional domains lack homology.

ORF2, predicted to encode a 575–amino acid protein, shares substantial homology with numerous genes encoding sensor kinase components of bacterial two-component regulatory systems (Albright *et al.* 1989; Bourret *et al.* 1991; Stock *et al.* 1989). Identities ranging from 18 to 27% at the level of the predicted amino acid sequence were observed with extensive regions of the following sensor genes: *rcsC* and *cheA* of *E. coli* (Mutoh and Simon 1986; Stout and Gottesman 1990), *frzE* of *M. xanthus* (McCleary and Zusman 1990), and *bvgS* of *Bordetella pertussis* (Arico *et al.* 1989). Significantly, conserved domains associated with the kinase portion of sensor genes (Albright *et al.* 1989), including a presumptive autophosphorylation site at His 210, were readily identifiable in ORF2 (Fig. 2). The C-terminal portion of the deduced ORF2 gene product (beginning at amino acid residue 451) contained an additional "receiver" or phosphoryl acceptor domain (Albright *et al.* 1989). Such receiver domains on sensor kinase molecules may serve to modulate the phosphorylation of transcription activators by the sensors (Stout and Gottesman 1990).

Approximately 1 kb downstream from ORF2, a cluster of genes transcribed in the orientation opposite that of ORF1 and ORF2 was identified (Fig. 1). The *glyW* tRNA gene of *P. fluorescens* BL915 is identical in sequence to the *glyW* tRNA locus of *E. coli* (Gopalakrishnan *et al.* 1986). ORF3 has homology (53% identity at the level of predicted amino acid sequence) to the *pgsA* gene of *E. coli* (Gopalakrishnan *et al.* 1986), which encodes phosphatidyl glycerophosphate syn-

**Table 1.** Characteristics of pleiotropic mutants derived from *Pseudomonas fluorescens* BL915

| Characteristic | *P. fluorescens* BL915 | Pleiotropic mutants |
|---|---|---|
| Pyrrolnitrin production | + | − |
| Cyanide production | + | − |
| Chitinase production | + | − |
| Gelatinase production | + | Reduced |
| Colony morphology | Circular, entire, convex, opaque | Circular, undulate, flat, translucent |
| *In vitro* inhibition of *Rhizoctonia solani* | + | − |
| Biocontrol activity against *R. solani* | + | − |

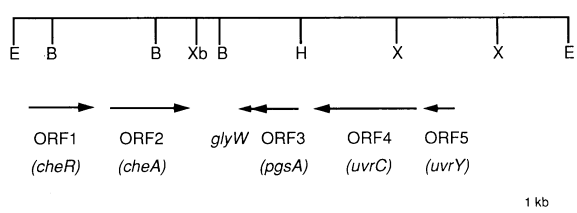


**Fig. 1.** Restriction endonuclease map of the approximately 11-kb *Eco*RI fragment from *Pseudomonas fluorescens* strain BL915. Arrows indicate the positions and directions of open reading frames and the *glyW* tRNA gene. *Escherichia coli* genes with sequence homology to each of the open reading frames are indicated in parentheses. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; X, *Xho*I; Xb, *Xba*I. The nucleotide sequence of a contiguous region including all of the open reading frames has been submitted to GenBank and assigned accession number L29642.

thase, an enzyme involved in phospholipid metabolism. ORF4 shares substantial homology (52% identity at the level of predicted amino acid sequence) with the *uvrC* gene of *E. coli,* which encodes a component of the UV damage repair excinuclease (Sancar *et al.* 1984). ORF5 shares homology with numerous genes encoding transcription activator components of bacterial two-component regulatory systems. In particular, ORF5 is very similar to the *gacA* gene of *P. fluorescens* strain CHA0 (Laville *et al.* 1992) (Fig. 3) and to the *uvrY* (*uvr-23*) gene of *E. coli* (Moolenar *et al.* 1987; Sharma *et al.* 1986) (89 and 60% DNA sequence identity, respectively). Examples of some additional bacterial transcription activator genes with more limited sequence similarity to ORF5 include *sacU* of *Bacillus subtilis* (Kunst *et al.* 1988), *bvgA* of *B. pertussis* (Arico *et al.* 1989), and *algR* of *Pseudomonas aeruginosa* (Deretic *et al.* 1989).

The organization of the gene cluster consisting of ORF5, ORF4, ORF3, and *glyW* is virtually identical to that of the *E. coli* gene cluster at map position 42 of the *E. coli* chromosome (Bachmann 1990), consisting of *uvrY, uvrC, pgsA,* and *glyW.* In *E. coli,* two additional tRNA genes, *cysT* and *leuZ,* are located immediately downstream from *glyW* (Bachmann 1990). These two tRNA genes were not identified at the corresponding location in *P. fluorescens* BL915. Instead, *glyW* in strain BL915 is followed, 6 bp downstream, by a potential stem-loop forming sequence, 5′-AAAAATGGACCTCTGA-AAAGAGGTCCATTTTTTTTT-3′, with features of a *rho*-independent transcription terminator. It should also be noted that Laville *et al.* (1992) demonstrated that *gacA* in *P. fluorescens* CHA0 is located adjacent to a *uvrC* homolog in that strain.

### Comparison of predicted amino acid sequences of ORF5, *gacA,* and *uvrY* gene products.

The predicted amino acid sequences of the *P. fluorescens* CHA0 *gacA* (Laville *et al.* 1992), *P. fluorescens* BL915 ORF5, and *E. coli uvrY* (Sharma *et al.* 1986) gene products are aligned in Figure 4. ORF5 and GacA are over 99% identical, differing at only two of 213 amino acid residues, despite the fact that their respective 639-bp DNA sequences differ at 73 positions (Fig. 3). The aligned ORF5 and UvrY amino acid sequences are 58% identical, and they are 75% similar when conservative amino acid substitutions are considered. In general, identical residues are evenly distributed over the en-

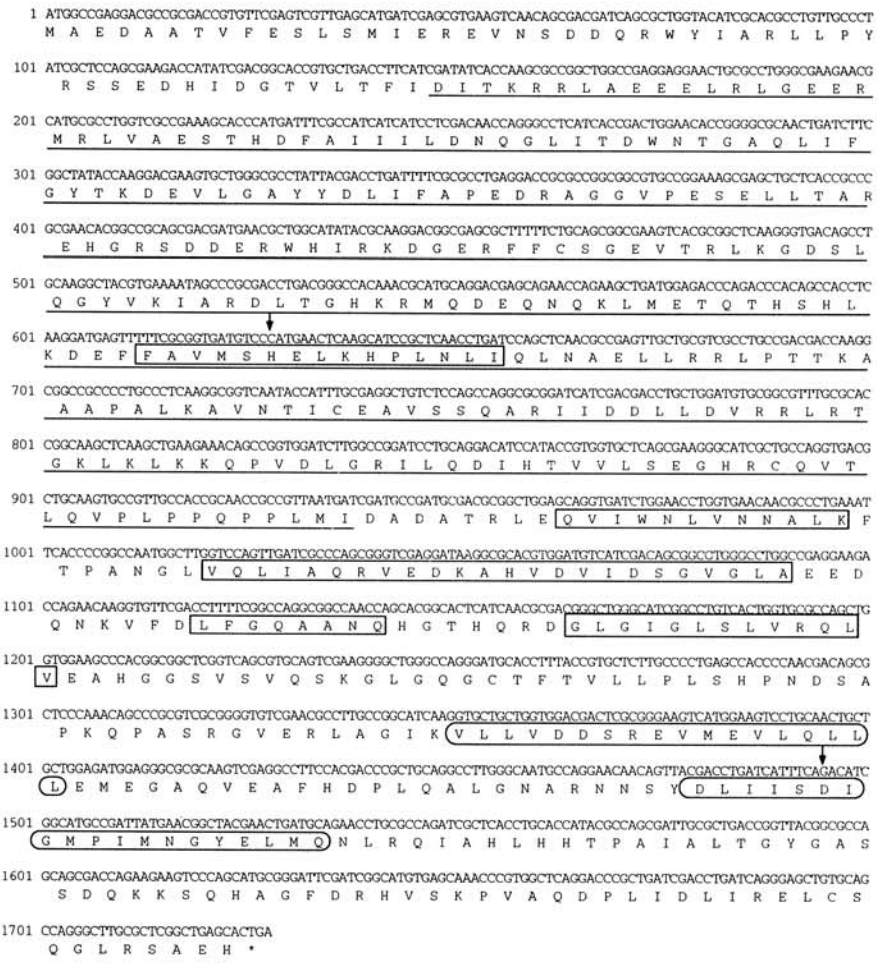


**Fig. 2.** Nucleotide sequence of ORF2 in the 11-kb *Eco*RI fragment from *Pseudomonas fluorescens* strain BL915. The deduced amino acid sequence of the putative ORF2 gene product is depicted below the nucleotide sequence. Amino acid stretches corresponding to highly conserved domains of bacterial sensor proteins (Albright *et al.* 1989) are enclosed in rectangular boxes. Amino acid stretches corresponding to conserved receiver domains found at the C-terminal end of a subset of bacterial sensor proteins are enclosed in rounded-corner rectangles. The conserved histidine residue of the presumptive autophosphorylation site and the conserved aspartic acid residue of the presumptive phosphoryl receiver domain are indicated with arrows. Amino acids not present in the truncated version of the ORF2 product, resulting from the deletion of an internal *Cla*I restriction site, are underlined.

tire lengths of ORF5 and UvrY. The N-terminal halves of these two proteins, which contain the presumptive phosphate acceptor (receiver) domains, are 59% identical. The C-terminal halves, which contain the presumptive DNA binding domains by analogy with other members of the FixJ transcription activator class (Kahn and Ditta 1991), are 57% identical.

### Sufficiency of ORF5 for pleiotropic mutant complementation and for activation of latent genes.

Subclones of the 11-kb *Eco*RI fragment of *P. fluorescens* BL915 were introduced into a pleiotropic mutant derivative of strain BL915 in broad-host-range plasmids and tested for their ability to complement the mutant. pCIB3344, containing a 3.7-kb *Eco*RI-*Xba*I fragment with ORF1 and ORF2, failed to restore any of the lost functions, while a 6.8-kb *Bam*HI-*Eco*RI fragment containing ORF5, ORF4, ORF3, and *glyW* cloned in pCIB161 restored the mutant to a wild-type phenotype. Further subcloning of the 6.8-kb *Bam*HI-*Eco*RI fragment revealed that pCIB137, a pVK100 derivative containing a 2.0-kb *Xho*I fragment carrying only ORF5, fully complemented the pleiotropic mutant, restoring cyanide production, chitinase production, pyrrolnitrin biosynthesis, gelatinase production, wild-type colony morphology, and biocontrol activity. Figure 5, lanes 2 and 3, demonstrates the restoration of cyanide production by pCIB137 in BL915-2, a spontaneous pleiotropic mutant.

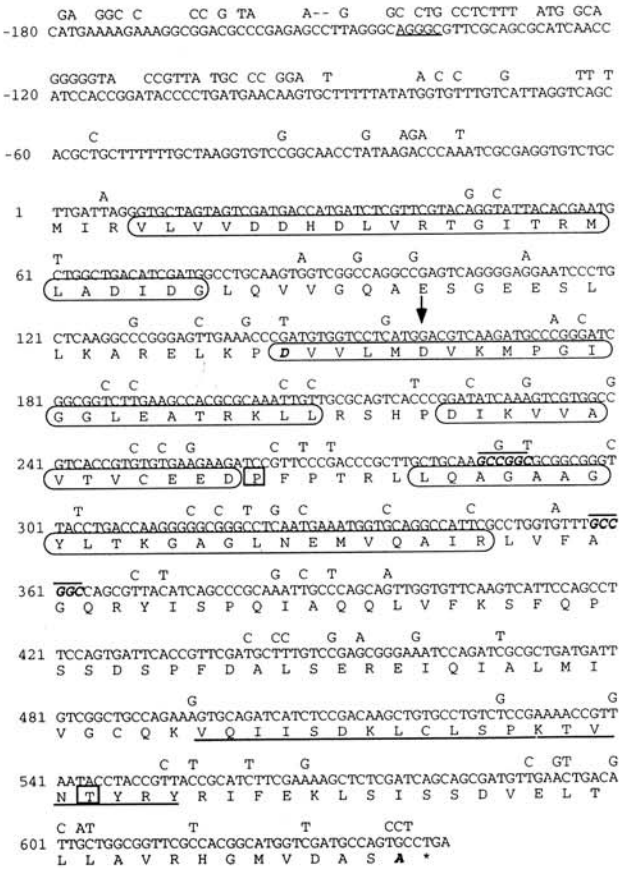


**Fig. 3.** Nucleotide sequence of ORF5 in the 11-kb *Eco*RI fragment from *Pseudomonas fluorescens* strain BL915. The deduced amino acid sequence of the putative ORF5 gene product is depicted below the nucleotide sequence. Nucleotide differences between ORF5 and the *gacA* gene of *P. fluorescens* strain CHA0 (Laville *et al.* 1992) are indicated above the ORF5 sequence. The only two predicted amino acid sequence differences between the ORF5 product and GacA are indicated in italics. Amino acid stretches corresponding to highly conserved phosphoryl receiver domains found in the regulator component of bacterial two-component systems (Albright *et al.* 1989) are enclosed in rounded-corner rectangles. The conserved aspartic acid residue presumed to be the phosphorylation site is indicated with an arrow. An amino acid stretch corresponding to a helix-turn-helix motif found in the C-terminal portion of FixJ class transcription activators (Kahn and Ditta 1991) is underlined. The two *Nae*I restriction sites (5′-GCCGGC-3′) used in generating a truncated version of ORF5 are italicized and overlined. The amino acids at positions 90 and 182 of the deduced amino acid sequence are boxed to indicate that these positions are affected by ORF5 mutations in the pleiotropic mutants BL915-1 and BL915-2, respectively. The 5-bp region approximately 140 bp upstream of ORF5 which is absent in the pleiotropic mutant BL915-3 is underlined.

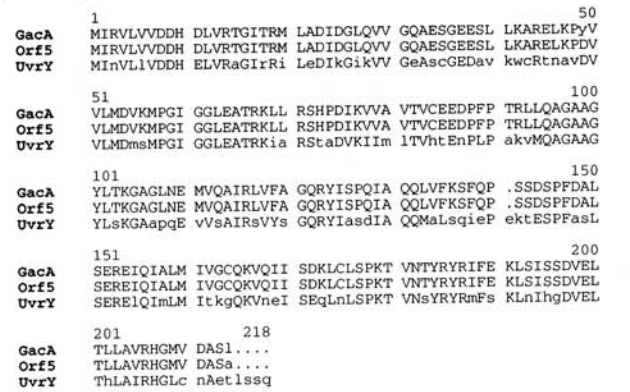


**Fig. 4.** Amino acid sequence alignment of GacA (Laville *et al.* 1992), ORF5, and UvrY (Sharma *et al.* 1986). Consensus amino acids and conservative replacements for consensus amino acids are indicated with uppercase letters.

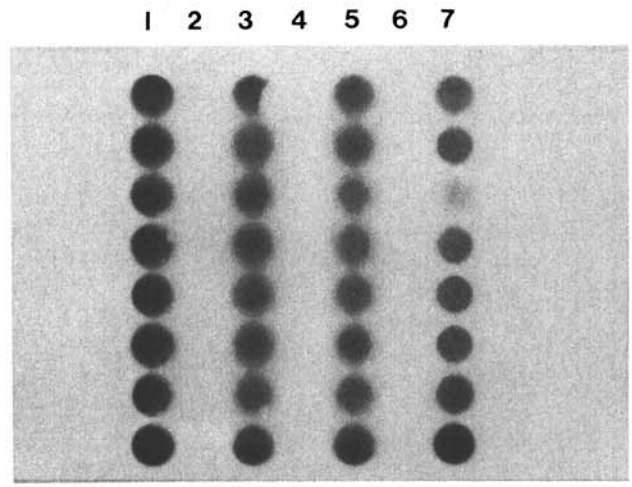


**Fig. 5.** Activation of cyanide production by pCIB137. Cyanide production by bacteria grown in 200 µl of Luria-Bertani broth in microtiter plate wells was detected by the method of Voisard *et al.* (1989), in which color development on reagent-soaked filter paper placed above the wells is a positive reaction indicating cyanide production. Eight individual colonies of each test strain were inoculated in vertical lanes of the microtiter plate. Lane 1, *Pseudomonas fluorescens* BL915; lane 2, *P. fluorescens* BL915-2, a spontaneous pleiotropic mutant; lane 3, *P. fluorescens* BL915-2-(pCIB137); lane 4, *P. fluorescens* BL915-4, a truncated chromosomal copy of ORF5; lane 5, *P. fluorescens* BL915-4(pCIB137); lane 6, *P. fluorescens* BL914; lane 7, *P. fluorescens* BL914(pCIB137).

Introduction of pCIB137 into the heterologous *P. fluorescens* strains BL914 and BL922 also activated the expression of latent genes in a pattern identical to that observed with the 11-kb *Eco*RI fragment (e.g., Fig. 5, lanes 6 and 7, for cyanide production by BL914). Introduction of ORF5 into strains BL914 and BL922 also conferred biocontrol activity upon them in a cotton-*Rhizoctonia* system (described in Materials and Methods), presumably as a result of antifungal gene activation. Data from representative biocontrol experiments are presented in Table 2.

An in-frame deletion was generated in ORF5 by cloning the 2.0-kb *Xho*I fragment into the *Xho*I site of pSP72, digesting with *Nae*I, and religating to accomplish the removal of a 72-bp *Nae*I fragment within ORF5 (see Fig. 3). Removal of this fragment is predicted to remove 24 amino acids from the ORF5 gene product, which should result in expression of a truncated protein. The approximately 1.9-kb *Xho*I fragment containing the truncated ORF5 was cloned into the *Xho*I site of pVK100 to generate pCIB149. pCIB149, which was identical to pCIB137 except for the 72-bp deletion in ORF5, was not capable of complementing the pleiotropic mutant. In addition, introduction of pCIB149 into *P. fluorescens* BL914 and BL922 failed to activate latent gene expression.

The truncated version of ORF5 was introduced into the genome of *P. fluorescens* BL915 by gene replacement as described in Materials and Methods. Most kanamycin-resistant colonies obtained after the introduction of pCIB156 (a pBR322-based replicon carrying the truncated ORF5) into strain BL915 resulted from homologous recombination between pCIB156 and the chromosome and contained the intact ORF5 as well as the truncated ORF5. Culture of one such transconjugant in the absence of kanamycin selection allowed identification of kanamycin-sensitive derivatives lacking the integrated pCIB156 and containing either intact ORF5 or the truncated version, as confirmed by Southern hybridization (data not shown). All derivatives containing the truncated version of ORF5 exhibited all the phenotypes associated with the pleiotropic mutants, while all derivatives containing intact ORF5 were indistinguishable from wild-type strain BL915. Introduction of pCIB137 into one such ORF5 deletion mutant, designated BL915-4, fully complemented the mutant, restoring chitinase and gelatinase activity, wild-type colony morphology, pyrrolnitrin and cyanide production, and biocontrol activity (e.g., Fig. 5, lanes 4 and 5, for cyanide production). In a biocontrol experiment in a cotton-*Rhizoctonia* system (described in Materials and Methods) with high disease pressure (i.e., only 28% final plant stand for the non-bacterized control versus a 93% stand for the no-pathogen control), the deletion mutant BL915-4 provided no biocontrol (18% final stand), while BL915-4 complemented with pCIB137 provided biocontrol equivalent to that provided by the wild-type strain BL915 (58 and 57% final stand, respectively).

**Table 2.** Effect of ORF5 of *Pseudomonas fluorescens* BL915 (carried on pCIB137) on biocontrol activity of *P. fluorescens* BL914 against *Rhizoctonia solani* on cotton

| Treatment | Disease rating[z] | |
|---|---|---|
| | Trial 1 | Trial 2 |
| BL914 | 3.57 a | 3.00 a |
| BL914 (pCIB137) | 1.65 b | 1.28 b |
| BL915 | 2.10 b | 1.40 b |
| No bacteria | 3.83 a | 2.95 a |
| Uninfested control | 2.32 b | 1.78 b |

[z] Each of the two trials consisted of 40 cotton seeds planted for each treatment. The values presented are mean ratings on the following scale: 1, no lesions; 2, minor lesions; 3, moderately severe lesions; 4, severe lesions with girdling of the stem; 5, dead plants and unemerged plants. Means within the same trial followed by the same letter are not significantly different at $P = 0.001$ according to Duncan's multiple-range test.

### DNA sequence analysis of ORF5 recovered from pleiotropic mutants.

*Xho*I fragments (2.0 kb) containing ORF5 were cloned from genomic DNA digests of three independently isolated spontaneous pleiotropic mutant derivatives of *P. fluorescens* BL915, designated strains BL915-1, BL915-2, and BL915-3, each of which could be complemented by the introduction of pCIB137. A single nucleotide change was observed in ORF5 of BL915-1, converting the CCG proline codon at position 90 (Fig. 3) of the deduced amino acid sequence to a CTG leucine codon. In ORF5 of BL915-2, the ACC threonine codon at position 182 (Fig. 3) was converted to an ATC isoleucine codon. In BL915-3, ORF5 itself was unaltered, but a 5-bp deletion was located 138 bp upstream from the ORF5 start codon (Fig. 3).

### Mutagenesis of ORF2.

Since ORF5, supplied *in trans,* proved to be sufficient to restore all lost functions to the pleiotropic mutants of *P. fluorescens* BL915 described above, we mutagenized the chromosomal copy of ORF2, a sensor kinase homolog, to determine whether ORF2 and ORF5 might be acting as a sensor-activator pair in a bacterial two-component system. A large in-frame deletion was created in ORF2 DNA by the removal of an approximately 800-bp *Cla*I restriction fragment. This deletion removed sequences encoding amino acid residues 50–313 of the putative sensor protein, including the predicted autophosphorylation site at His 210 of the kinase domain (Fig. 2). pCIB3346 was used as a vehicle for introducing the truncated version of ORF2 into the *P. fluorescens* BL915 chromosome by homologous recombination as described in Materials and Methods. Southern hybridizations were performed to identify BL915 derivatives in which a replacement of full-length ORF2 by the truncated version had occurred. Two such derivatives were selected for further analysis. Neither of these ORF2 deletion strains were deficient in any of the characteristics associated with the strain BL915 pleiotropic mutants described in Table 1. Since ORF2 shares some sequence homology with the *E. coli cheA* gene (Mutoh and Simon 1986), which, like the *uvrY-uvrC-pgsA-glyW* gene cluster, is located near *E. coli* map position 42, we examined the chemotactic behavior of the ORF2 deletion mutants on both LB agar and nutrient agar swarm plates. The behavior of the mutants was identical to that of wild-type strain BL915 on such plates (data not shown).

## DISCUSSION

The expression of a notably diverse set of genes is altered in pleiotropic mutants of the biological control bacterium *P. fluorescens* strain BL915, suggesting that these genes are

subject to global regulation. The majority of mutants of this strain identified on the basis of lacking activity of any single gene in this set have proven to be pleiotropic mutants. An 11-kb genomic DNA fragment, isolated from strain BL915 on the basis of its ability to restore antibiotic production to an antibiotic-deficient strain BL915 derivative (which subsequently proved to be a pleiotropic mutant), restored all of the lost or altered functions to the pleiotropic mutants described in this study. This finding, in conjunction with the ability of the 11-kb fragment to activate the expression of multiple genes in heterologous *Pseudomonas* strains, suggested that the fragment would contain at least one regulatory gene involved in a global regulatory network. The generation of spontaneous pleiotropic mutants of *P. fluorescens* BL915 is, in a general sense, similar to the phenotypic conversion phenomenon observed in the phytopathogen *Pseudomonas solanacearum* (Brumbley and Denny 1990), although regulatory genes identified in the strain BL915 genomic fragment are not strictly analogous to those identified thus far in the *P. solanacearum* system.

DNA sequence analysis of the 11-kb genomic fragment from strain BL915 led to the identification of five open reading frames and a tRNA gene (*glyW*). Strikingly, the gene cluster consisting of ORF5, ORF4, ORF3, and *glyW* corresponds, on the basis of DNA sequence homology, to the *E. coli* gene cluster at map position 42 of the chromosome, consisting of *uvrY, uvrC, pgsA,* and *glyW. uvrY,* which corresponds to ORF5 in this gene cluster, is a putative transcription activator gene of unknown function in *E. coli.* Comparison of ORF5 with sequences contained in the GenBank database revealed that the ORF5 gene product is nearly identical in amino acid sequence to GacA, a proposed transcriptional activator of genes involved in the synthesis of the antifungal factors cyanide and 2,4-diacetylphloroglucinol in the biocontrol strain *P. fluorescens* CHA0 (Laville *et al.* 1992). ORF2, another potential regulatory gene with homology to sensor kinase components of bacterial two-component systems, is also located within the 11-kb *Eco*RI fragment from strain BL915.

ORF5 is essential to the 11-kb BL915 genomic fragment's ability to complement the spontaneous pleiotropic mutants described here and to activate expression of latent genes in other *P. fluorescens* isolates. A 2.0-kb subclone of the 11-kb fragment containing only ORF5 fully complemented the mutants, while a derivative of this subclone carrying a truncated version of ORF5, generated by removing a 72-bp *Nae*I restriction fragment, was inactive. In addition, marker exchange of the truncated ORF5 into the *P. fluorescens* chromosome resulted in the generation of pleiotropic mutants indistinguishable from the original spontaneous mutants. Characterization of ORF5 recovered from spontaneous pleiotropic mutants in each case revealed nucleotide differences associated with this regulatory gene. One such mutant contained a nucleotide change predicted to convert Pro 90 of ORF5 to a leucine residue, presumably affecting protein conformation. A second mutant contained a nucleotide change predicted to convert Thr 182 of ORF5 to an isoleucine residue. Since Thr 182 lies within the predicted helix-turn-helix motif of the ORF5 transcription activator, a mutation affecting this region might impact the DNA binding ability of the protein. A third mutant had a 5-bp deletion 138 bp upstream from the ORF5 start codon, conceivably affecting transcription of the gene.

In contrast, our data do not indicate a role for ORF2 in the global regulatory network described here. A strain BL915 derivative with a large deletion in ORF2 was not altered with respect to any of the characteristics normally affected in biocontrol-deficient pleiotropic mutants. However, this finding does not rule out the possibility that ORF2 is a functionally redundant sensor involved in some aspect of biocontrol gene regulation. Nitrate regulation in *E. coli* K-12 involves functionally redundant sensors (Rabin and Stewart 1992), although such examples are extremely rare.

The predicted ORF5 gene product and the *P. fluorescens* CHA0 GacA protein differ in only two of 213 amino acids, despite the fact that the nucleotide sequences have diverged so that the two genes differ in 73 of 639 nucleotides. This may indicate that optimal function of the ORF5 and *gacA* gene products in *P. fluorescens* is compromised even by minor changes in amino acid sequence. Over the same stretch of 213 amino acids, the ORF5 gene product and UvrY from *E. coli* differ in 89 amino acid residues. A potentially interesting difference between the ORF5 product and GacA occurs at amino acid residue 49. GacA is reported to contain tyrosine at this position, while the predicted ORF5 product has an aspartic acid residue. Transcription activators of bacterial two-component regulatory systems are normally converted to an active form via phosphorylation by a sensor component upon receipt by the sensor of some environmental signal (Albright *et al.* 1989; Bourret *et al.* 1991; Stock *et al.* 1989). The ORF5 product and GacA each possess a typical conserved receiver domain, which presumably can be phosphorylated at a consensus aspartic acid residue at position 54. The presence of an additional charged residue within the phosphoryl receiver domain at a position nearby the consensus aspartic acid residue may have implications for the activity of the ORF5 product. For example, one type of constitutive mutant of the VirG transcription activator in *Agrobacterium tumefaciens* underwent a change from asparagine to aspartic acid near the consensus phosphorylation site of that molecule (Pazour *et al.* 1992).

Proteins with chitinase activity have been identified in numerous bacteria, including pseudomonads (Jones *et al.* 1986; Robbins *et al.* 1988; Roberts and Selitrennikoff 1988). We are unaware of any examples of bacterial chitinase expression being absolutely dependent upon a functional global regulatory system, as is apparently the case with the chitinase of *P. fluorescens* strain BL915. The finding that the strain BL915 regulatory region allowed expression of otherwise latent chitinase genes in certain heterologous *P. fluorescens* soil isolates indicates that additional chitinases are likely to be subject to this type of regulation.

The pleiotropic mutants derived from *P. fluorescens* BL915 are ineffective in biocontrol, and it is likely that many of the functions we have identified which are regulated by the global regulatory network described here will prove to play a role in fungal inhibition. However, the finding that the organization of the gene cluster to which ORF5 belongs is conserved in *E. coli,* in which the role of the ORF5 homolog *uvrY* is unknown, suggests a broader role for this class of transcription activator in regulating genes expressed during stationary phase. The identification of additional ORF5-regulated genes may extend the catalogue of genes associated

with this global regulon beyond those associated with biological control, and it may shed light on some aspects of the role of *uvrY* in *E. coli*. Likewise, in light of recent evidence that *uvrY* can activate biocontrol gene expression in *Pseudomonas* (Gaffney *et al.* 1993), elucidation of the *E. coli* regulatory network involving *uvrY* may ultimately reveal some common features between the *Pseudomonas* and *E. coli* regulons.

## MATERIALS AND METHODS

### Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used in this study are described in Table 3. *E. coli* strains were grown either in liquid LB medium (Bertani 1951) or on LB agar plates incubated at 37° C. *P. fluorescens* strains were grown at 28° C either on LB medium or on minimal medium consisting of 35 mM $K_2HPO_4 \cdot 3H_2O$, 22 mM $KH_2PO_4$, 8 mM $(NH_4)_2SO_4$, 25 mM sodium succinate, and 1.2 mM $MgSO_4$. When required for maintenance of plasmids, antibiotics were added at the following concentrations: ampicillin, 50 μg/ml; tetracycline, 15 μg/ml; and kanamycin, 50 μg/ml.

### Bacterial conjugation.

Plasmid DNA was introduced by conjugation from *E. coli* into *P. fluorescens* recipients by either the triparental mating procedure (Ditta *et al.* 1980) or a biparental procedure utilizing a donor host with *tra* genes integrated into the donor strain chromosome (Simon *et al.* 1983).

### DNA manipulations.

Plasmid DNA was purified on preparative columns supplied by Promega (Madison, WI). Restriction endonuclease digestions and DNA ligations were performed according to standard procedures (Maniatis *et al.* 1982). Biotinylated DNA probes for use in Southern hybridizations (Southern 1975) were prepared with a Flash labeling system supplied by Stratagene (La Jolla, CA).

Generation of an in-frame deletion in the chromosomal copy of ORF2 was accomplished as follows. An approximately 3.7-kb *Eco*RI-*Xba*I fragment containing ORF1 and ORF2 (Fig. 1) was subcloned in pUC19 (Yanisch-Perron *et al.* 1985). The resulting plasmid was digested with *Cla*I and religated. The loss of an approximately 800-bp fragment internal to ORF2 was detected in one resulting plasmid and confirmed by DNA sequencing. This plasmid was digested with *Xba*I and *Hin*dIII and ligated with an approximately 1.9-kb *Xba*I-*Hin*dIII fragment containing sequences adjacent to ORF2, to provide additional flanking sequence for the subsequent homologous recombination steps. This plasmid was designated pCIB3345. To introduce the truncated version of ORF2 into the *P. fluorescens* BL915 chromosome, pCIB3346 was generated, consisting of the approximately 4.8-kb *Eco*RI-*Hin*dIII fragment of pCIB3345, the approximately 3.7-kb *Eco*RI-*Sal*I portion of pBR322 (Bolivar *et al.* 1977), and an approximately 1.5-kb *Sal*I-*Hin*dIII fragment containing the kanamycin resistance gene of Tn*5* (Rothstein and Reznikoff 1981). pCIB3346, which cannot replicate in *Pseudomonas,* was mobilized by triparental mating into *P. fluorescens* BL915. Several resulting kanamycin-resistant colonies, containing both intact and truncated versions of ORF2 as a result of a single crossover event between pCIB3346 and the chromosome (confirmed by Southern hybridization), were grown without selection in LB broth. After two successive 24-hr subcultures, cells were plated on LB agar and screened for loss of kanamycin resistance. Kanamycin-sensitive colonies

**Table 3.** Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| *Pseudomonas fluorescens* | | |
| BL914 | Soil isolate | Texas |
| BL915 | Soil isolate | Texas |
| BL915-1 | Spontaneous pleiotropic mutant derivative of BL915 | This study |
| BL915-2 | Spontaneous pleiotropic mutant derivative of BL915 | This study |
| BL915-3 | Spontaneous pleiotropic mutant derivative of BL915 | This study |
| BL915-4 | BL915 derivative with truncated ORF5 | This study |
| BL922 | Soil isolate | Texas |
| *Escherichia coli* | | |
| HB101 | Restriction-minus; *recA* background | Maniatis *et al.* 1982 |
| DH5α | Provides α-complementation of cloning vector *lacZ* gene | Gibco BRL (Gaithersburg, MD) |
| S17-1 | *tra* genes for plasmid mobilization integrated in chromosome | Simon *et al.* 1983 |
| Plasmids | | |
| pPRN-E11 | 11-kb genomic *P. fluorescens* BL915 *Eco*RI fragment cloned in pRK290 | Hill *et al.*, in press |
| pRK2013 | $Km^r$; *tra* genes mobilize broad-host-range plasmids | Ditta *et al.* 1980 |
| pBR322 | $Ap^r$, $Tc^r$ *E. coli* cloning vector | Bolivar *et al.* 1977 |
| pUC19 | $Ap^r$ *E. coli* cloning vector | Yanisch-Perron *et al.* 1985 |
| pLAFR3 | $Tc^r$ broad-host-range cloning vector | Staskawicz *et al.* 1987 |
| pSP72 | $Ap^r$ *E. coli* cloning vector | Promega (Madison, WI) |
| pVK100 | $Km^r$, $Tc^r$ broad-host-range cloning vector | Knauf and Nester 1982 |
| pCIB137 | 2.0-kb *Xho*I fragment containing ORF5 cloned in pVK100 | This study |
| pCIB149 | 1.9-kb *Xho*I fragment containing truncated ORF5 in pVK100 | This study |
| pCIB150 | 1.9-kb *Xho*I fragment containing truncated ORF5 in pSP72 | This study |
| pCIB156 | Truncated version of ORF5 cloned in $Km^r$ pBR322 derivative | This study |
| pCIB161 | 6.8-kb *Eco*RI-*Bam*HI pPRN-E11 fragment cloned in pLAFR3 | This study |
| pCIB3344 | 3.7-kb *Eco*RI-*Xba*I pPRN-E11 fragment cloned in pLAFR3 | This study |
| pCIB3345 | Truncated version of ORF2 cloned in pUC19 | This study |
| pCIB3346 | Truncated version of ORF2 cloned in $Km^r$ pBR322 derivative | This study |

[a] $Ap^r$, ampicillin resistance; $Km^r$, kanamycin resistance; $Tc^r$, tetracycline resistance.

represented approximately 0.4% of the total colonies examined in this experiment. Southern hybridization with a probe containing ORF2 was performed on genomic *Eco*RI digests of DNA recovered from kanamycin-sensitive clones to confirm the presence of the truncated version of ORF2 in the chromosome.

The same strategy was used to obtain a derivative of *P. fluorescens* BL915 containing a truncated chromosomal version of ORF5. A 2-kb *Xho*I fragment containing ORF5 was cloned into the *Xho*I site of pSP72 (Promega). The resulting plasmid was digested with *Nae*I and religated in order to generate a 72-bp in-frame deletion within ORF5. Recovery of a plasmid, pCIB150, with the appropriate deletion was confirmed by Southern hybridization and DNA sequencing. To introduce the truncated version of ORF5 into the *P. fluorescens* BL915 chromosome, pCIB156 was generated, consisting of the approximately 3.7-kb *Eco*RI-*Sal*I portion of pBR322, an approximately 1.5-kb *Sal*I-*Hin*dIII fragment containing the kanamycin resistance gene of Tn*5*, and an approximately 5-kb *P. fluorescens* BL915 *Hin*dIII-*Eco*RI fragment in which an internal 2-kb *Xho*I fragment containing ORF5 had been replaced with the 1.9-kb *Xho*I fragment of pCIB150 containing the truncated version of ORF5. pCIB156 was transformed into *E. coli* strain S17-1 and mobilized by conjugation into *P. fluorescens* BL915 with selection for kanamycin resistance. Marker exchange was accomplished as described above for ORF2.

**DNA sequencing.**

Portions of the 11-kb *Eco*RI fragment contained in pPRN-E11 were subcloned into pBluescript SK II+ (Stratagene) and sequenced by the dideoxy chain termination method (Sanger *et al.* 1977) using double-stranded DNA templates with either commercially available –20 and reverse sequencing primers (New England Biolabs, Beverly, MA) or oligonucleotide primers synthesized by β-cyanoethylphosphoramidite chemistry on an Applied Biosystems 380A DNA synthesizer. Sequence analysis and comparisons with sequences contained in databases was accomplished with software from the Genetics Computer Group (Devereux *et al.* 1984). The same approach was used to sequence DNA fragments containing ORF5 recovered from *P. fluorescens* BL915 pleiotropic mutants. The nucleotide sequence of a contiguous region including all of the open reading frames described here has been submitted to GenBank and assigned accession number L29642.

**Cyanide assay.**

Cyanide production by bacterial cultures grown in LB broth in microtiter plate wells was detected by the method described by Voisard *et al.* (1989).

**Chitinase assay.**

Chitinase activity was detected essentially as described by Robbins *et al.* (1988), with the substrate 4-methylumbelliferyl β-D-N,N′-diacetylchitobioside. Bacterial cultures were grown in LB broth in microtiter plate wells and subjected to a freeze-thaw step before addition to the assay. Chitinase activity was also detected in cell extracts by the use of a tritiated chitin substrate (New England Nuclear) essentially as described by Molano *et al.* (1977).

**Pyrrolnitrin determination.**

High-pressure liquid chromatography and thin-layer chromatography were used to detect pyrrolnitrin synthesized by bacterial cultures essentially as described by van Pée *et al.* (1983).

**Gelatinase assay.**

Gelatinase activity was determined by measuring the diameters of zones of clearing surrounding colonies growing on nutrient agar supplemented with 3.0% gelatin.

***In vitro* fungal growth inhibition assay.**

Bacterial strains were tested for their ability to inhibit growth of *R. solani* on LB agar plates by streaking the bacterial isolate across the diameter of a plate and placing a small agar plug containing *R. solani* hyphae at an edge of the plate (i.e., at a position farthest away from the bacterial growth). The plates were incubated at 28° C for approximately 3 days, and fungal growth toward the bacterial streak was monitored.

**Biological disease control assays.**

Cotton seeds (cultivar Coker 310, mechanically delinted) were added to overnight cultures of *P. fluorescens* which had been diluted to an optical density of 0.2 at 600 nm. The seeds were allowed to soak for 10 min. They were then planted, at a depth of approximately 2.5 cm, in pots (one seed per pot), each containing 180 ml of potting mixture infested with *R. solani*. This potting mixture was prepared by autoclaving a 1:1 mix of sand and vermiculite for 70 min at 250° F (121° C) on each of two consecutive days. Millet seed infested with *R. solani* was then added in a large drum mixer at the rate of 5 mg per 180 ml of the potting mixture. Distilled water was also added to the mix at the rate of 50 ml per 180 ml of the potting mixture. Pots containing the bacterized seeds were placed in a phytotron, and growth conditions were set at 27 and 21° C for daytime and nighttime temperatures, respectively, 70% humidity, and 14 hr of light per 24-hr period. The plants were watered on day 7 by adding 15 ml of distilled water to each pot. The cotton seedlings were removed from the pots on day 12, and the crown area of each plant was observed for evidence of brown lesions characteristic of post-emergence damping-off disease caused by *R. solani*. Numerical disease severity ratings were assigned as follows: 1, no lesions; 2, minor lesions; 3, moderately severe lesions; 4, severe lesions with girdling of the stem; 5, dead plants and unemerged plants. Disease severity data in each experiment were analyzed according to Duncan's multiple-range test (SAS Institute, Cary, NC). Alternatively, biocontrol data were expressed in some experiments as the ratio of the number of plants standing at the end of the experiment to the number of seeds planted for each treatment. A minimum of 40 seeds were planted for each treatment in all biocontrol experiments.

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