Phenotypic comparison between rhizosphere and clinical isolates of *Burkholderia cepacia*

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The phenotypic characteristics of four *Burkholderia cepacia* strains isolated from the rhizosphere and the clinical environment were compared. Tests included optimum growth temperature, utilization of carbon sources, production of HCN, indole-3-acetic acid (IAA) and siderophores, proteolytic activity, nitrogen fixation, inhibition of some phytopathogenic fungi, adherence to human mucosal and plant root epithelia, and greenhouse-based plant-growth promotion experiments using cucumber (*Cucumis sativus*). Results indicated that the strains of *B. cepacia* isolated from the rhizosphere differ markedly from their clinical counterparts. Strains isolated from the rhizosphere grew over a wider temperature range, fixed nitrogen and produced IAA, did not produce proteases, displayed a wider antibiosis against the phytopathogenic fungi studied, did not adhere to human uroepithelial cells, promoted growth of *C. sativus* and only produced a hydroxamate-like siderophore. In contrast, clinical isolates could not fix nitrogen or produce IAA, produced proteases, adhered to human uroepithelial cells, did not promote the growth of *C. sativus* and, in addition to a hydroxamate-like siderophore, produced pyochelin and salicylate siderophores. All four isolates exhibited the ability to adhere to the root tissue of *C. sativus* and were unable to produce HCN.

Keywords: *Burkholderia* (*Pseudomonas*) *cepacia*, phenotype, siderophores, pathogenicity, adherence

INTRODUCTION

Burkholderia cepacia (basonym *Pseudomonas cepacia*), formerly included in the rRNA homology group II of the genus *Pseudomonas* (Palleroni, 1984), is now recognized as the type species of the new genus *Burkholderia* on the basis of cellular lipid and fatty acid composition, 16S rRNA sequences, DNA–DNA homology values, and phenotypic characteristics (Yabuuchi *et al.*, 1992). Although originally described as the causal agent of soft rot of onions (Burkholder, 1950), *B. cepacia* has later been reported to be a widely spread species in different soil and root samples (Hebbar *et al.*, 1992a). In recent years, there has been considerable interest in using *B. cepacia* as a biocontrol agent because of its ability to antagonize and repress soilborne plant pathogens (Homma *et al.*, 1990; Hebbar *et al.*, 1992a, b, c; McLoughlin *et al.*, 1992). Some biotypes of *B. cepacia* have been implicated in biodegradation of pesticides (Folsom *et al.*, 1990; Kilbane *et al.*, 1983). Occurrence and distribution of *B. cepacia* are not confined solely to environmental sources. This species has

also been isolated from clinical specimens of human origin, particularly from cystic fibrosis patients (Goldmann & Klinger, 1986), as well as from contaminated medical devices and solutions employed in hospital practice (Gilardi, 1983). *B. cepacia* is characterized by an extreme resistance to antimicrobial agents (Gilardi, 1971), and by the ability to grow in minimal media including purified water systems (Carson *et al.*, 1973). These factors have led to the emergence of *B. cepacia* as a serious problem in health care product contamination (Craven *et al.*, 1981), nosocomial infections (Martone *et al.*, 1987), and recalcitrant infections of cystic fibrosis patients (Isles *et al.*, 1984; McKevitt & Woods, 1984; Thomassen *et al.*, 1985).

Strains of *B. cepacia* isolated from plant, soil and human samples have previously been distinguished according to differences in bacteriocin production, maceration of onion slices, hydrolysis of low pectate agar and the size of resident plasmids (Gonzalez & Vidaver, 1979). Lennon & DeCicco (1991) compared strains of *B. cepacia* from

clinical, pharmaceutical-industrial, and environmental origin for the presence of plasmid DNA. In contrast to the environmental group, the clinical isolates varied considerably in the number and size of harboured plasmids which were cryptic and unrelated to the source of strains. The aim of the present study was to compare *B. cepacia* strains of clinical and rhizosphere origin for characters relevant to virulence in the human host as well as factors involved in biological control and plant-growth promotion. This will enable the selection of strains that may be used safely and effectively as biocontrol agents.

METHODS

Microbial cultures. The different *B. cepacia* strains used in this study were: *B. cepacia* PHP7 and TVV75, isolated in France and Vietnam from the rhizosphere of maize and rice, respectively (obtained from T. Heulin and Tran Van Van, Centre Pedologie Biologique, CNRS, Nancy, France), and the clinical isolates 7/25 and 9/27, isolated, respectively, from urine and sputum of two patients with cystic fibrosis from the University Hospital 'Policlinico Umberto I' (Rome, Italy). All the strains were identified with the API 20NE (Bio-Mérieux) system. *Pseudomonas aeruginosa* PAO1 (ATCC15692) was obtained from the American Type Culture Collection (Rockville, MD, USA).

Fungal cultures of *Fusarium culmorum* ISPAVE F125, *F. moniliforme* ISPAVE F44, *F. oxysporum* ISPAVE F45 and *F. graminearum* ISPAVE F202 were kindly provided by L. Corazza, Phytopathology Institute, MAF, Rome, Italy. The plant-pathogenic fungi *F. solani* EF5 and *Rhizoctonia solani* EF9 were obtained from the Plant Pathology Institute, University of Naples, Italy. Fungi were maintained on potato dextrose agar (PDA, Difco) with monthly transfers.

Glassware preparation. For experiments requiring low-iron [Fe(III)] conditions, the glassware was rendered Fe(III)-free by 24 h treatment with 6 M HCl, rinsed once in distilled water and finally rinsed in deionized water from a Milli Q system (Millipore) before sterilization. This high quality deionized water was also used in all growth media and in the preparation of all solutions.

Effect of temperature on growth rates and utilization of carbon sources. For growth rate determinations, cultures of *B. cepacia* strains were grown in M9 (Guterman, 1973) with glucose (10 g l^{-1}) as the carbon source. Cultures were incubated at 21 °C, 28 °C, 37 °C and 42 °C, with rotary shaking at 200 r.p.m. in a Kühner Clim-O-Shake, model ISF-1-V. Growth was monitored by measuring optical density at 620 nm at 1 h intervals.

Carbon source utilization assays were performed by adding 20 mM organic substrates to the M9 basal salt solution.

Siderophore synthesis. Siderophore production was preliminarily detected using chrome azurol-S (CAS) plates as described by Schwyn & Neilands (1987). The CAS assay is a universal assay for Fe(III)-chelators and allows detection of siderophores independently of their structure. The presence of a Fe(III)-chelator is indicated by the colour change of a blue-Fe(III)-CAS complex, resulting in a yellow-gold halo around colonies growing on CAS agar plates.

Siderophore production was also tested in culture supernatants by means of specific colorimetric tests. Cultures of *B. cepacia* were grown for about 48 h at 37 °C and 150 r.p.m. either in DCAA (Visca *et al.*, 1992b) or in SM9 (Visca *et al.*, 1992c) media. Removal of trace amounts of Fe(III) was achieved by treatment

with Chelex-100 resin (Bio-Rad). The activated resin was added to the medium at 5 g l^{-1} , stirred at 4 °C for 20 h, and removed by filtration through Whatman no. 4 filter paper (Clifton). Hydroxamate-like siderophores were detected using the test of Csáky (1948), with hydroxylamine hydrochloride as the standard. Catechol-type siderophores were measured using the test developed by Arnow (1937), with 2,3-dihydroxybenzoic acid as the standard. Pyochelin, cepabactin and salicylic acid were extracted and visualized as previously described by Cox & Graham (1979), Meyer *et al.* (1989) and Visca *et al.* (1993), respectively. For pyochelin and salicylic acid detection, supernatants from cultures grown in DCAA were adjusted to pH 2.0 with HCl, extracted twice with 0.4 vols ethyl acetate and concentrated by rotary evaporation. For cepabactin determination, supernatants from cultures in succinate mineral medium (Meyer *et al.*, 1989) were acidified with HCl to pH 3.0, and extracted twice with 0.2 vols chloroform. The chloroform extracts were washed twice with water, filtered on anhydrous sodium sulphate, and evaporated under vacuum to dryness. The dry residues obtained by both procedures were dissolved in a small volume of methanol and applied to a silica gel G thin layer chromatography plate (TLC), using chloroform/acetic acid/ethanol (90:5:2.5, by vol.) as the development solvent. Siderophores were characterized by chromatographic mobility (R_F), fluorescence emission, and chemical reactivity when sprayed with 0.1 M FeCl_3 in 0.1 M HCl or with the ammoniacal silver nitrate reagent for thiazolidine rings (Visca *et al.*, 1992c, 1993).

Indole-3-acetic acid (IAA) production. *B. cepacia* strains were grown for 48 h in SM9 supplemented with $100 \mu\text{g}$ of L-tryptophan ml^{-1} and $100 \mu\text{M}$ FeCl_3 . The supernatant was recovered by cell centrifugation for 20 min at 5500 g, adjusted to pH 2.5, and applied to a C-18 Sep-Pak cartridge (Sagee *et al.*, 1986). The cartridge was washed with distilled water and IAA was eluted with methanol containing $10 \mu\text{g}$ butylated hydroxytoluene ml^{-1} , as described by Forni *et al.* (1992). The eluate containing IAA was evaporated under vacuum to dryness and dissolved in 200 μl methanol. IAA was determined using the Salkowski reagent (Ehmann, 1977).

Cyanide production. Cyanogenesis was detected according to Castric & Castric (1983), using HCN-sensitive Whatman 3MM paper disks soaked in the HCN detection reagent of Feigl & Anger (1966). *P. aeruginosa* PAO1 was used as the cyanogenic control.

Protease activity. Protease activity was first qualitatively assayed in the API 20NE test. Quantitative determinations were made on supernatants of 30 h cultures in SM9 using the colorimetric assay described by Tomarelli *et al.* (1949), with azoalbumin as the substrate. One unit of proteolytic activity was defined as the amount of enzyme hydrolysing $1 \mu\text{g}$ of azoalbumin in 1 min at 37 °C and was calculated assuming a $A_{440}^{1\%}$ for azoalbumin of 34.

N_2 fixation. Nitrogenase activity was determined by monitoring the reduction of acetylene to ethylene by gas chromatography (Hardy *et al.*, 1973; Lifshitz *et al.*, 1986). Bacterial cultures were grown in 125 ml stoppered flasks containing 15 ml of the following medium (g l^{-1}): $1.0 \text{ KH}_2\text{PO}_4$, $1.74 \text{ K}_2\text{HPO}_4$, 0.2 MgSO_4 , 0.1 NaCl , 0.02 CaCl_2 , 0.015 FeCl_3 , 0.002 NaMoO_4 , $0.08 \text{ yeast extract}$ (Difco), 5.0 glucose , pH 7.0. Cultures were grown on a rotary shaker (150 r.p.m.) at 28 °C for 24 h, under an atmosphere of 99.9% (v/v) N_2 and 0.1% O_2 , and cells were collected by centrifugation (10000 g, 4 °C, 20 min). The pellets were washed twice with the same medium and 100 μl aliquots were transferred to 15 ml of fresh medium to give an initial concentration of approximately $5.5 \times 10^5 \text{ c.f.u. ml}^{-1}$. Following an additional 4 h incubation, 10% of the volume of the

atmosphere of each flask was replaced with acetylene, and the incubation was continued for an additional 48 h under the same conditions. The ethylene concentration in the flasks was assayed by means of a gas chromatograph (model 8410, Perkin-Elmer) equipped with a flame ionization detector and a Porapak N 80/100 mesh column [1 m by 0.125 in. (ca. 3.2 mm)]. Aliquots of 250 µl were used for the assay. The operating conditions of the gas chromatograph were as follows: oven temperature, 90 °C; injection-detector temperature, 190 °C; carrier gas, nitrogen at a flow rate of 40 ml min⁻¹. All cultures were assayed in triplicate and compared with a standard ethylene curve.

Screening for *in vitro* antibiosis. An *in vitro* assay was performed to test the ability of *B. cepacia* strains to suppress fungal phytopathogens on PDA and King's B (KB) (King *et al.*, 1954) media. An agar plug with mycelium was placed at the centre of the agar plate and bacterial strains were streaked near the edge of the petri dish at fixed positions. Plates were incubated at 28 °C, and the growth of fungal phytopathogens was measured at 48 h intervals. The percentage inhibition of the fungal growth was calculated with the following formula: $[(R_1 - R_2)/R_1] \times 100$, where R_1 is the farthest radial distance grown by the fungus in the direction of the antagonist (a control value), and R_2 is the distance grown on a line between the inoculation positions of the fungal phytopathogen and the antagonist strain of *B. cepacia* (Whipps, 1987).

Adherence to human uroepithelial cells. The adherence assay was performed with P-phenotype uroepithelial cells collected by centrifugation (2000 g, 10 min, 4 °C) from fresh morning urine of a single female healthy donor and washed three times in phosphate buffered saline (PBS, Flow Laboratories), pH 7.2. The number of epithelial cells per ml was calculated by counting in a Burkner haemocytometer and adjusted to a concentration of about 2×10^5 cells ml⁻¹ with PBS. The adherence of *B. cepacia* to uroepithelial cells was examined by mixing together 0.5 ml samples of a standard bacterial suspension in PBS (10^8 c.f.u. ml⁻¹) and 0.5 ml of epithelial cells (10^5 cells ml⁻¹) in a water bath for 45 min at 37 °C. Cells were separated from unattached bacteria by continuous vacuum washing with PBS through 8 µm Nucleopore filters. The filter membranes were gently pressed against glass slides which had previously been coated with a thin albumin layer. Slides were fixed in absolute methanol and stained with Giemsa. The total adherent bacteria per epithelial cell were microscopically determined. In each experiment, 40 epithelial cells were examined and duplicate determinations were performed. Strains showing 30 or more attached bacteria per epithelial cell were classified as adhesive (Visca *et al.*, 1992a). Whenever this procedure was used, control epithelial cell preparations without exogenous bacteria were treated in the above manner to detect any indigenous organism which may have been present.

Seed sterilization. Commercial cucumber seeds (*Cucumis sativus* L. cv. Marketmore) were obtained from Four s.r.l. (Bolzano, Italy). Surface sterilization was obtained as follows: seeds were submerged for 2 min in 95% (v/v) ethanol, rinsed five times with sterile distilled water, treated with 0.56% H₂O₂ for 16 h, and finally washed three times with sterile distilled water. Surface-sterilized seeds were germinated aseptically in the dark on nutrient agar (NA, Difco) plates for 48 h at 25 °C, to check for the presence of contaminating micro-organisms.

Adherence studies to *C. sativus* roots. Seedlings were transferred onto Petri dishes containing a filter paper soaked with sterile distilled water. After 3 d of growth in a controlled environment growth chamber at 25 °C with a 16/8 h light/dark cycle and 80% relative humidity, the roots were used in the attachment assay. The assay system consisted of a glass tube

(160 × 22 mm diam.) filled with 50 g of a sandy soil-vermiculite mixture (3:1, v/v) saturated with 10 ml of water. This mixture was used to facilitate soil removal from the roots of seedlings and minimize root damage. The tubes were sterilized by autoclaving at 127 °C for 30 min on two consecutive days. One seedling was transferred to each tube, and inoculated with 10^6 *B. cepacia* cells which had been grown in KB for 24 h at 28 °C on a gyratory shaker (150 r.p.m.). The tubes were capped and incubated in a growth chamber at a constant temperature of 25 °C, with a 16/8 h light/dark cycle, and an average light intensity of 340 µE m⁻² s⁻¹. After 1 week, the seedlings were aseptically removed from the tubes, and each root was sequentially immersed into a series of 5 50 ml tubes containing 25 ml PBS, pH 6.0. Samples (0.1 ml) of each washing step were appropriately diluted and plated on NA. Bacterial colonies were counted after 24 h incubation at 28 °C. After the last PBS wash, the roots were excised with sterile forceps, crushed in a sterile mortar, and the number of bacteria was determined by plate counts on NA. To study bacterial penetration into the root, the seedlings were inoculated as described above, and after one week of growth were removed from the tubes. To kill attached bacteria, roots were immersed in 3% (w/v) calcium hypochlorite for 3 min, washed thoroughly in sterile distilled water, and crushed in a sterile mortar. The number of bacteria was estimated as above.

Pot experiments. Three-day-old *C. sativus* sterile seedlings were transferred aseptically to sterile 3 l pots with a diameter of 20 cm (4 seeds per pot), and inoculated with 10^6 viable cells of *B. cepacia* grown to the late exponential phase in SM9. The soil utilized was an alkaline Fe(III)-poor substrate consisting of a mixture of calcareous soil and sand (3:1, w/w), pH 8.8, containing 0.07% nitrogen, 63% calcium, 0.47% potassium, traces of phosphorus, no carbon, no assimilable iron. To study the plant-growth-promoting activity of *B. cepacia* strains in the absence of competing microflora, the soil was sterilized by autoclaving twice at 127 °C for 30 min. Greenhouse treatments were as follows: (i) uninoculated controls, (ii) inoculated with *B. cepacia* PHP7, (iii) inoculated with *B. cepacia* TVV75, (iv) inoculated with *B. cepacia* 7/25, (v) inoculated with *B. cepacia* 9/27. Pots were watered twice a week alternately with 250 ml of sterile deionized water, and 250 ml of mineral solution (Mitchell & Livingstone, 1968) for a total of 10 applications. The plants were grown in a greenhouse at 25 °C during the day and 16 °C at night with a 16/8 h light/dark period. Each treatment was replicated 16 times and plants were harvested 40 d after germination. The root and shoot tissues of each plant were dried at 85 °C for 24 h and weighed. Data were analysed using Duncan's Multiple-Range Test.

RESULTS

Parameters influencing the growth of *B. cepacia* strains

To study the carbon source utilization profile of *B. cepacia* strains a number of carbon sources released by the roots of plants (Rovira, 1965) were added to the M9 basal salt solution at a final concentration of 20 mM. All the strains were able to utilize succinic acid, malic acid, citric acid, sucrose, xylose, arabinose, cellobiose, mannose, mannitol, adipate, caprate, phenylacetate, lactose, galactose, glucose, gluconate, *N*-acetyl-D-glucosamine, leucine, asparagine, glutamic acid, aspartic acid, proline, arginine, but did not grow on oxalic acid and maltose as the sole carbon source. Lysine utilization was shown for clinical strains only.

Fig. 1. Effect of temperature on growth rates (μ) of *B. cepacia* strains. ■, PHP7; ▨, TVV75; ▩, 7/25; □, 9/27.

The growth rates of the *B. cepacia* isolates at temperatures ranging from 21 °C to 42 °C were also determined. Fig. 1 shows that the two clinical isolates of *B. cepacia* display maximum growth at 42 °C, whereas rhizosphere strains gave different responses to increasing temperatures. The highest growth rate of *B. cepacia* PHP7 was at 37 °C, whereas for *B. cepacia* TVV75 the maximum growth rate was observed at 42 °C. It is worth noting that the growth rates at 21 °C were significantly higher for rhizosphere isolates than for clinical ones.

Synthesis of siderophores

The four *B. cepacia* strains of clinical and rhizosphere origin appeared to synthesize Fe(III)-chelator(s) as shown

by a strong positive reaction in the assay of Schwyn & Neilands (1987). Culture supernatants of the *B. cepacia* strains grown for 48 h in Chelex-100-treated DCAA or succinate mineral medium were tested for the presence of catechol- and hydroxamate-type siderophores. Strains PHP7, TVV75, 7/25 and 9/27 did not release catechol-like compounds, but produced a Fe(III)-repressible, hydroxamate-like molecule, the level of which approached 210 μ M, 156 μ M, 220 μ M and 240 μ M, respectively. The addition of 100 μ M FeCl₃ to the Fe(III)-poor media strongly repressed hydroxamate synthesis. TLC analysis of acidified ethyl acetate extracts from culture supernatants in DCAA of strains PHP7, TVV75, 7/25 and 9/27 showed that, similarly to the *P. aeruginosa* PAO1 control, clinical *B. cepacia* isolates produced pyochelin and its biosynthetic precursor salicylic acid, in response to conditions of limiting Fe(III) (Fig. 2). In contrast, rhizosphere isolates were unable to synthesize either pyochelin or salicylate. Pyochelin (R_F values approximately 0.35 and 0.40), migrated as two distinct bands (Visca *et al.*, 1993), displayed grey-green fluorescence emission under UV light, turned brown-red when sprayed with acidic FeCl₃, and reacted with the ammoniacal silver nitrate reagent for thiazolidine groups yielding black silver mercaptide salts. Salicylic acid (R_F approximately 0.70, in agreement with Visca *et al.*, 1993) had a blue-fluorescence under UV light and turned violet after spraying with FeCl₃, but did not react with ammoniacal silver nitrate reagent. Salicylate and, to a lesser extent, pyochelin production were repressed in succinate mineral medium, as already reported for pyochelin synthesis by *P. aeruginosa* PAO1 (Cox & Graham, 1979). The R_F value of the hydroxamate-positive compound in the system used was 0.18–0.20 as determined by fractionation of the silica gel and analysis in the Csáky assay (1948) of the methanol-eluted compound.

Fig. 2. Thin-layer chromatograms of acidified ethyl acetate and chloroform extracts from cultures of *P. aeruginosa* PAO1 and *B. cepacia* strains grown in Fe(III)-deficient and Fe(III)-rich media. In Fe(III)-rich conditions FeCl₃ was added to DCAA and succinate medium at a final concentration of 100 μ M (see Methods). Lanes: 1 and 1*, *P. aeruginosa* PAO1 in Fe(III)-poor DCAA and succinate medium, respectively; 2 and 2*, *B. cepacia* PHP7 in Fe(III)-poor DCAA and succinate medium, respectively; 3 and 3*, *B. cepacia* PHP7 in Fe(III)-rich CAA and succinate medium, respectively; 4 and 4*, *B. cepacia* TVV75 in Fe(III)-poor DCAA and succinate medium, respectively; 5 and 5*, *B. cepacia* TVV75 in Fe(III)-rich CAA and succinate medium, respectively; 6 and 6*, *B. cepacia* 7/25 in Fe(III)-poor DCAA and succinate medium, respectively; 7 and 7*, *B. cepacia* 7/25 in Fe(III)-rich CAA and succinate medium, respectively; 8 and 8*, *B. cepacia* 9/27 in Fe(III)-poor DCAA and succinate medium, respectively; 9 and 9*, *B. cepacia* 9/27 in Fe(III)-rich CAA and succinate medium, respectively. (a) Chromatograms were visualized by exposure to UV light; (b) chromatograms developed by spraying with the ammoniacal silver nitrate reagent for thiazolidine groups; (c) chromatograms developed by spraying with 0.1 M FeCl₃ in 0.1 M HCl. Abbreviations: O, origin of migration; Pchl and PchlI, two forms of pyochelin; Sal, salicylate.

Fig. 3. Antagonistic activity of *B. cepacia* strains against fungal phytopathogens on PDA (a) and KB (b) media. Inhibition by *B. cepacia* PHP7 (■), TVV75 (▨), 7/25 (▩) and 9/27 (□). The percentage inhibition of the growth of plant-pathogenic fungi was calculated according to Whipps (1987). F.s., *Fusarium solani*; F.g., *F. graminearum*; F.c., *F. culmorum*; F.o., *F. oxysporum*; F.m., *F. moniliforme*; R.s., *Rhizoctonia solani*.

Table 1. Adherence of *B. cepacia* strains to cucumber roots

Each value represents mean $\pm$ SD ($n = 3$)

Strain	Origin	No. of cells per seedling initially ($10^{-6} \times$ c.f.u. $\pm$ SD)	No. of cells per fresh weight of root* ($10^{-7} \times$ c.f.u. g $^{-1}$ $\pm$ SD)
PHP7	Rhizosphere	1.7 $\pm$ 0.4	1.4 $\pm$ 0.8
TVV75	Rhizosphere	5.8 $\pm$ 0.7	2.3 $\pm$ 0.7
7/25	Human	1.8 $\pm$ 0.4	2.1 $\pm$ 0.6
9/27	Human	1.5 $\pm$ 0.3	3.1 $\pm$ 0.7

* No bacteria were detected inside the cucumber roots after $\text{Ca}(\text{ClO})_2$ surface sterilization.

Production of IAA, HCN and protease(s)

Differentiation of strains by production of IAA revealed production in the rhizosphere isolates only; the level of IAA in late-stationary-phase cultures approached 0.6 and 0.4 $\mu\text{g ml}^{-1}$ for strains PHP7 and TVV75, respectively. None of *B. cepacia* strains produced HCN, whereas the

control strain *P. aeruginosa* PAO1 did. Moreover, no proteolytic activity was detected in culture supernatants of rhizosphere isolates PHP7 and TVV75; conversely, clinical strains 7/25 and 9/27 showed relatively high proteolytic activity. The level of protease(s) in culture supernatants of strains 7/25 and 9/27 grown in SM9 were 0.69 and 0.39 U ml^{-1} , respectively.

Nitrogenase activity

Under standard conditions the rhizosphere isolates PHP7 and TVV75 showed a N_2 -fixing activity of 620 and 715 $\text{nmol C}_2\text{H}_4 \text{ h}^{-1}$ produced per flask when grown in suspension culture under 0.1% O_2 . C_2H_2 reduction was not shown by clinical strains 7/25 and 9/27 under the same experimental conditions.

Suppression of fungal pathogens

The antagonistic activity of *B. cepacia* PHP7, TVV75, 7/25 and 9/27 against six fungal phytopathogens was tested in a plate assay using KB and PDA media. The results shown in Fig. 3 indicate that all *B. cepacia* strains were able to restrict the growth of several fungal pathogens on both KB and PDA, although to differing degrees. The inhibition was more evident on KB, whereas on PDA the effect was strongly reduced. Only strain PHP7 inhibited all six of the fungi tested and exerted a generally higher antagonistic effect in comparison with the other *B. cepacia* strains. The most sensitive fungi were *F. graminearum*, *R. solani*, *F. oxysporum*, and *F. culmorum* on KB, and *F. culmorum* and *F. graminearum* on PDA.

Adherence to human uroepithelial cells

Adherence to uroepithelial cells was demonstrated for the clinical strains 9/27 and 7/25 only. Strain 7/25 appeared to be more adhesive than strain 9/27 (approximately 50 and 30 attached bacteria per epithelial cell, respectively). Cell-associated bacteria were not observed with rhizosphere isolates of *B. cepacia*.

Adherence to *C. sativus* roots

Table 1 shows comparative data on the ability of *B. cepacia* strains to adhere to *C. sativus* roots under sterile conditions. Bacterial adherence was assessed by evaluating the bacterial population associated with the roots 7 d after the inoculation. It was observed that most of the root-associated bacteria were removed during the first wash (approximately 10^6 c.f.u. ml^{-1}), whereas in the second through to the fifth washes approximately 10^4 and 10^3 c.f.u. ml^{-1} were removed, respectively (data not shown). Thus, the first wash was effective in removing bacteria from the roots and multiple wash steps did not cause a significant decrease in the number of attached organisms. On this basis we assumed that after five washes the number of bacteria present on the root corresponded to the number of bacteria which were irreversibly associated with the root. Under these experimental conditions all four *B. cepacia* strains became

Table 2. Effect of *B. cepacia* strains on root dry weight of *C. sativus*

In each case inoculation was with approx. 10^6 c.f.u. *B. cepacia*. Values are the means of 16 replicates. Values designated with the same letter (a, b or c) are not significantly different ($P = 0.05$) according to Duncan's Multiple-Range Test.

Strain	Root dry wt (g)
None (control)	0.35 c
PHP7	0.51 a
TVV75	0.44 b
7/25	0.35 c
9/27	0.37 c

irreversibly attached to the roots, reaching values of approximately 10^7 c.f.u. g⁻¹ fresh weight of root. Furthermore, bacteria were not detected inside the cucumber roots as deduced from plate counts of viable cells obtained from an homogenate of Ca(ClO)₂ surface-sterilized roots.

Greenhouse studies

Table 2 shows that inoculation of *C. sativus* seedlings with rhizosphere isolates PHP7 and TVV75 results in significantly higher dry weight values of plant roots by comparison with clinical strains 7/25 and 9/27, and with the uninoculated control ($P = 0.05$). *B. cepacia* PHP7 and TVV75 exerted a positive effect on root dry weight only, as they did not cause any significant change in shoot dry weight (data not shown). Under identical conditions *B. cepacia* strains of clinical origin (7/25 and 9/27) did not cause any apparent change in root and shoot dry weight, as compared with the uninoculated controls.

DISCUSSION

In agriculture, application of plant-growth-promoting rhizobacteria (PGPR) is desirable to improve crop production and to decrease utilization of chemical pesticides (de Freitas & Germida, 1991; Kloepper *et al.*, 1988; Suslow & Schroth, 1982). The widespread use of PGPR in field experiments has been hampered by the possibility that massive introduction of exogenous micro-organisms in fields may be a risk factor for the balance of the ecosystem and for animal and human health. Thus, when a potentially pathogenic species has to be tested for plant-growth-promoting (PGP) activity in field experiments, any environmental and health risk must be ruled out. Therefore, investigations designed to compare rhizosphere and clinical isolates of *B. cepacia* for some relevant virulence characteristics (e.g., adhesiveness to human uroepithelial cells, synthesis of protease(s), and siderophores) and for some traits associated with known PGP activity (e.g., root colonization, synthesis of siderophores, antibiosis against fungal phytopathogens, N₂-fixation, and production of IAA and HCN) were carried out.

B. cepacia is recognized as one of the most nutritionally

versatile species among Pseudomonadaceae (Stanier *et al.*, 1966; Palleroni & Holmes, 1981; Yabuuchi *et al.*, 1992). Initially the ability of *B. cepacia* strains to grow on different carbon sources normally present in root exudates was explored. These organic substances are released by the plant roots and used as nutritional sources for root-colonizing microbial populations (Rovira, 1956). The results obtained confirm the ability of *B. cepacia* to multiply using a wide range of organic compounds and indicate that rhizosphere and clinical strains differed for one biochemical trait only (L-lysine utilization). It was also observed that the optimal growth temperature of rhizosphere isolates was in the range 37–42 °C, rather than 25–28 °C which is the generally accepted growth temperature of soil bacteria. In contrast, clinical strains grew as well at high temperatures, but at lower temperatures their growth rates were significantly lower than those of rhizosphere isolates. This indicates that rhizosphere strains are better adapted to grow at low temperatures than clinical strains, which preferentially develop at body temperature. We also observed differences in the levels of extracellular protease(s) released by the strains studied. Only clinical strains showed proteolytic activity, in agreement with the role proposed for this extracellular enzyme(s) in the pathogenicity mechanism towards the human host (Janda & Bottone, 1981).

It is now recognized that PGPR may benefit plant growth by providing nutrients and growth factors or by producing antibiotics, siderophores, and cyanide, which may inhibit pathogenic fungi and bacteria (Davidson, 1988). Phytohormone-like substances have been reported in fluorescent pseudomonads (Kloepper *et al.*, 1989), but up to now no study has been published on production of plant hormones by *B. cepacia*. Results show that rhizosphere isolates are capable of synthesizing detectable amounts of IAA, while clinical isolates do not. The behaviour of rhizosphere and clinical *B. cepacia* strains examined in greenhouse studies clearly shows that rhizosphere isolates exert growth promotion of *C. sativus* roots independently of native root zone microflora; the clinical strains, in contrast, did not have any effect on *C. sativus* growth. In addition, nitrogen fixation allowed a differentiation between rhizosphere and clinical isolates of *B. cepacia*. The nitrogen fixing capability was observed for rhizosphere isolates only.

The ability of *B. cepacia* to inhibit the *in vitro* growth of several fungal phytopathogens was investigated. Differences in the level of antagonism shown by the rhizosphere isolate PHP7 with respect to the other *B. cepacia* strains were observed. The antagonistic activity was affected by the medium composition, as it was higher on KB rather than PDA plates. It is generally assumed that the inhibition of fungal pathogens on PDA, being a C-, N- and Fe(III)-rich medium, may be due to the production of antibiotic-like substances, and on KB, an Fe(III)-deficient medium, the inhibition may occur by production of siderophores in addition to antibiotics (Hebbbar *et al.*, 1991). Since *B. cepacia* strains displayed higher inhibition of fungal growth in Fe(III)-poor medium, it can be hypothesized that siderophores could be involved in the

antagonistic response. It is known that to counter the effect of Fe(III) limitation in the environment or within the mammalian host tissue, most micro-organisms have developed high-affinity systems for Fe(III) uptake, consisting of low-molecular-mass chelators, called siderophores, and their cognate membrane receptors (Cox, 1989). It has been established that release of siderophores may be partly responsible for enhanced plant growth (Kloepper *et al.*, 1980), biocontrol (Leong, 1986) and morbidity and mortality in animal infections (Sokol, 1986; Sokol & Woods, 1988). Clinical isolates of *B. cepacia* have been reported to produce pyochelin (Sokol, 1986), a salicyl-derived thiazo-compound, chemically unrelated to pyoverdins and pseudobactins, previously described in the fluorescent pseudomonads (Abdallah, 1991). Pyochelin has been shown to be a potent siderophore for clinical isolates of *B. cepacia*, and a correlation was found between its production and the severity of infection in cystic fibrosis patients (Sokol, 1986). We found that the clinical isolates of *B. cepacia*, 7/25 and 9/27, synthesize pyochelin, while the rhizosphere isolates PHP7 and TVV75 do not. In addition to pyochelin, clinical isolates of *B. cepacia* released detectable amounts of salicylic acid, the biosynthetic precursor of pyochelin. This molecule, formerly termed azurechelin (Sokol *et al.*, 1992), has been shown to function as a siderophore in *B. cepacia* and fluorescent pseudomonads (Visca *et al.*, 1993). Salicylic acid is a plant hormone known to exert a variety of positive functional effects on plants, e.g. induction of systemic resistance against pathogen attack (Enyedi *et al.*, 1992; Raskin, 1992), so that it appears quite surprising that production of such a molecule was characteristic of clinical, rather than rhizosphere isolates. Moreover, both clinical and rhizosphere isolates synthesize a hydroxamate-like compound which displays a strong reactivity in the Schwyn & Neilands (1987) assay for siderophores. TLC analysis did not allow identification of this compound as cepabactin (Meyer *et al.*, 1989) as had been previously described for *B. cepacia*. It will be of interest to investigate whether the hydroxamate-like compound can be related to the new ornibactin family of siderophores from a non-fluorescent *Pseudomonas* strain which resemble pyoverdins in their peptide structure, but lack the chromophore moiety (Stephan *et al.*, 1993). The existence of additional Fe(III)-transport systems in the pyochelin-producing clinical isolates of *B. cepacia* could be an advantage for human pathogenicity.

Another characteristic to be considered was the adhesion to uroepithelial cells, as the attachment of bacteria to the mucosal epithelia represents an essential step in the colonization of the host (Prince, 1992). Adhesiveness was demonstrated for clinical strains 7/25 and 9/27, while rhizosphere isolates were unable to adhere to uroepithelial cells. Thus, rhizosphere isolates appear to lack specific structures involved in the adhesion to human mucosa. In contrast, the screening of *B. cepacia* strains for the ability to attach to plant root surfaces indicated that all the strains adhere to cucumber roots in the absence of competition, but are unable to penetrate inside the roots. It should be stressed that the root-adherence assay was performed

in vitro under sterile conditions, i.e. in the absence of competing microflora, and therefore does not predict whether the bacterium will actually colonize a root surface, but only determines the potential ability to adhere and/or proliferate at the rhizoplane (James *et al.*, 1985). Our results suggest that the adherence of *B. cepacia* strains to cucumber roots does not involve specific recognition events and that the *B. cepacia* isolates probably share similar surface properties which do not allow the discrimination between rhizosphere and clinical bacteria for their root adhesion ability. Thus, while clear differences between rhizosphere and clinical isolates are evident in the adhesion test to the human epithelium, no distinction can be made between the two groups when the target is the root surface.

Differences between human- and plant-pathogenic *B. cepacia* strains have been reported previously (Gonzalez & Vidaver, 1979); these were based on bacteriocin production, maceration of onion slices, pectolytic activity, and plasmid profile. However, the extent of variation of phenotypic traits between PGP and clinical *B. cepacia* isolates was still not clear. In the present study, relevant characteristics related to virulence and PGP activity were analysed in clinical and rhizosphere isolates of *B. cepacia*. It has been observed that *B. cepacia* strains of clinical origin lack factors involved in PGP activity as compared to rhizosphere isolates. Conversely, the rhizosphere isolates appear to have reduced virulence potential by comparison with PGP isolates. Such an evolutionary divergence probably results from the selection exerted by extremely diverse ecological niches on colonizing strains and leads us to hypothesize that the PGP *B. cepacia* isolates may have a very limited capacity for acting as pathogens. However, due to the limited number of strains examined to date, further work is still required to determine whether this divergent trend is universal within the species.

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