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Trehalose Induces Antagonism towards *Pythium debaryanum* in *Pseudomonas fluorescens* ATCC 17400†

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***Pseudomonas fluorescens* ATCC 17400 shows in vitro activity against *Pythium debaryanum* under conditions of iron limitation. A *lacZ* reporter gene introduced by transposon mutagenesis into the *P. fluorescens* ATCC 17400 trehalase gene (*treA*) was induced by a factor released by the phytopathogen *Pythium debaryanum*. The induction of the *lacZ* gene was lost upon treatment of the *Pythium* supernatant with commercial trehalase. A trehalose concentration as low as 1 μM could induce the expression of *treA*. The mutation did not affect the wild-type potential for fungus antagonism but drastically decreased the osmotolerance of the mutant in liquid culture and suppressed the ability of *P. fluorescens* ATCC 17400 to utilize trehalose as a carbon source. A subsequent transposon insertion in *treP*, one of the trehalose phosphotransferase genes upstream of *treA*, silenced the *lacZ* gene. This double mutant restricted fungal growth only under conditions of high osmolarity, which probably results in internal trehalose accumulation. These data confirm the role of the disaccharide trehalose in osmotolerance, and they indicate its additional role as an initiator of or a signal for fungal antagonism.**

Certain interactions between plants and microorganisms, as well as between different microbial populations, are initiated through specific signaling molecules produced by plants and/or microorganisms (2). Such interactions are critically important in establishing both beneficial and detrimental relationships that affect plant growth and development (7). Inducers for virulence genes in phytopathogens are most commonly reported. The genes of *Agrobacterium tumefaciens* that are involved in virulence (*vir*) are induced by acetosyringone (20, 44, 45), and the *hrp* genes of *Erwinia amylovora* (42), *Pseudomonas solanacearum* (1), and *Pseudomonas syringae* (34) are induced by a broad range of compounds.

The study of relationships between microbial populations in the rhizosphere has focused mainly on the identification of compounds and genetic loci involved in the biocontrol of phytopathogens by plant growth-promoting rhizobacteria (PGPR), among which the fluorescent pseudomonads constitute the most intensively investigated group (12, 25, 26, 37, 38). Molecular signals have also been implicated in certain host plant-phytopathogen-PGPR interactions; for example, transcription of *aggA* in *Pseudomonas putida*, whose gene product is involved in the agglutination and rapid adhesion of the bacterium to the root, is induced by a broad range of compounds (5). *Pseudomonas* sp. strain WCS417r induces the accumulation of phytoalexin in carnations through an unidentified signal (40). The induction of systemic resistance of cucumber to *Colletotrichum orbiculare* (41) and of tobacco to tobacco necrosis virus by *P. fluorescens* CHAO (19) has previously been described; however, the signaling molecules involved have not yet been identified. Salicylic acid is involved in systemic acquired resistance in plants (14) and is known to be produced by *P. fluorescens* CHAO, where it serves as a siderophore (21). However, the role of the salicylic acid produced by *P. fluorescens* in the induction of plant systemic resistance remains to be established. The production of bioactive compounds by PGPR pseudomonads is regulated by different systems. These include the global regulator GacA (15) and the cell density-dependent regulation of phenazine biosynthesis controlled by the *phzI-phzR* two-component system (27). The production of pyrrolnitrin by *P. fluorescens* Pf-5 has previously been shown to be under the control of the stationary-phase sigma factor σ^s (30), whereas the housekeeping sigma factor RpoD was found to be essential for the production of the antibiotics pyoluteorin and phloroglucinol in *P. fluorescens* CHAO (31). Reporter gene operon fusions have been used successfully to study gene responses to different environmental inducers (11). The promoter probe system Tn*5*-B20, with a promoterless *lacZ* reporter gene, was used to construct operon fusions in *P. fluorescens* R2F that were induced by root exudates (39). Most of these mutants responded to a broad range of compounds, such as D-mannitol, sucrose, D-trehalose, organic acids, and amino acids, but one mutant responded specifically to L-proline (39). To our knowledge, transposon-mediated reporter gene transcription fusion probes have not been used so far to identify genes in PGPR that respond to molecules produced by phytopathogens. Here, we report the identification of such a molecule from the phytopathogen *Pythium debaryanum* by using *lacZ* and *luxAB* operon fusions in the *Pythium debaryanum*-antagonistic strain *P. fluorescens* ATCC 17400.

MATERIALS AND METHODS

Strains and growth conditions. Wild-type *P. fluorescens* ATCC 17400 was maintained in Casamino Acids (CAA) medium (9). *P. fluorescens* ATCC 17400 mutants were maintained in CAA medium supplemented with 600 μg of kanamycin per ml or 50 μg of tetracycline per ml. Sugars from a filter-sterilized stock were added after autoclaving. Unless otherwise indicated, 50-ml cultures were inoculated from an overnight preculture and incubated at 28°C at 100 rpm (Innova 4000 shaker; New Brunswick Scientific) for 48 h. *Pythium debaryanum* is a soilborne pathogen isolated from the beet rhizosphere (P. Lepoivre, Gem-

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† This paper is dedicated to the memory of H.K.

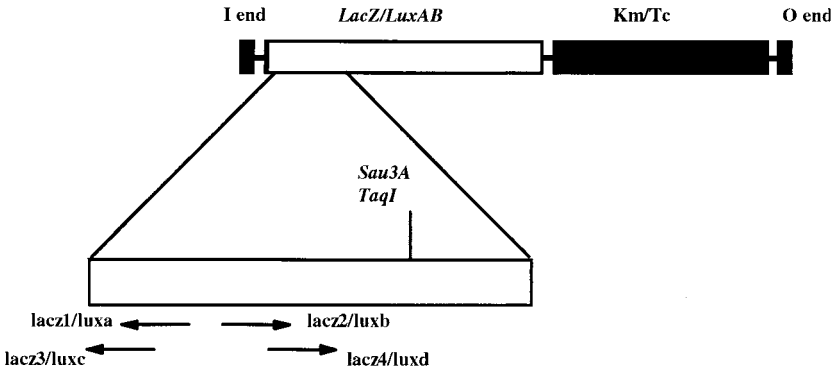

FIG. 1. Organization of mini-Tn*5* *lacZ1* Km^r and mini-Tn*5* *luxAB* Tc^r, indicating the *Sau*3A and *Taq*I restriction sites used for IPCR of genomic DNAs of mutants constructed with the two transposons, respectively. Primers lacz1 and lacz2 or luxa and luxb were used for IPCR, and primers lacz3 and lacz4 or luxc and luxd were used as nested primers with IPCR products as the template.

bloux, Belgium). *Escherichia coli* Sm10 λpir(pUT mini-Tn*5*) (10) was grown at 37°C in Luria broth with 100 μg of ampicillin per ml.

Preparation of the *Pythium* culture supernatant. *Pythium debaryanum* was grown in CAA medium for 3 days, the fungal hyphae were removed by filtration through a glass filter (Whatman GF/A), and the supernatant was sterilized by filtration (0.45 μm-pore-size filter). The sterilized supernatant was stored at 4°C and used within 1 week. Fresh and stored supernatants gave the same results.

Transposon mutagenesis. A transcription fusion, mini-Tn*5* *lacZ1* Km^r (10), was mobilized to *P. fluorescens* ATCC 17400 (streptomycin resistance up to 25 μg/ml) by conjugation with *E. coli* SM10 λpir harboring the pUT suicide vector with the transposon by the method of Cornelis et al. (9). Transconjugants were selected on CAA-agar (1.5% [wt/vol]) supplemented with 20% (vol/vol) *Pythium* supernatant, 600 μg of kanamycin per ml, 25 μg of streptomycin per ml, and 100 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Transconjugants expressing β-galactosidase, as indicated by blue colonies with X-Gal, were replica plated on CAA medium with kanamycin supplemented or not with *Pythium* supernatant (10% [vol/vol]). Clones expressing β-galactosidase only in the presence of the *Pythium* supernatant were selected and retested at least three times on separate plates. A second transposon mutagenesis was carried out by the method of Cornelis et al. (9) with the mini-Tn*5* *luxAB* Tc^r transposon (10), and with a mutant resulting from the first mutagenesis (Pyt6) as a recipient.

IPCR. In order to identify the sequences flanking the inserted transposon, an inverse PCR (IPCR) (24) was performed (Fig. 1). In brief, genomic DNA of the mutant was isolated by the method of Wilson (43). One microgram of DNA was digested with *Taq*I and ligated in a total reaction volume of 50 μl to favor intramolecular ligation. The IPCR mixture consisted of different volumes of the ligation mixture (2, 4, or 8 μl), 50 pmol of primer lacz1 (5′-GGGAATTCAAA GCGCCATTCGCCATTCAG-3′), 50 pmol of primer lacz2 (5′-GGAAGCTTT ATGGCAGGGTGAAACGCAGG-3′), 200 μM (each) deoxynucleoside triphosphates, and 0.5 U of Gold Star DNA polymerase (Eurogentec) in 50 μl of polymerase buffer. The PCR was carried out for 35 cycles of 94, 53, and 72°C for 1 min each. The gel-purified IPCR product was reamplified with nested primers lacz3 (5′-GGTCTAGACGTTTTCCCAGTCACGAC-3′) and lacz4 (5′-GCGG ATCCTTTCGGCGGTGAAATTATCG-3′) in a 100-μl reaction mixture as described above. IPCR with the double mutant was done with primers in the *luxA* region under the same conditions as those used with primers in the *lacZ* region except that annealing was carried out at 55°C. The IPCR primers used were luxa (5′-CGGAATTCATCACTTCGGTCTGAGA-3′) and luxb (5′-GCTCTAGAA AAGCTCAATGTTGGCAC-3′), and reamplification of IPCR products was done with nested primers luxc (5′-CGGGATCCATCAGGTGGCTGATAAGT GA-3′) and luxd (5′-ATTGTATTGCCGACTGCCCAT-3′).

Sequencing. Direct sequencing of PCR fragments was carried out with a Sequenase PCR sequencing kit (version 2.0 T7 DNA) according to the manufacturer's (U.S. Biochemicals) instruction. Due to the high GC content of *Pseudomonas* DNA, false stops of polymerase were removed by a terminal transferase treatment. At the end of PCR cycles, 1 μl of a mixture containing 4 mM deoxynucleoside triphosphates, 8 mM $CoCl_2$, 1× terminal transferase buffer, and 0.75 U of terminal transferase per ml was added to each tube. After incubation at 37°C for 1 h, the reaction was terminated by adding sequencing stop solution. DNA fragments were separated with a Bio-Rad electrophoresis system.

Amplification of part of *treA* and *treP* from *P. fluorescens* 17400. Primers tre1 (5′-GCACCAGAACAGCGCATTCCA-3′) and tre2 (5′-GATCAACCTGATG GCCAACAC-3′) were constructed on the basis of the sequences of the IPCR fragments obtained from the single mutant and double mutants, respectively. PCR amplification was done for 35 cycles of 94 and 60°C for 1 min each and 72°C for 2 min with *P. fluorescens* ATCC 17400 genomic DNA as the template and with *Taq* polymerase. The amplified fragment was analyzed by agarose gel electrophoresis and DNA sequencing (Biozyme; Sequitherm) after cloning in the AT cloning vector pCRII (Invitrogen).

β-Galactosidase activity measurements. β-Galactosidase activity was measured in the Pyt6 mutant by the method of Miller (22) with ONPG (*o*-nitrophenyl-β-D-galactopyranoside) as the substrate. The results shown are the averages of three independent reactions.

TLC. Samples (10 μl) of *Pythium* supernatant and different sugar standards were spotted on silica gel-coated thin-layer chromatography (TLC) plates (Polygram; Sil G/UV, Macherey-Nagel, Düren, Germany) and developed in 1-propanol–water (6:1 [vol/vol]). Plates were incubated at 80°C for 10 min after being sprayed with 10% sulfuric acid.

Trehalase treatment. Five milliliters of *Pythium* supernatant medium was incubated with 1 U of trehalase (Sigma) at 37°C for 2 h. Samples were used for TLC or β-galactosidase induction.

***Pythium* antagonism.** *Pythium debaryanum* was inoculated as a plug of the margin of actively growing mycelium at the center of a CAA plate, and bacterial strains, both the wild type and mutants, were inoculated (5 μl from an overnight culture of 5×10^8 bacteria/ml; four inoculations per plate), 3 cm from the *Pythium* inoculum. Plates were incubated at 28°C for 2 days, and the degree of antagonism was evaluated by measuring the radius of the fungal-growth-inhibition zone around bacterial growth. All experiments were done with five replicates, and the data were averaged. Control plates were inoculated with *Pythium debaryanum* only.

RESULTS

Transposon mutagenesis with mini-Tn*5* *lacZ1*. A mini-Tn*5* carrying a *lacZ* reporter gene and a Km^r gene (10) was used to construct operon fusions in *P. fluorescens* ATCC 17400, a strain that shows antagonism in vitro against *Pythium debaryanum* under iron limitation (9). Of 2,400 clones tested, 5 were confirmed to produce β-galactosidase only in the presence of *Pythium* culture supernatant. Of these five, two (Pyt5 and Pyt6) showed iron-independent β-galactosidase production; the other three (Pyt7, Pyt8, and Pyt9) showed iron-repressed production (in the presence of 100 μM $FeCl_3$) of the reporter enzyme (data not shown). One clone (Pyt6) which showed β-galactosidase activity in an iron-independent manner (both in the presence and absence of 100 μM ferric chloride) was used for further analysis.

IPCR sequence analysis. IPCR with primers in the *lacZ* region of the transposon resulted in the amplification of a single DNA fragment of 0.4 kb, indicating a single transposon insertion, whereas the wild-type genomic DNA used as a control did not give any amplification with the same primers. DNA fragments were sequenced in both orientations. A computer-assisted similarity search between the predicted amino acid sequence of the Pyt6 IPCR fragment and sequences in the database revealed 87% identity to the trehalase of *Bacillus subtilis* encoded by *treA* (Fig. 2) (17). *B. subtilis treA* is induced by trehalose, repressed in the presence of 0.1% (wt/vol) glu-

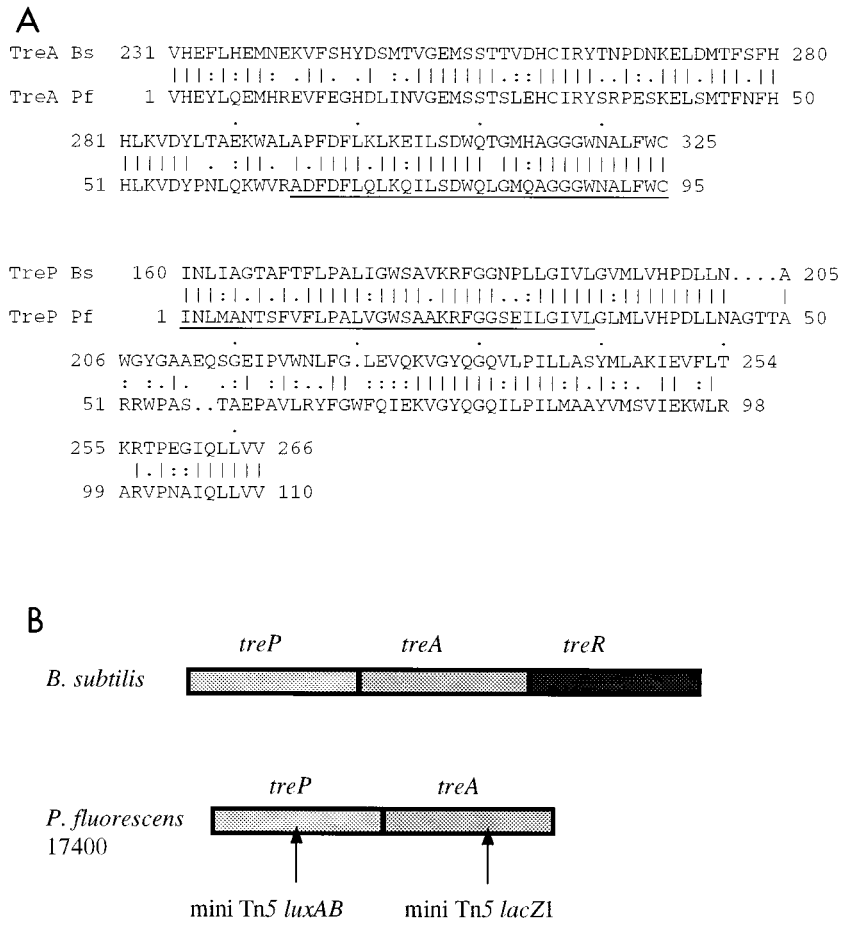

FIG. 2. (A) Similarity between TreA and TreP of *B. subtilis* and the amino acid residues encoded by the partial sequence obtained from the 2-kb PCR fragment which was amplified with primers designed from the IPCR sequences obtained from Pyt6 and Pyt61 mutants, respectively. The underlined sequences correspond to the residues encoded by the sequenced IPCR fragments from Pyt6 and Pyt61. Vertical lines indicate identity. Bs, *B. subtilis*; Pf, *P. fluorescens* ATCC 17400. (B) Organization of the *treP*, *treA*, and *treR* cluster in *B. subtilis* and the proposed organization of *P. fluorescens* ATCC 17400 *treP* and *treA* open reading frames, indicating the two transposon insertions.

cose, and not induced under conditions of high osmolarity (17). The *treA* gene from *E. coli* with 70% identity to the Pyt6 sequence is induced under conditions of high osmolarity but is not induced by trehalose (4).

Physiological characterization of Pyt6 mutant. In contrast to the wild type, the Pyt6 mutant was not able to grow in the presence of trehalose as the sole carbon source or under conditions of high osmolarity in liquid medium, as induced by 500 mM NaCl or mannitol, although it was able to grow on solid medium with the same concentration of NaCl or mannitol.

β-Galactosidase in Pyt6 was induced by all of the concentrations of trehalose tested down to 1 μM and by high osmolarity (250 to 500 mM NaCl or mannitol), and it was not repressed by glucose (2% [wt/vol]). The levels of β-galactosidase production in Pyt6 in the presence of 0.2% trehalose (13.25 Miller units at the optical density at 600 nm [OD_{600}]) and 10% *Pythium debaryanum* culture supernatant (13.68 Miller units at OD_{600}) were comparable, whereas the level in the control medium was 0.98 Miller units at OD_{600}. The treatment of the *Pythium* supernatant with commercial trehalase resulted in the disappearance of the spot on the TLC plate with the same R_f as that of trehalose and in a 1,000-fold decrease in its potential for *treA* induction with respect to that of untreated supernatant (data not shown).

Since sugars, such as glucose, sucrose, and maltose, did not induce the expression of β-galactosidase at the same concentration, the induction of *lacZ* is not a general response to sugars but is considered to be specific to trehalose.

Transposon mutagenesis by using mini-Tn*5* *luxAB* and IPCR. To characterize a possible sensory or transport pathway of trehalose in *P. fluorescens* ATCC 17400, a second transposon mutagenesis was done in Pyt6 with a mini-Tn*5* *luxAB* Tc^r transposon (10). A double mutant (Pyt61) that did not produce β-galactosidase in the presence of trehalose was obtained. This mutant also failed to produce β-galactosidase under conditions of high osmolarity (in CAA-agar and in the presence of 500 mM mannitol or NaCl). The flanking region of the mini-Tn*5* *luxAB* was isolated by IPCR (Fig. 1), and the amino acid sequence (34 residues) encoded by the IPCR fragment showed 67.6% identity to enzyme II^{tre} (TreP) of *B. subtilis* (Fig. 2), which encodes a subunit of the trehalose phosphotransferase which participates in the transport of trehalose in the cell (28). In *B. subtilis*, the gene encoding TreP is located upstream of the *treA* gene, which encodes trehalase (17). IPCR from either the Pyt6 (with the *lacZ* primers) or Pyt61 (with the *lux* primers) mutant resulted in single-band amplification, indicating a single insertion for each transposon.

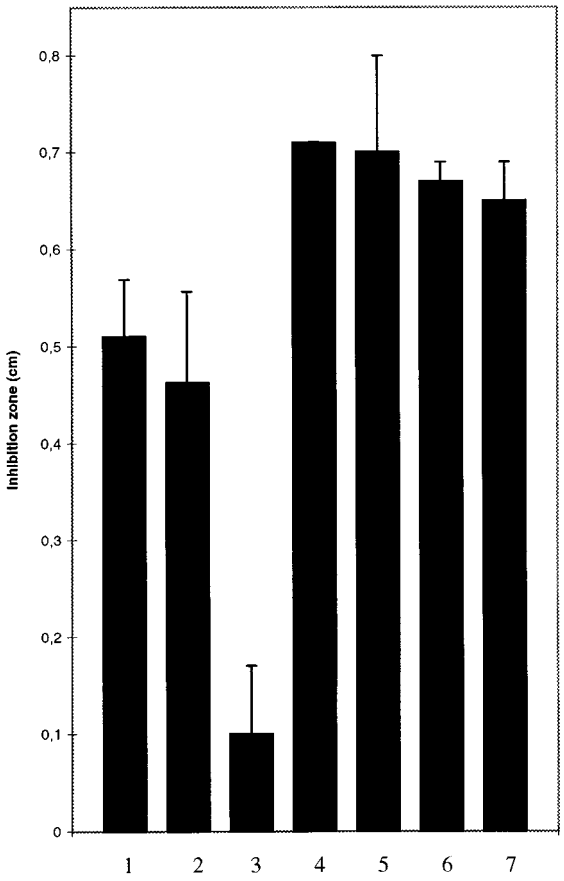

FIG. 3. *Pythium debaryanum* growth inhibition by wild-type *P. fluorescens* ATCC 17400 (lanes 1, 4, and 5), Pyt6 (lanes 2 and 6), and Pyt61 (lanes 3 and 7) under conditions of normal osmolarity (lanes 1 through 3), in the presence of trehalose (lane 4), and under high-osmolarity conditions induced by 500 mM mannitol (lanes 5 through 7). Error bars indicate standard errors.

Amplification of part of *treA* and *treP*. In order to explicitly define the arrangement of the two transposon insertions and the identity of the second insertion, PCR amplification with primers constructed from the sequences of the two IPCR fragments was done with *P. fluorescens* ATCC 17400 DNA as the template. PCR resulted in the amplification of a 2-kb fragment, which corresponds in size to the distance between the regions with similarity to the primers used and to the arrangement of *B. subtilis treA* and *treP* genes. The 2-kb fragment was cloned into pCRII and sequenced partially from both ends. The sequence from each end was identical to the sequences obtained from IPCR fragments; furthermore, the amino acid sequences encoded by both ends showed 80 and 79% similarity with TreP and TreA of *B. subtilis*, respectively (Fig. 2).

***Pythium* antagonism.** The ability of wild-type *P. fluorescens* ATCC 17400 to restrict the growth of *Pythium debaryanum* was increased by 40% in CAA medium containing trehalose; a similar increase in antagonism was observed for the wild type in a medium containing 500 mM mannitol (Fig. 3). The mutation in *treA* showed no significant effect on the ability of Pyt6 to restrict the growth in vitro of the phytopathogen *Pythium debaryanum*, whereas the double mutant (Pyt61) completely lost its antagonizing activity against the phytopathogen (Fig. 3). However, the in vitro antagonizing capacity of Pyt61 was fully restored under the high osmolarity induced by 500 mM mannitol (Fig. 3). Mannitol was used because *Pythium debaryanum* did not grow in the presence of 500 mM NaCl.

DISCUSSION

Fluorescent *Pseudomonas* spp. can produce several compounds that contribute to the suppression of plant root pathogens (12, 25). Several of these compounds or the genetic loci essential for their production have previously been characterized; these include pyoluteorin from *P. fluorescens* Pf-5 and *P. fluorescens* CHAO (18, 32), 2,4-diacetylphloroglucinol from various *Pseudomonas* strains (3, 33), and phenazine from *P. fluorescens* (38) and *Pseudomonas aureofaciens* (26). An unidentified antifungal compound from *P. aureofaciens* was also reported (6). The production of these compounds was found to be under the regulation of common activators, such as GacA from *P. fluorescens* CHAO (29) and ApdA (LemA) from *P. fluorescens* Pf-5 (8); these proteins seem to be part of a classical two-component sensor-activator regulatory system. The fact that the production of an antifungal compound(s) was regulated through these activators suggested the existence of a sensory pathway in *Pseudomonas* spp., although the signals in these cases have not yet been identified. In the case of phenazines, it has previously been shown that their production is induced by the cell density signal molecule homoserine lactone via the *phzI* and *phzR* genes (27). The existence of a signaling-sensory pathway is advantageous for the bacterium because it ensures that the antifungal compound is produced only under specific conditions (high cell density or presence of a competitor).

The mutants generated in this study expressed β-galactosidase only in the presence of *Pythium debaryanum* growth culture supernatant. Isolation and sequencing of the flanking region of the transposon insertion in mutant Pyt6 by IPCR led to the identification of a gene, *treA*, that codes for trehalase. Trehalose is a nonreducing sugar and the most widely distributed disaccharide in fungi, where it is considered to be one of the essential storage compounds in vegetative cells and spores (36). Many organisms accumulate trehalose when they are exposed to adverse growth conditions, such as desiccation, high osmolarity, frost, and heat (35). As such, trehalose is a good indicator of a particularly fungal presence; it might also be exploited as a carbon source by rhizobacteria. In this regard, a recent study (13) demonstrated that the mycorrhizosphere of Douglas fir exerted positive selection for *P. fluorescens* type I strains. They hypothesized that trehalose, produced by the mycorrhiza, was responsible for this preferential association of type I strains, which are able to utilize trehalose (13).

The transcription of *treA* in *P. fluorescens* ATCC 17400 was found to be regulated by high osmolarity and by the presence of external trehalose. In *E. coli*, *treA* is induced only under conditions of high osmolarity (4, 16), whereas the *B. subtilis treA* gene is induced only by external trehalose (17). Unlike the *treA* genes of both *E. coli* and *B. subtilis*, *P. fluorescens* ATCC 17400 *treA* was not repressed by glucose (2% [wt/vol]). These observations indicate that the regulation of trehalose degradation in *Pseudomonas* spp. is different. Endogenously synthesized trehalose acts as an osmoprotectant in *E. coli* grown under conditions of high osmolarity, and osmotolerance in *E. coli* is also associated with induction of the *treA* gene (35). Unlike the wild type, our *P. fluorescens* Pyt6 *treA* mutant was not able to grow under the same osmotic conditions, indicating the involvement of trehalose and the *treA* gene in protection against high osmolarity in *P. fluorescens* ATCC 17400.

Trehalose was detected in the *Pythium debaryanum* culture. Its presence may have been due to the lysis of fungal hyphae,

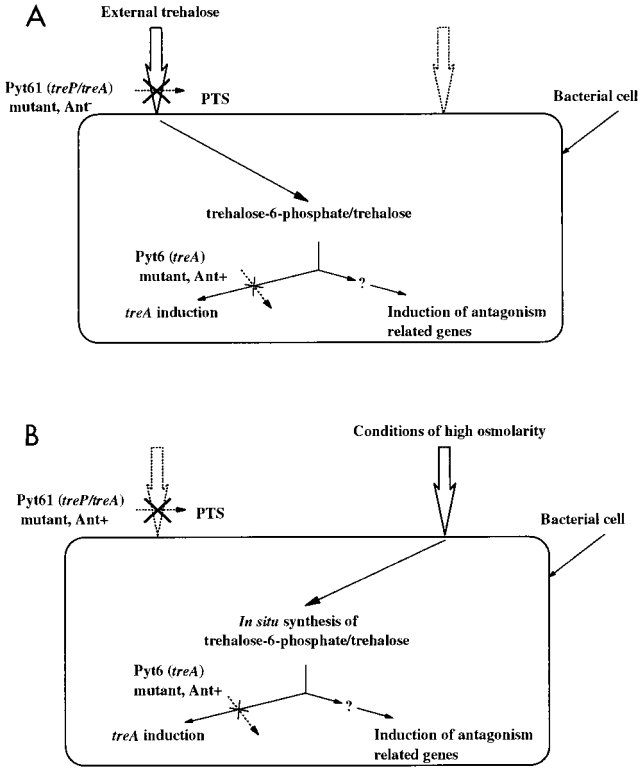

FIG. 4. Models for the role of trehalose in the induction of antagonism-related genes in *P. fluorescens* ATCC 17400 in the presence of external trehalose (A) and under conditions of high osmolarity (B). Crossed dotted arrows indicate the effects of mutations. PTS, phosphotransferase transport system. See text for details.

resulting in its release to the surrounding environment. The reduced ability of the trehalase-treated *Pythium* supernatant to induce *treA* suggests that the trehalose released from the phytopathogen is the major inducer of *treA* in the *Pythium* supernatant. A second mutation in *treP*, whose product may be required for trehalose uptake by the cell, yielded a nonantagonistic mutant. This suggests a role for trehalose in the induction of antagonism. Moreover, the low concentration of trehalose needed for *treA* induction (1 μM) is consistent with its possible role in initiating an induction pathway.

The ability of Pyt6 to antagonize *Pythium debaryanum* shows that trehalase has no direct role in the antagonism mechanism; however, it has roles in the utilization of trehalose as a carbon source and in osmotolerance. The antagonistic activity of Pyt6 excludes the presence of any open reading frame downstream of *treA* which could initially be involved in *Pythium* inhibition. Our results show that the *treP* gene is upstream of *treA*, similar to what is found in *B. subtilis* (17); this explains why the insertion of Tn*5* in *treP* precludes the transcription of *treA*, resulting in the inability of the Pyt61 mutant to express the *lacZ* reporter gene under any of the inducing conditions tested (trehalose and high osmolarity). The complete inability of the double mutant Pyt61 (*treA treP*) to inhibit the growth of *Pythium debaryanum*, except under conditions of high osmolarity, suggests that the presence of trehalose in the cell either by active internalization, for which *treP* expression would be needed (17), or via the induction of its biosynthesis under conditions of high osmolarity is essential for the induction of genes involved in *Pythium* antagonism (Fig. 4). This was corroborated by the 40% increase in the ability of the wild type to restrict *Pythium debaryanum* growth in the presence of trehalose. Bacteria that use trehalose as an osmoprotectant produce and excrete trehalose to the medium (4). This means that the amount of trehalose produced under conditions of high osmolarity is enough to induce the trehalase gene and most probably other genes. Moreover, in the double mutant Pyt61, the level of internal trehalose under conditions of high osmolarity must be higher than that in the wild type since it cannot produce trehalase. Although the function of *treP* is supported only by sequence data, the fact that the antagonizing ability of Pyt61 can be restored under conditions of high osmolarity (when trehalose is produced and accumulated and the organization of *treP* is conserved in relation to *treA*) supports the suggested function for TreP in trehalose transport.

The dual roles of trehalose in osmoprotection and in increasing the ability of *P. fluorescens* ATCC 17400 to restrict the growth of possibly competitive thizosphere microorganisms are in accordance with the observation that rhizosphere osmolarity exceeds that of bulk soil (23). These results indicate that trehalose may act as a signal and as an internal sensor in the cell which initiates signal transduction in *P. fluorescens*, leading to the induction of antagonism towards *Pythium debaryanum.*

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