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Isolation of a Gene from *Burkholderia cepacia* IS-16 Encoding a Protein That Facilitates Phosphatase Activity

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Abstract. A genomic library from *Burkholderia cepacia* IS-16 was constructed in *Escherichia coli* by partial *Sau*3AI digestion of the chromosomal DNA, with the plasmid vector Bluescript SK. This library was screened for clones able to grow as green stained colonies on selective medium developed for detecting phosphatase-positive colonies. Three green-stained clones (pFS1, pFS2, and pFS3) carried recombinant plasmids harboring DNA inserts of 5.0, 8.0, and 0.9 kb, respectively. DNA hybridization experiments demonstrated the presence of overlapping DNA fragments in the three clones and that these three clones were all derived from *Burkholderia cepacia* IS-16 genomic DNA. DNA sequence analysis, together with polyacrylamide gels of proteins encoded by *E. coli* containing pFS3, suggested that the isolated 0.9-kb DNA fragment encodes the functional portion of a phosphate transport protein.

Rhizosphere bacteria can enhance plant growth by a number of different mechanisms [3, 4]. One of these mechanisms involves the solubilization of inorganic and organic phosphates from soil, making phosphorus available for plant assimilation [5, 13]. A substantial fraction of the phosphorus in the soil is in the form of poorly soluble mineral phosphate and organic matter, which must be converted to soluble phosphate to be assimilable by the plant. As a consequence of the secretion of organic acids by certain rhizosphere bacteria, which causes a localized lowering of the soil pH and a concomitant enhancement of phosphate diffusion [1], inorganic phosphates in the soil may become more available for uptake by the roots of plants. The solubilization of organic phosphate is carried out by bacteria with the help of phosphatase enzymes, especially acid phosphatases, which play the major role in organic phosphate solubilization in soil [5, 13]. Bacterial acid phosphatases show a wide spectrum of activities among different species [14, 17]. In addition, some of the genes encoding phosphatases from different bacterial species have been isolated and characterized [6, 7, 9, 10, 16].

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The cloning of genes encoding proteins involved in phosphate uptake and phosphatase activity is relatively straightforward. It should be possible to overexpress some of these genes in soil bacteria and thereby obtain bacterial strains with an increased ability to solubilize organic phosphate; such superior phosphate-solubilizing bacteria should have utility in terms of improving plant growth. In this publication, we report the cloning and characterization of a gene from *Burkholderia cepacia* IS-16 that, when it is expressed in *E. coli*, appears to increase the uptake of organic phosphate and hence phosphatase activity.

Materials and Methods

Strains, plasmids and culture conditions. *Burkholderia cepacia* IS-16 was isolated from rhizosphere soil at the Cuban Institute of Soil Sciences, Havana, Cuba (Martínez et al., personal communication) and characterized during the course of this study with a Bio-Merieux kit (Bio-Merieux, Marcy-l'Etoile, France). *Escherichia coli* DH5α [15] was used as the host for genetic vectors and recombinant plasmids. The plasmid Bluescript SK (pBSK, Stratagene) was used for library construction and subcloning procedures.

B. cepacia IS-16 was grown in nutrient broth (NB) medium with shaking [15] at 30°C (unless otherwise specified); *E. coli* strains were grown at 37°C in Luria Bertani (LB) medium with shaking [15]. Cultures of *B. cepacia* were plated on NB-agar, and *E. coli* strains were plated on LB-agar. For *E. coli* strains harboring plasmids, ampicillin

(Sigma Chemical Co., St. Louis, MO) was used in the culture medium at 250 μg · ml^{-1} final concentration.

Purification of plasmid DNA, restriction analysis, and gel electrophoresis. For most analytical purposes, plasmids were prepared from overnight cultures by the alkaline lysis method as described [15]. Restriction enzymes were purchased from Boehringer-Mannheim (Montreal, QC) and used with the buffers supplied by the manufacturer. The digestions of plasmid DNA were carried out for 4 h or overnight, at 37°C, and the restriction fragments analyzed by agarose gel electrophoresis [15].

Preparation and analysis of the *B. cepacia* IS-16 genomic DNA library. *B. cepacia* IS-16 chromosomal DNA was isolated as described [15] and then redissolved in TE buffer. Chromosomal DNA was partially digested with 0.05 U/mg DNA of restriction endonuclease *Sau*3AI at 37°C for 30 min. These conditions gave the highest concentration of DNA fragments of the desired size (i.e., 2–9 kb). The *Sau*3AI partial digestion was electrophoresed in 0.5% low-melting-point agarose, the region of the gel containing fragments from 2 to 9 kb in size was excised, and the DNA was extracted and purified [15].

Plasmid pBSK was digested with restriction endonuclease *Bam*HI and dephosphorylated by treatment with calf intestinal alkaline phosphatase (Boehringer-Mannheim) under conditions recommended by the manufacturer. Upon completion of the dephosphorylation reaction, the enzyme was inactivated by heat at 65°C for 15 min, and the vector was purified from a 0.8% agarose gel.

The fractionated chromosomal DNA and the alkaline phosphatase-treated vector DNA were mixed in a 7:1 molar ratio and ligated together, with T4 DNA ligase (Boehringer-Mannheim), by overnight incubation at 16°C. The ligation mixture was used to transform *E. coli* DH5α cells by electroporation. Colonies of *E. coli* transformants were selected on ampicillin-containing LB agar plates and then plated onto selective medium to screen for phosphatase positive clones using tryptose phosphate agar (PTA; Difco Laboratories, Detroit, MI), pH 7.2, supplemented with 2 mg · ml^{-1} of the phosphatase substrate phenolphthalein diphosphate (tetrasodium salt; Sigma), and 0.05 mg · ml^{-1} methyl green (Sigma). On this medium, the presence of phosphatase activity (i.e., the Pho^+ phenotype) is indicated by a green staining of the bacterial colony [12].

DNA hybridization. Probes were [^{32}P]-labeled according to the random oligodeoxynucleotide primers extension protocol [2]. Southern hybridization was performed at 60°C as described [15]. Radiolabeled bands were visualized by autoradiography.

DNA sequence analysis. DNA sequencing was done at the MOBIX Central Facility, McMaster University, Hamilton, Ontario. Sequencing reactions utilized the modified Taq-FS enzyme (Perkin-Elmer) with fluorescently labeled dideoxy terminator chemistry [11]. The sequencer, an Applied Biosystems 373A Stretch, was used for running the gel and detecting the fluorescent bands. Sequence information was analyzed with the computer program DNA Strider v.1.2 [8].

Results and Discussion

Screening the *B. cepacia* IS-16 gene library. After construction of the *B. cepacia* genomic library and its transformation into *E. coli*, three putative phosphatase-positive clones were identified out of approximately 15,000 colonies screened with the PTA indicator medium. The three clones that were isolated displayed a pale green color on selective medium. This is in contrast with what was observed with *Morganella morganii*, which appeared much darker on the same medium [16]. The lighter color on selective medium could reflect a somewhat lower activity of a cloned *B. cepacia* IS-16 phosphatase compared with that of *Morganella morganii*, or it might indicate that the selected clones do not encode *B. cepacia* IS-16 acid phosphatase.

On the basis of their mobility after electrophoresis in agarose gels, recombinant plasmids isolated from the three selected clones, namely pFS1, pFS2 and pFS3, appeared to be different sizes (data not shown). It was confirmed that all three of these plasmids have the ability to retransform *E. coli* DH5α cells to ampicillin resistance and to the Pho^+ phenotype, consistent with the conclusion that these plasmids encode a protein that confers the Pho^+ phenotype upon *E. coli* DH5α cells.

The banding patterns of the three selected plasmids were compared by agarose gel electrophoresis after restriction enzyme digestion of these plasmids (data not shown). This analysis indicated that plasmids pFS1, pFS2, and pFS3 carried inserts of 5.0, 8.0, and 0.9 kb, respectively. These sizes are consistent with the fact that the gene library was originally constructed of partially digested chromosomal DNA fragments that were primarily 2–9 kb in size.

Cross-hybridization experiments. Restriction endonuclease analysis of the three selected clones suggested that there was overlap between the *B. cepacia* IS-16 chromosomal DNA inserts in the three clones. Moreover, digestion of clone pFS3 was found to liberate a 0.9-kb insert (data not shown). Considering that the 0.9-kb insert retained the Pho^+ phenotype, this clone (pFS3) was subsequently used as a probe for hybridization with the other recombinant plasmids and genomic DNA.

Cross-hybridization experiments confirmed that the three clones carried partially overlapping DNA fragments, that the insert in pFS3 was derived from *B. cepacia* IS-16 genomic DNA, and that the 0.9-kb insert existed as one contiguous piece of DNA on the *B. cepacia* IS-16 chromosome (data not shown). These results suggested that a putative phosphatase gene or a phosphatase-enhancing gene from *B. cepacia* IS-16 was encoded within the 0.9 kb insert. Therefore, this clone was selected for further characterization.

Restriction mapping and subcloning. Several restriction endonucleases were used to construct a restriction endonuclease map of pFS3 (Fig. 1). When DNA fragments were isolated from the chromosomal DNA insert within plasmid pFS3, spliced into plasmid pBSK, and tested for the Pho^+ phenotype, none of the subclones retained the ability to turn the PTA indicator medium

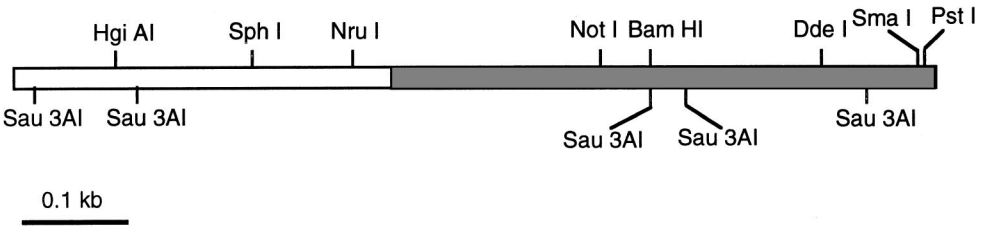

Fig. 1. Restriction endonuclease map of the 0.9-kb DNA fragment from *Burkholderia cepacia* IS-16 carried in plasmid pFS3. The region that encodes a putative outer membrane protein is colored grey.

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1/1                                         31/11
ATG ATG AAG TCG ATG ACA TCC TCC GCC GAG GCG GGC GCA CGC GCG AGC GTC GGC GTC GCG
Met met lys ser met thr ser ser ala glu ala gly ala arg ala ser val gly val ala
61/21                                       91/31
CGC CTT TCG GCC CGT CTT CCG GCC AGT TTC CCG GCA CTC GCC GCG GCC TGC GCC TTC GTG
arg leu ser ala arg leu pro ala ser phe pro ala leu ala ala ala cys ala phe val
121/41                                      151/51
TTC GCG CTG GCC GGC TGC GCG GTC GGC CCC GAC TAC AAG CCG CCC GCG GCC GAG CTG GCG
phe ala leu ala gly cys ala val gly pro asp tyr lys pro pro ala ala glu leu ala
181/61                                      211/71
CCG TTC CAG CAC GTG CCG GCT TCG ACA TCG GCG GCG TCG CAA GCG CAG GGC GCG GCC GCC
pro phe gln his val pro ala ser thr ser ala ala ser gln ala gln gly ala ala ala
241/81                                      271/91
GCG CCG GCG CCC TCG CTC GAT ACC TGG TGG ACC GGG TTC CAG GAT CCG ATG CTG GTG TCG
ala pro ala pro ser leu asp thr trp trp thr gly phe gln asp pro met leu val ser
301/101                                     331/111
ATC GTC GAT CGC GCG CTG GCG CAG AAC CTC GAC CTG GCT GCC GCC TTC GCG CGG GTT CGG
ile val asp arg ala leu ala gln asn leu asp leu ala ala ala phe ala arg val arg
361/121                                     391/131
CAG GCT CGC GCC GCC GCC TCG GCG GCC GGT GCC GAG CTG CTG CCG ACC TTC GAC CTG GAC
gln ala arg ala ala ala ser ala ala gly ala glu leu leu pro thr phe asp leu asp
421/141                                     451/151
GGC TCG GCC AGC GAG CAG CAC CAG AGC GAG CTG AGC CCG ACC GGC TCG CTG GCC AAG GTC
gly ser ala ser glu gln his gln ser glu leu ser pro thr gly ser leu ala lys val
481/161                                     511/171
TTC CCC GGC TAC GAT CGC AAC CAG CGC GAA TAC ACG CTG GGC GCG GCG GCG AGC TGG GAG
phe pro gly tyr asp arg asn gln arg glu tyr thr leu gly ala ala ala ser trp glu
541/181
ATT TCC CGG GCT GCA GGA ATT
ile ser arg ala ala gly ile
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Fig. 2. DNA and protein sequence of the *Burkholderia cepacia* IS-16 protein encoding the Pho$^+$ phenotype.

green. This indicates that all or most of the 0.9-kb fragment is required for the observed activity.

DNA sequence analysis. DNA sequence analysis of the 0.9 kb DNA fragment from plasmid pFS3 (GenBank accession number is AF190626) revealed that this DNA encoded a 187-amino acid protein with a predicted molecular mass of 19,081 Da (Fig. 2). Moreover, this DNA fragment does not encode the C-terminal end of this protein. In fact, the DNA sequences encoded within plasmids pFS1 and pFS2, both of which confer the Pho$^+$ phenotype on *E. coli* cells, also do not encode the C-terminal end of this protein. Despite missing a portion of its C-terminus, this protein nevertheless is active in conferring a Pho$^+$ phenotype on the host *E. coli* cells. Of note is the fact that the 187-amino acid protein includes 87 hydrophobic amino acid residues (glycine, alanine, leucine, isoleucine, and valine) including 47 alanines, an amino acid composition consistent with its being a membrane protein. In fact, comparison of the amino acid sequence of the protein encoded by plasmid pFS3 with the GenBank database of known proteins revealed that the protein encoded by this plasmid was most similar to several bacterial outer membrane proteins (Table 1).

Protein characterization. DNA sequence analysis of the *B. cepacia* IS-16 chromosomal fragment, which confers the Pho$^+$ phenotype onto *E. coli* DH5α, suggested that this fragment encoded an outer membrane protein rather than an acid phosphatase. Thus, it was surmised that the

Table 1. The similarity of the amino acid sequence of the *B. cepacia* IS-16 protein conferring the Pho$^+$ phenotype to *E. coli* to other known proteins

Protein	% Identity	% Similarity	Accession #
Pseudomonas aeruginosa outer membrane protein	36	46	L11616
Pseudomonas aeruginosa outer membrane protein	36	46	Q51487
Positively regulated protein involved in multi-drug efflux in *Pseudomonas aeruginosa*	28	40	X99514
Pseudomonas putida outer membrane channel protein	33	43	AF029405
Burkholderia cepacia outer membrane lipoprotein involved in multiple antibiotic resistance	37	46	U38944
Neisseria gonorrhoeae efflux pump channel protein	27	41	X95635

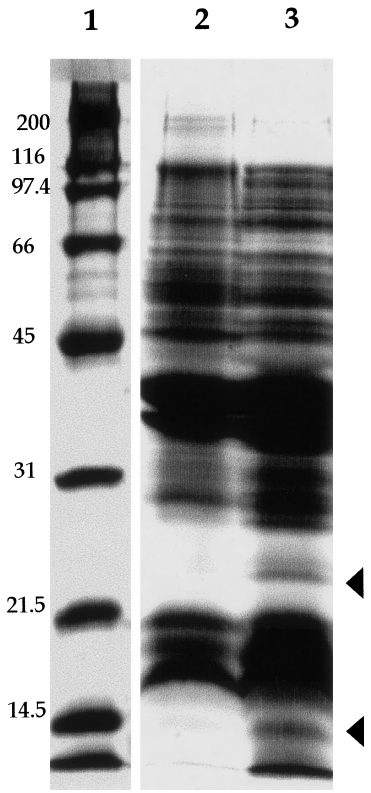

Fig. 3. Polyacrylamide gel electrophoresis of Coomassie blue-stained outer membrane proteins from *E. coli* DH5α/pBSK (lane 2) and *E. coli* DH5α/pFS3 (lane 3). Lane 1 shows the positions of protein molecular mass markers. The arrows to the right of the gel indicate the positions of pFS3 encoded 23.5-kDa and 14-kDa protein bands. The band in lane 3 that runs with the dye front and slightly ahead of the 14 kDa protein band is believed to include partially degraded protein fragments.

cloned protein is most probably a porin that is specifically involved in the uptake of phosphorylated compounds and provides the periplasmic phosphatase with increased access to its substrate, which is normally external to the bacterium. In order to ascertain that plasmid pFS3 did in fact direct the synthesis of an outer membrane protein, protein extracts of various cellular compartments of *E. coli* DH5α transformed with this plasmid were examined by polyacrylamide gel electrophoresis. Following staining of the gel with Coomassie blue, the data from this experiment indicate clearly that, after the introduction of plasmid pFS3, *E. coli* DH5α synthesizes both a 23.5-kDa and a 14-kDa protein, both localized in the *E. coli* cell outer membrane (Fig. 3). It is believed that the 23.5-kDa protein band probably corresponds to the 22.5-kDa protein that is predicted to be encoded by the fusion of the putative 19-kDa outer membrane protein and the amino acids encoded by the vector pBSK, while the 14-kDa protein band may be a partial degradation product. Moreover, the synthesis of the 23.5-kDa protein is increased in the absence of phosphate in the growth medium (data not shown).

In *E. coli*, alkaline phosphatase activity is regulated by the outer membrane protein PhoE (a porin), the synthesis of which is induced upon phosphate starvation [18]. The gene encoding a *B. cepacia* outer membrane protein, whose isolation is reported here, could play a similar role to the *E. coli* PhoE protein. As far as we are aware, this is the first report of a phosphate-regulated gene from *B. cepacia* influencing phosphatase activity. The manipulation of this gene in *B. cepacia* may provide a means for increasing by genetic engineering the efficiency of phosphate accumulation in *B. cepacia* and other similar plant growth-promoting bacteria.

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