8. JENDL-3.2, The Japanese Evaluated Nuclear Data Library by the JAERI Nuclear Data Centre and the Japanese Nuclear Data Committee, Summary of Contents, Lemmel, H. D. (ed.) IAEA-NDS-110, Rev. 5 August 1994, IAEA Nuclear Data Section, Vienna, Austria.
9. Rubbia, C., Buono, S., Kadi, Y. and Rubio, J. A., Report No. CERN/LHC/97-01 (EET), 17 February 1997.
10. Clayton, E. D., Anomalies of Nuclear Criticality, PNL-SA-4868, Rev. 5, Pacific Northwest Laboratory, 1979.
11. Lathrop, K. D., DTF-IV: A Fortran Program for Solving Multigroup Transport Equation with Anisotropic Scattering, LA-3373, 1965.
12. Garg, S. B. and Sinha, A., Report No. BARC-1222, Bhabha Atomic Research Centre, 1984.
13. Sinha, A. *et al.*, Report No. NDE (BARC)-7, Bhabha Atomic Research Centre, 1987.
14. American Nuclear Society, American National Standard Report ANSI/ANS-8.15-1981, 1982.
15. Wu, G. C. and Ruby, L., *Nucl. Sci. Eng.*, 1978, **68**, 349–368.
16. Dunford, C. L. and Burrows, T. W., IAEA-NDS-150 Rev. 98/9, September 1998, Nuclear Data Section, IAEA, Vienna, Austria. See the current newsletter of the IAEA Nuclear Data Section at Web-site: http:\\www-nds.iaea.org
17. McLane, V., Dunford, C. L. and Rose, P. F., *ENDF-6 Formats Manual*, US National Nuclear Data Centre, Brookhaven National Laboratory, Upton, NY, USA, Available as report IAEA-NDS-76, Rev. 5, June 1997, Vienna, Austria.
18. Shibata, K. *et al.*, Report JAERI-1319, 1990, Japan Atomic Energy Research Institute, Japan.
19. Ganesan, S. and MacLaughlin, P. K., Report INDC (NDS)-256, Nuclear Data Section, IAEA, Vienna, Austria.
20. Halsall, M. J. and Taubmann, C. J., The 1981 WIMS Nuclear data Library, AEEW-R1442, 1983.
21. MacFarlane, R. E., The NJOY Nuclear Data Processing Code System, Version 91, LA-12740-M, 1994. The 1997 version of this code available at the IAEA-NDS was run at the IAEA Nuclear Data Section.
22. Rose, P. F., ENDF-201-ENDF/B-VI Summary Documentation, US National Nuclear Data Centre, Brookhaven National Laboratory, Upton, NY, USA. See also, Lemmel, H. D. (ed.), Report IAEA-NDS-107, Rev. 11 March 1996, Vienna, Austria.
23. Krishnani, P. D. and Huria, H. C., Report BARC-1313, 81, 1986.
24. Huria, H. C., *Ann. Nucl. Energy*, 1981, **8**, 183–198.
25. Jagannathan, V. and Jain, R. P., Simplified input to MURLI code for analysis of uniform lattice experiments, unpublished, 1998.
26. Breismeister, J. F. (ed.), MCNP-A General Monte Carlo Code for Neutron Photon Transport, LA7396-M, 1986.
27. Bois, R. and Frehaut, J., Report CEA-R-4791, Bruyeres-Le-Chatel, France, 1976. The 'nubar' evaluation for JENDL-3.2 is described in Ohsawa, T., Proc. Int. Conf. Nucl. Data for Science and Tech., Mito, p. 565, Japan, 30 May–3 June 1988.
28. Howerton, R. J., *Nucl. Sci. Engg.*, 1977, **62**, 438.

ACKNOWLEDGEMENT. This work was partially supported by a Research Contract (No. 10612/R0) of the International Atomic Energy Agency within the scope of an IAEA Co-ordinated Research Programme entitled, 'Final Stage of the WIMS Library Update Project'. We thank P. K. MacLaughlin and D. W. Muir, IAEA Nuclear Data Section for their collaboration on the NJOY97 runs.

Received 23 December 1998; revised accepted 2 July 1999

Molecular basis of antifungal toxin production by fluorescent *Pseudomonas* sp. strain EM 85 – A biological control agent

K. N. Anith*,†, K. V. B. R. Tilak and S. P. S. Khanuja‡**

†Regional Agricultural Research Station, Ambalavayal, Wayanad 673 593, India
**Division of Microbiology, Indian Agricultural Research Institute, Pusa, New Delhi 110 012, India
‡Biotechnology and Genetic Research Division, Central Institute of Medicinal and Aromatic Plants, Lucknow 226 015, India

A fluorescent *Pseudomonas* sp. (strain EM 85) was found to inhibit growth of many soil-borne plant pathogenic fungi and effectively control fungal diseases. The genetics governing this character was probed by mutagenesis followed by a functional complementation analysis after construction of a genomic library of the wild-type strain using the cosmid vector pLAFR 1. A cosmid clone, pANF 17 was able to complement the antifungal toxin production in a defective mutant. The chromosomal origin of the DNA fragment in the cosmid clone was confirmed by a Southern blot. Sub-clones of the three *Eco*RI fragments from pANF 17, however, failed in the complementation test. The complementing cosmid was found to be stably maintained and expressed in the defective mutant under laboratory plate assay and *in vivo* conditions as evidenced by the extraction and detection of the toxin and biological control experiment.

RHIZOSPHERE-competent bacteria and fungi are the most preferred candidates as biological control agents. Soil pseudomonads are important among them as they colonize the underground growing plant organs efficiently and survive in a variety of diverse conditions[1]. The biological activities by which these pseudomonads bring about disease control include rhizosphere colonization, antibiosis, iron chelation by siderophore production, production of volatile compounds, induction of systemic resistance and competition for nutrients[2].

*For correspondence.

Genetic improvement as well as modification of these biocontrol strains for improved efficiency and consistency of performance in different conditions require a thorough understanding of the mechanism of biocontrol at the molecular level. Complementation of specific mutants defective in antifungal property, generated either through transposon or NTG mutagenesis have been employed for identification of gene(s) responsible for antifungal toxin biosynthesis in *Pseudomonas* spp. by many workers[3–8].

Pseudomonas sp. strain EM 85 was isolated from maize rhizosphere, at the Division of Microbiology, IARI, New Delhi, and is reported to have biological control property against charcoal rot, foot rot and collar rot of maize caused by *Macrophomina phaseolina*, *Fusarium graminearum* and *F. moniliforme* respectively[9]. The strain also controlled damping-off of cotton caused by *Pythium ultimum* and *Rhizoctonia solani*. It is also reported that this isolate possesses multiple antifungal properties and produces siderophores, HCN, fluorescent pigments and ammonia[9]. Possession of multiple antifungal properties makes any biological control agent a better candidate for efficient field performance. However, understanding the exact mechanism of fungal growth inhibition is essential to improve the strain through genetic manipulation. Here, an attempt is made to understand the molecular basis of antifungal property of the fluorescent *Pseudomonas* sp. strain EM 85 through mutagenesis and functional complementation.

Materials and methods

Media, culture conditions, bacterial strains, fungal cultures and plasmids

Bacterial strains were grown in Luria-Bertani (LB)/ Agar (LA) at 30 or 37°C. Antibiotics were added into the medium as and when required, at the following concentrations: ampicillin, 50 mg; tetracycline, 10 mg; streptomycin, 100 mg; kanamycin 50 mg per one litre of the medium. Fungi were grown on potato dextrose agar (PDA). The bacterial strains, fungal cultures, plasmids and their derivatives used in this study are described in Table 1.

Identification of the biological control bacteria

The taxonomic position of the bacterial strain was identified by carrying out biochemical tests for bacterial identification[10]. The GC content of the total DNA of the bacterial strain was found by observing the melting temperature of the DNA[11].

Screening of Pseudomonas strain EM 85 for antagonism against plant pathogenic fungi

Screening was done on PDA medium according to the method of Carruthers *et al.*[7] Actively growing *Pseudo-*

Table 1. Bacterial strains, fungal cultures, plasmids and plasmid derivatives used

Organisms	Relevent character	Reference
Bacterial strains		
Escherichia coli		
HB 101	F^-, Pro, Leu, thi, LacY, recA, LamB, Str^r	25
DH5α	F^-, lacZAM15, hsdR1, rec A 1, gyr A 96, thi-1, rel A	12
Pseudomonas		
Strain EM 85	Antifungal toxin producing (Aft^+), Amp^r	9
Strain AN 21	NTG derivative of EM 85 (Aft^-), Amp^r	This study
Plasmids		
pLAFR 1	Wide host range cosmid vector, Tet^r, Ori (RK 2), Tra^-	26
pRK 2013	Kan^r, Tra^+, mobilizing plasmid	27
pKT 230	RSF 1010 derivative Km^r Str^r	21
pANF 17	pLAFR 1 cosmid clone from EM 85 gene bank complementing AN 21 mutant phenotype	This study
pANF 17–1	pKT 230 carrying 11.5 kb *Eco*RI fragment from pANF 17	This study
pANF 17–2	pKT 230 carrying 9 kb *Eco*RI fragment from pANF 17	This study
pANF 17–3	pKT 230 carrying 3 kb *Eco*RI fragment from pANF 17	This study
Fungal cultures (All virulent strains obtained from ITCC, New Delhi)		
Rhizoctonia solani		
Fusarium oxysporum f. sp. *ciceri*		
Fusarium moniliforme		
Fusarium solani		
Macrophomina phaseolina		

monas EM 85 culture was spotted using a loop on the edge of a PDA plate. A fungal agar disc of 5 mm diameter was cut out from an actively growing fungal culture grown on PDA and kept at the centre of the PDA plate inoculated with bacterial culture. Plates were incubated for two to five days and observed for zone of fungal growth inhibition around the bacterial streak.

Chemical mutagenesis of Pseudomonas strain EM 85 and screening for defective mutants

Pseudomonas strain EM 85 was incubated in 10 ml LB to log phase. To this NTG @ 100 µg/ml was added and incubated at 30°C for 2 h while shaking. The cells were washed thrice with sterile water and resuspended in 10 ml LB. Mutations were fixed by incubating the broth overnight and the mutant bank was stored at – 70°C after addition of glycerol to a final concentration of 25%. *R. solani* and single colonies of *Pseudomonas* from the mutant bank were co-inoculated on PDA plates for screening defective mutants. Wild-type strain served as a control in the screening tests.

Construction of genomic library of the wild-type Pseudomonas strain EM 85

Plasmid extraction, restriction digestion, agarose gel electrophoresis, preparation and transformation of competent cells, etc. were done according to Sambrook *et al.*[12], unless otherwise mentioned. Total genomic DNA of strain EM 85 was isolated from four 1 ml log phase culture aliquots using Wizard™ genomic DNA isolation kit (Promega Corporation, Madison, USA). One mg each of genomic DNA was restricted with varying concentrations of *Eco*RI (0.125, 0.25, 0.5 and 1 unit) in a total reaction volume of 20 µl, for 1 h at 37°C to find out the enzyme dose which can produce maximum amount of DNA having 17–23 kb size. Restriction digestion was stopped by addition of 10 mM EDTA, followed by heat inactivation at 65°C for 15 min. After standardizing the dose of the enzyme, 200 µg of genomic DNA was restricted with appropriate quantity of *Eco*RI. The partially restricted genomic DNA was carefully layered over a 10–40% sucrose density gradient in a 38 ml polypropylene tube. It was subjected to centrifugation in a Beckman SW 28 rotor for 24 h at 24,000 rpm at 20°C in a Beckman ultracentrifuge. The gradient tube was punctured at the bottom and fractions of 500 µl were collected. Fractions containing DNA fragments of 17–23 kb were pooled after analysing the fractions on a 0.4% agarose gel and subjected to dialysis for 24 h against $T_{10}E_1$ buffer at room temperature with seven changes of buffer. The dialysed DNA was then extracted with butanol and ethanol precipitated and dissolved in 200 µl of TE buffer.

Cosmid vector pLAFR1 was restricted to completion with *Eco*RI and dephosphorylated with calf intestine alkaline phosphatase (CIAP). Two µg each of genomic DNA fragments and linearized, dephosphorylated vector DNA were allowed to ligate at 16°C for 18 h. *Giga Pack II* (Stratagene) was used for packaging cosmid clones in λ heads and the instructions given by the manufacturer were followed. *E. coli* HB 101 strain was incubated for 16 h at 37°C in 50 ml of LB containing 10 mM $MgSO_4$ and 0.2% (w/v) maltose. The cells were harvested in 10 mM $MgSO_4$ and the OD of the suspension adjusted to 0.5 with the same. A 1 : 1 dilution of the λ phage mixture was made with SM buffer. 100 µl each of the diluted phage and HB 101 cell suspension were mixed and incubated at room temperature for 30 min. 200 µl of LB was added into this and incubated at 37°C for 1 h. The mixture was pelletted and resuspended in 50 µl of fresh LB. The suspension was serially diluted and spread plated onto LB plates containing tetracycline. Colonies were pooled after incubation, in small amounts of LB and stored at – 70°C after addition of DMSO @ 88 µl/ml.

Screening of genomic library for cosmid clones complementing Aft⁻ phenotype

Cosmid clones were conjugally mobilized into the Aft⁻ mutant by triparental mating. Individual colonies of AN 21 and HB 101 (pRK 2013) were incubated in 5 ml each of LB containing antibiotics. Cells from 1 ml culture of AN 21 and HB 101 (pRK 2013) were harvested, washed twice with fresh LB, and dissolved in 0.5 ml LB. 100 µl of genomic library stock was spotted onto LA Tet plates and incubated overnight. The patch was scrapped and dissolved in 0.5 ml LB and washed once with fresh LB. The mutant, helper and the genomic library were mixed well in a ratio of 1 : 1 : 1 and spotted on a fresh plain LA plate and incubated for 16 h at 37°C. Different dilutions of the conjugal mixture were plated on LA plates, containing ampicillin and tetracycline. The transconjugants of AN 21 coming up after 24 h of incubation were individually checked for production of antifungal toxin against *R. solani* on PDA.

Isolation of cosmid DNA complementing Aft⁻ phenotype and sub-cloning

Total plasmid was extracted from transconjugant of AN 21 exhibiting Aft⁺ activity by alkaline lysis plasmid *miniprep* method[12]. This was used to transform competent cells of DH5α and the transformants were selected on LA Tet plates. A few transformed colonies were picked up and the plasmids in them were individually mobilized into the mutant strain AN 21 by triparental mating, as described earlier. Individual transconjugants were again

checked for antifungal toxin production against *R. solani*. Plasmid isolation was carried out from the tranformants of DH5α containing the cosmid carrying *aft*$^+$ DNA fragment. For sub-cloning, pANF 17 was restricted to completion with *Eco*RI and subjected to electrophoresis on a 0.75% low melting point agarose under cold room condition. The three genomic DNA fragments of molecular weight 11.5, 9 and 3 kb were eluted separately using DNA cleaning kit (Bangalore Genei (P) Ltd). Plasmid vector pKT 230 was used for sub-cloning. Isolation of plasmid, linearization with *Eco*RI, dephosphorylation of the vector and ligation reaction were performed according to Sambrook *et al.*[12] and the procedure described earlier.

Southern hybridization

1 µg each of total genomic DNA and the native plasmid DNA of the wild-type strain *Pseudomonas* EM 85 was electrophoresed on a 0.4% agarose gel. The DNA in the gel was depurinated in 0.2 N HCl for 10 min and then denatured with several volumes of 0.2 N NaOH and 0.6 M NaCl for 20 min with gentle shaking. The gel was then soaked in 25 mM sodium phosphate buffer, pH 6.8 as described by Bittner *et al.*[13]. The denatured DNA was transferred to nylon membrane in LKB 2005 transphor unit (Pharmacia, UK). The nylon membrane was air dried and baked in vacuum at 80°C for 2 h using a gel drier and prehybridized at 65°C in a hybridization oven (Amersham Life Sciences, UK) in 25 ml of prehybridization buffer with constant rotation for 6 h. The 11.5 kb *Eco*RI fragment from pANF 17 was eluted and radiolabelled with [α^{32}P]dCTP using the labelling kit supplied by Promega Corporation, USA, as per the instructions given by the manufacturer. The labelled probe DNA was added to the prehybridization buffer and hybridization was carried out at 65°C with constant rotation for 18 h. The membrane was washed under stringent conditions, i.e. thrice with solutions containing 2 × SSC and 0.2% SDS at 65°C. The membrane was exposed to X-ray film in a cassette for 3 days at – 70°C.

Extraction of antifungal compound from Pseudomonas strain EM 85 and derivatives

Antifungal toxin was extracted according to the method given by Levy and Eyal[14] with slight modification. Ten PDA plates each were heavily inoculated with EM 85, AN 21 and AN 21 (pANF 17) by cross streaking on all directions. After incubation, agar was scrapped from plates, made into small pieces, put inside a 1 l flask and 2 volumes of acetone was added. After shaking for a period of 24 h, the contents were passed through muslin cloth and centrifuged. The supernatant was collected and acetone evaporated in a rotary evaporator. The aqueous fraction was extracted twice with diethyl ether and the ether fraction evaporated to dryness. The dried material was dissolved in 1 ml of acetonitrile and used for bioassay against *R. solani* on PDA. Wells of 10 mm diameter were made towards the edge in PDA plates by removing agar disc from the medium using a cork borer. The wells were then partially sealed with molten soft agar. When the agar solidified, 100 µl of crude antifungal toxin was put carefully into the well and allowed to percolate. Bioassay against *R. solani* was performed by co-inoculating agar disc of *R. solani* growth on the centre of the plate. Growth inhibition was observed after 2 days of incubation.

In vivo biological control activity

The ability of the *Pseudomonas* strains EM85 (Aft$^+$), AN 21 (Aft$^-$) and AN 21 carrying pANF 17 (Aft$^+$) to control *R. solani* induced damping-off of cotton was assessed. A completely randomized design was employed. The treatments were (i) *R. solani* alone, (ii) *R. solani* + EM 85, (iii) *R. solani* + AN 21, (iv) *R. solani* + AN 21 (pANF 17), and (v) No inoculation.

Pots of 12 inch diameter were filled with soil @ 12 kg/pot. One week prior to sowing, fungal inoculum @ 100 g/pot was applied to the upper 10 cm of the pots. Fungal inoculum was prepared in sand, maize powder mixture (9 : 1). Bacterial inoculum was prepared in LB. Seeds were soaked in water for 10 h prior to sowing. 10 seeds/pots were sown. Four replications were kept in each treatment. 3 ml of bacterial inoculum was added to each seed after sowing and covered with soil. Observations on damping-off were made one week after sowing. The data were statistically analysed[15].

Results and discussion

The results of the various biochemical, morphological and physiological tests showed that the strain belongs to the genus *Pseudomonas* and falls in the fluorescence group. The organism was gram negative, motile rod, having positive reactions for fluorescent pigment production, oxidase, catalase, urease and negative reactions for ONPG, arginine hydrolase, indole, MR and VP. The strain was able to utilize carbohydrates like glucose, galactose, arabinose, xylose, fructose, sucrose, trehalose, mannose and unable to use rhamnose, ribose, lactose and inulin. The T_m value of the DNA was found to be 82 ± 1°C and the GC content of the total genomic DNA was 68.6 mole%, which falls under the GC content range of the genus *Pseudomonas*[16].

Inhibition of fungal growth on a nutritionally rich medium like PDA by the bacterial strain is an indication of strong antagonistic property. The strain EM 85 was found to inhibit the growth of many soil-borne fungi under PDA plate assay (Table 2). For further studies, *R.*

solani was selected due to its faster growth on PDA medium. The phenotype of the wild-type strain was designated as Aft$^+$ (Antifungal toxin positive) with respect to the fungal growth inhibition.

Incubation with nitrosoguanidine @ 100 µg/ml of the *Pseudomonas* EM 85 culture for 2 h was found to give a percentage killing of 99.42 and hence probability of finding a particular type of mutant in the surviving population was high. Though nitrosoguanidine induces multiple notoriously close-linked mutations[17], it has been used for generation of bacterial mutants in genetic experiments[3,6]. On screening the mutant bank, several colonies showed varying degrees of fungal growth inhibition as measured by the distance between the bacterial streak and the growing edge of the fungus. A colony was found to be completely defective in antifungal toxin production and was designated as AN 21. This mutant was subjected to detailed analysis for finding out the nature of the mutation, and has been found to be isogenic to the wild-type strain in all characters examined, like colony morphology, cell shape and size, growth rate, production of siderophores, fluorescent pigments, HCN and ammonia, except for fungal growth inhibition[18]. It was found that the fungus grew over the mutant bacterial streak after two days of incubation, whereas the wild-type strain showed a clear zone of inhibition around the colony even after one week of incubation (Figure 1). This showed that mutation affected the antifungal toxin biosynthesis gene(s) in AN 21 and the same was selected for further genetic analysis.

Construction of gene banks employing cosmid cloning is advantageous because concatamers of foreign DNA having 37–52 kb size, i.e. 75 to 105% the size of the lambda DNA can be packaged into λ phage heads. Thus, the total genomic library can be represented in lesser number of independent cosmid clones[19]. Wide range cosmid vector pLAFR 1 was used here for the construction of the genomic library since the wild-type and the mutant *Pseudomonas* strains were susceptible to tetracycline, a marker possessed by the comid vector, enabling easy selection. Out of the four different enzyme concentrations used for partial restriction of the total genomic DNA, 0.25 units of enzyme/µg of the DNA was found to yield maximum amount of fragments of size 17–23 kb. Packaging of the ligated vector-insert molecule in λ heads and subsequent transfection of the phage to *E. coli* HB 101 resulted in the formation of around 25,000 bacterial colonies. Plasmid isolation and restriction analysis of eight randomly picked clones showed presence of insert fragments in all of them. The average insert size has been calculated as 26.06 kb (Table 3). Assuming that the EM 85 genome is similar to *E. coli* genome (approximately 4000 kb), the number of clones to be screened to get a specific DNA fragment of interest in the constructed genomic library with 99% probability was found to be 704.5 or around 705 colonies[20].

Mobilization of individual gene bank clones separately into the mutant requires lot of manpower as well as material. So *en masse* mobilization of the genomic library into AN 21 was performed for complementation experiments. A colony of AN 21 carrying a cosmid clone was thus obtained which possessed antifungal toxin

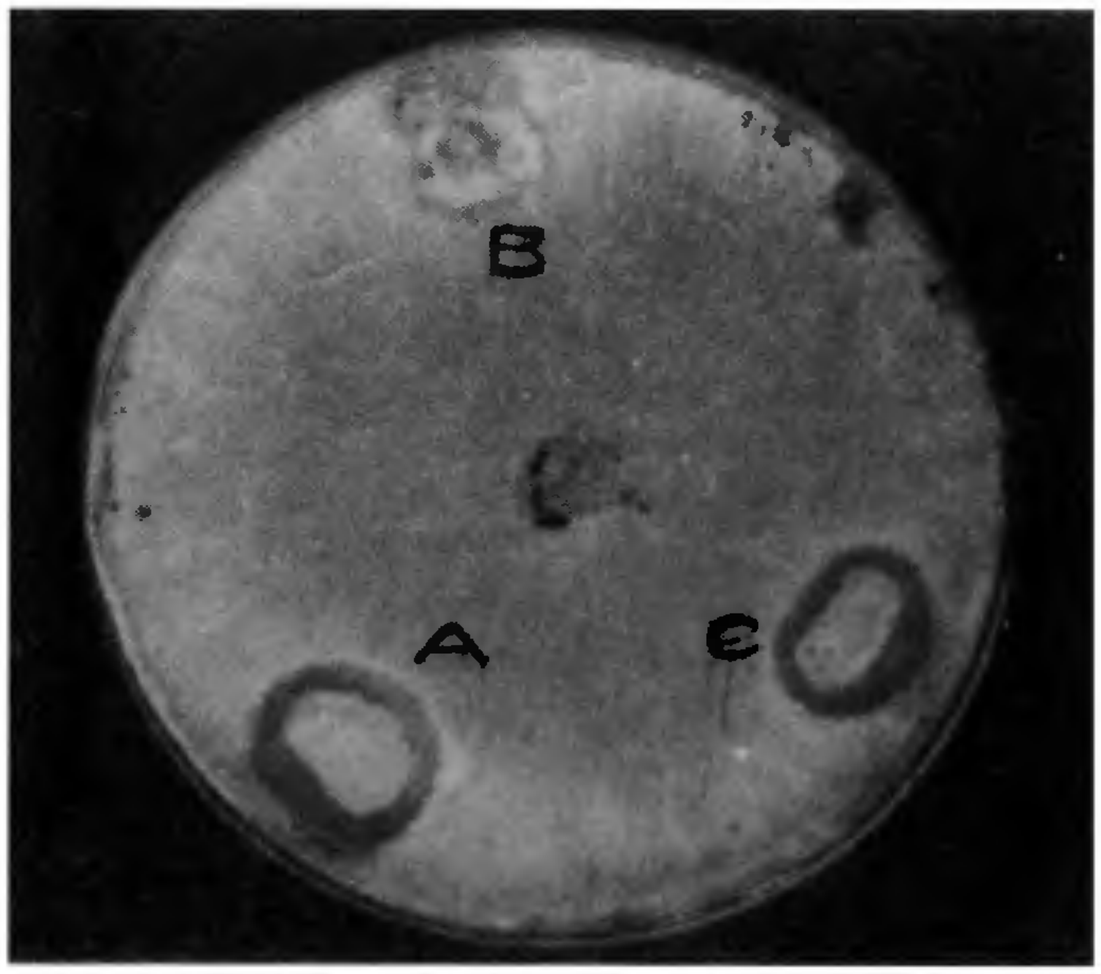

Figure 1. Growth inhibition of *Rhizoctonia solani* by *Pseudomonas*. A, Wild-type strain EM 85; B, Mutant strain AN 21; C, Mutant strain AN 21 carrying complementing cosmid pANF 17.

Table 2. Distance of inhibition zone on phytopathogenic fungi by strain EM 85

Fungal culture	Distance of inhibition (mm)* (from the growing edge of the fungus)
Rhizoctonia solani	7.0
Fusarium oxysporum f. sp. *ciceri*	6.5
Fusarium moniliforme	6.5
Fusarium solani	6.0
Macrophomina phaseolina	5.5

*Average of 10 independent measurements.

Table 3. DNA insertion in randomly picked cosmid clones from the genomic library

Clone no.	No. of DNA fragments	Total molecular weight (kb)
1	4	32.86
2	2	25.74
3	2	24.53
4	2	25.05
5	2	26.06
6	3	19.78
7	2	28.72
8	2	26.06
Average		26.06

production. The complementing clone was named as pANF 17. A new approach was used to isolate the cosmid clone responsible for complementing the defective character. Total plasmid from the transconjugant of AN 21 showing Aft$^+$ phenotype, i.e. AN 21 carrying the *aft*$^+$ cosmid clone, was isolated, used for transformation of competent cells of *E. coli* DH5α and selected on LA Tet plates. It was made sure that the cosmid clone was brought back to the *E. coli* background. It was important to prove that the restoration of Aft$^+$ activity by the transconjugant of AN 21 was not by any reversion, but by the complementation of the character by the cosmid clone inside. Hence, the complemented cosmid clone pANF 17 in *E. coli* background was again conjugally mobilized into the Aft$^-$ phenotype AN 21 and the transconjugant was tested for production of antifungal toxin against *R. solani*. The suppression of fungal growth by the transconjugant thus obtained confirmed the ability of pANF 17 to functionally complement the antifungal toxin production by the defective mutant AN 21 (Figure 1).

Plasmid DNA was extracted from *E. coli* DH5α harbouring pANF 17 and restricted to completion with *Eco*RI. It was found that the cosmid contained a DNA fragment of size 23.5 kb and had two internal cut sites of *Eco*RI yielding DNA fragments of molecular weights 11.5, 9 and 3 kb (Figure 2). Restriction digestion with *Sal*I, *Bgl*II and *Bam*HI showed that there were 3, 2 and 3 internal cut sites respectively, in the inserted DNA fragment in the complementing clone.

The origin of the clone pANF 17 was ascertained by a Southern blot experiment. The strain EM 85 carried a native plasmid of size approximately 25 kb. Since the total DNA extraction may also include this, for confirming the origin of the complementing cosmid clone, it was necessary to take the plasmid DNA also for Southern blot experiment. The labelled 11.5 kb *Eco*RI fragment of pANF 17 cosmid clone was found to hybridize with the total genomic DNA of the wild-type strain EM 85. There was no signal on the lane with the native plasmid (Figure 3). It was thus confirmed that the gene(s) responsible for Aft$^+$ phenotype was carried by the chromosome of the *Pseudomonas* strain EM 85 and the clone pANF 17 was originally derived from the wild-type genome of EM 85.

pANF 17 on restriction digestion with *Eco*RI released the inserted DNA fragment in pLAFR 1. The gene(s) responsible for antifungal toxin production might be located anywhere in the 23.5 kb fragment. Sub-cloning is an important molecular tool in finding out the smallest possible DNA segment conferring any character. Plasmid pKT 230, a derivative of RSF 1010 which is having wide host range, has been used for sub-cloning since it was able to multiply in all gram negative bacterial backgrounds[21]. Biparental mating was done to mobilize the sub-cloned plasmids pANF 17–1, 17–2 and 17–3 into AN 21 and the transconjugants were tested for antifungal toxin production. No complementation of Aft$^-$ phenotype was observed in these transconjugants. This may be due to a break in the continuous coding region of the *aft* gene, i.e. *Eco*RI might be having a cut site within the *aft* gene, thus the individual sub-clones are not in a position to functionally complement the defective character. Sub-cloning with DNA fragments obtained by restriction digestion of pANF 17 with other enzymes or combination of two enzymes may give further details in this regard.

The crude antibiotic from EM 85 and AN 21 (pANF 17) inhibited the mycelial growth of *R. solani* in PDA plate assay, whereas that from AN 21 had no inhibitory effect. This shows that the cosmid clone was stable in the mutant background and expressed. This again affirms that the cosmid clone pANF 17 carried gene(s) or any essential part(s) of gene(s) which determine antifungal toxin production by the *Pseudomonas* strain EM 85.

Pre-emergent damping-off was observed on the emerging seedlings of cotton after one week of sowing. The diseased plants failed to grow. Hence, the number of

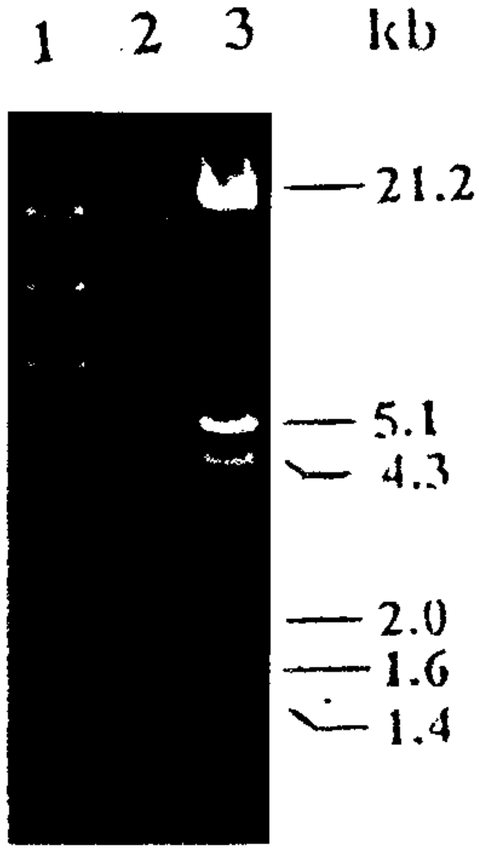

Figure 2. Agarose gel electrophoresis of complementing clone. Lane 1, pANF 17 restricted with *Eco*RI; lane 2, *Eco*RI linearized pLAFR 1; and lane 3, λ *Hin*dIII + *Eco*RI MW marker.

Figure 3. Southern blot of DNA from *Pseudomonas* strain EM 85 with radiolabelled 11.5 kb *Eco*RI fragment from pANF 17; A, Total genomic DNA; B, Native plasmid DNA.

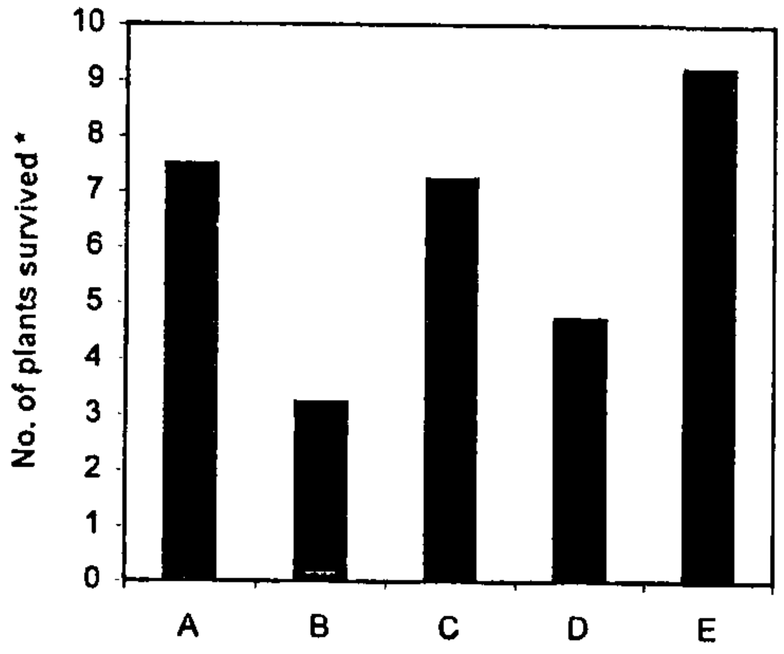

Figure 4. Damping-off suppression by *Pseudomonas* on cotton seedlings. A, *R. solani* + EM 85; B, *R. solani* + AN 21; C, *R. solani* + AN 21 carrying pANF 17; D, *R. solani* alone; E, No inoculation with pathogen (control).
*Mean of four replications having 10 seeds each. CD at *P* (0.05) 1.23.

surviving plants in each treatment was noted for the assessment of disease suppressive ability of the strains (Figure 4). *In vivo* biocontrol efficiency of the mutant and the wild-type strain differed significantly compared to the control treatment. The disease suppression was 57.89% in the case of the wild-type strain and 52.63% in the case of AN 21 carrying the complementing clone pANF 17. There was no significant difference between the disease-controlling ability of the wild-type strain EM 85 and AN 21 having pANF 17. *In vivo* biological control ability of antifungal toxin defective mutants carrying complementing clones have been reported earlier[22–24]. The results show that the complementing clone pANF 17 was stably maintained in the mutant background and was expressed in the pot culture conditions efficiently. The strain EM 85 was reported to produce multiple antifungal compounds and a preliminary study of the chemical nature of the antifungal compound was done by Pal[9]. A detailed analysis of the structure of the compound will further help identify the relative importance of the same in biological control conditions by detecting the chemical in *in vivo* systems, especially in the rhizosphere.

The possibility of using the cosmid clone pANF 17 to engineer Aft$^-$ *Pseudomonas* strains to Aft$^+$ phenotype by heterologous expression is open when finer regulatory mechanisms as well as expression studies are made on the *aft* genes.

1. Bahme, J. B. and Schroth, M. N., *Phytopathology*, 1987, **77**, 1093–1100.
2. Lam, S. T. and Gaffney, T. D., in *Biotechnology in Plant Disease Control* (ed. Chet, I.), Weiley Liss Inc., USA, pp. 291–320.
3. Gutterson, N. I., Layton, T. J., Ziegle, J. S. and Warren, G. J., *J. Bacteriol.*, 1986, **165**, 696–703.
4. Poplawsky, A. R., Peng, Y. F. and Ellingboe, A. H., *Phytopathology*, 1988, **78**, 423–432.
5. Vincent, M. V., Harrison, L. A., Brackin, M. J., Kovacevic, P. A., Mukerji, P., Weller, D. M. and Pierson, E. A., *Appl. Environ. Microbiol.*, 1991, **57**, 2928–2934.
6. Hill, D. S., Stein, J. I., Torkewitz, N. R., Morse, A. M., Howell, C. R., Pachalatko, J. P., Becker, J. O. and Ligon, J. M., *Appl. Environ. Microbiol.*, 1994, **60**, 78–85.
7. Carruthers, F. L., Corner, A. J. and Mahanthy, H. K., *Appl. Environ. Microbiol.*, 1994, **60**, 71–77.
8. Pierson, L. S. and Thomashow, L. S., *Mol. Plant Microbe Interact.*, 1992, **5**, 330–339.
9. Pal, K. K., Ph D thesis, IARI, New Delhi, 1996.
10. Kreig, N. R., Williams, H. J. G. and Wilkins, *Bergey's Manual of Systematic Bacteriology*, 9th edn, 1984, vol. 1.
11. Mandel, M. and Marmur, J., *Methods Enzymol.*, 1968, **B12**, 195.
12. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbour, New York, 1989, 2nd edn.
13. Bittner, M., Kupferer, P. and Morris, C. F., *Ann. Biochem.*, 1980, **102**, 459.
14. Levy, E. and Eyal, Z., *Plant Pathol.*, 1988, **37**, 551–557.
15. Panse, V. G. and Sukhatme, P. V., in *Statistical Methods for Agricultural Workers*, ICAR, New Delhi, 2nd edn, 1967.
16. Palleroni, N. J., in *Bergye's Manual of Systematic Bacteriology* (eds Krieg, N. R., Holt, J. G. and Williams, H. J. H.), 9th edn, 1984, vol. 1.
17. Cerda-Olmedo, E., Hanwalt, P. C. and Guerola, N., *J. Mol. Biol.*, 1968, **33**, 715–719.
18. Anith, K. N., Tilak, K. V. B. R., Khanuja, S. P. S. and Saxena, A. K., *World J. Microbiol. Biotechnol.*, 1998, **14**, 939–941.
19. Hohn, B., *J. Mol. Biol.*, 1975, **98**, 93–106.
20. Clarke, L. and Carbon, J., *Cell*, 1976, **9**, 91–99.
21. Bagdasarian, M., Lurz, R., Ruckert, B., Francklin, F. C. H., Bagdasarian, M. M., Frey, J. and Timmis, K. N., *Gene*, 1981, **16**, 237–247.
22. Keel, C., Schinder, U., Maurhofer, M., Voisard, C., Laville, J., Murger, U., Wirthner, P., Hass, D. and Defago, G., *Mol. Plant Microbe Interact.*, 1992, **5**, 4–13.
23. Laville, J., Voissard, C., Keel, C., Maurhofer, U., Defago, G. and Hass, D., *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 1562–1566.
24. Costa, J. M. and Loper, J. E., *Mol. Plant Microbe Interact.*, 1994, **7**, 440–448.
25. Boyer, H. B. and Roulland-Dussoix, D., *J. Mol. Biol.*, 1969, **41**, 459.
26. Freidman, A. M., Long, S. R., Brown, S. E., Buikema, W. J. and Ausubel, F. M., *Gene*, 1982, **18**, 289.
27. Figuruski, D. H. and Helinski, D. R., *Proc. Natl. Acad. Sci. USA*, 1979, **76**, 1648.

Received 17 December 1998; revised accepted 22 June 1999