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Influence of biocontrol strain *Pseudomonas fluorescens* CHA0 and its antibiotic overproducing derivative on the diversity of resident root colonizing pseudomonads

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Abstract

Non-target effects of biocontrol strains of *Pseudomonas* on the population of resident pseudomonads should be assessed prior to their large scale application in the environment. The rifampicin resistant bacterium *P. fluorescens* CHA0-Rif and its antibiotic overproducing derivative CHA0-Rif/pME3424 were introduced into soil microcosms and the population of resident pseudomonads colonizing cucumber roots was investigated after 10 and 52 days. Both CHA0-Rif and CHA0-Rif/pME3424 displaced a part of the resident pseudomonad population after 10 days. To investigate the population structure, utilization of 10 carbon sources and production of two exoenzymes was assessed for 5600 individual pseudomonad isolates and 1700 isolates were subjected to amplified ribosomal DNA restriction analysis of the spacer region (spacer-ARDRA). After 10 days, only the proportion of pseudomonads able to degrade L-tryptophan was reduced in treatments inoculated with either biocontrol strain. In parallel the phenotypic diversity was reduced. These effects were only observed 10 days after inoculation, and they were similar for inoculation with CHA0-Rif and CHA0-Rif/pME3424. Changes in the population structure of resident pseudomonads on cucumber roots during plant growth were more pronounced than changes due to the inoculants. The inoculants did not affect the genotypic diversity detected with spacer-ARDRA, but the genotypic fingerprints corresponded only partially to the phenotypic profiles. Overall CHA0-Rif had a small and transient impact on the population of resident pseudomonads and the effect was essentially the same for the genetically engineered derivative CHA0-Rif/pME3424.

Keywords: *Pseudomonas*; Biocontrol; Antibiotic overproduction; Effects on resident bacteria; Diversity; Amplified ribosomal DNA restriction analysis

1. Introduction

Biological control of plant diseases with beneficial *Pseudomonas* strains is receiving increased attention [1,2]. In many cases, the capacity of beneficial pseudomonads to suppress various soil-borne fungal pathogens relies on their ability to produce antibiotic

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metabolites [1,2]. Several research groups have succeeded in genetically engineering strains with improved disease control by conferring on them the ability to produce enhanced amounts of or additional antibiotic metabolites [3–7]. Pseudomonads are common members of the microflora in most soils [8–12], and introduced pseudomonads may not only affect the target pathogen(s) but may also compete with indigenous bacteria, particularly with related pseudomonads, for colonization of favorable sites on the plant roots. Introduced strains may alter both the abundance and the diversity of the resident *Pseudomonas* population. Prior to large scale release into the environment of either wild-type or genetically engineered biocontrol agents, non-target effects on the closely related resident bacteria should therefore be evaluated.

The population of pseudomonads in the soil environment is shaped by many factors. Latour et al. [12] showed that the population structure of resident pseudomonads is influenced by both soil type and plant species. In this context, it is interesting that specific pseudomonads have been found to be associated with natural disease suppressive soils [13–16] and with soils which became suppressive to take-all after monoculture with cereals and heavy infections with *Gaeumanomyces graminis* [17,18]. The structure of a *Pseudomonas* population can be investigated with several methods. Most common are tests for the utilization of different carbon sources by single isolates [12,19]. Recently, genotypic fingerprinting methods such as REP-PCR or ERIC-PCR based on the amplification of repetitive sequences [20,21] and restriction analysis of amplified 16S ribosomal DNA (ARDRA) [22] have been developed. A variation of the ARDRA technique with a higher resolution uses primers to amplify a region which includes the spacer DNA located between the genes coding for the 16S and the 23S RNA. This approach has successfully been applied for molecular typing of bacterial strains belonging to the family of Comamonadaceae [23].

Several authors have investigated the effects of an introduced *Pseudomonas* strain on the abundance of resident pseudomonads. Transient reductions of the resident fluorescent pseudomonads have been reported on wheat roots [24] and wheat seeds [10] after inoculation with *Pseudomonas* spp. Fukui et al. [25] observed antagonism between coinoculated strains of *Pseudomonas fluorescens* when one strain was inoculated at high densities and they suggested that competition for carbon was the primary factor affecting antagonism.

Here we describe the effects of soil inoculation with *P. fluorescens* CHA0 and its antibiotic overproducing derivative CHA0/pME3424 on the resident population of root-associated pseudomonads. Strain CHA0 is an effective biocontrol agent, which protects plants from a range of root diseases [2,15,26]. Strain CHA0/pME3424 contains an extra copy of the *rpoD* gene that encodes the housekeeping sigma factor σ^{70} on an oligo-copy plasmid resulting in several-fold increased production of the antibiotics 2,4-diacetylphloroglucinol (Phl) and pyoluteorin (Plt) and improved disease control [6,27,28]. In previous studies, the ecological behavior and fate of strain CHA0 has been monitored under growth chamber and field conditions [9,29–31]. The objectives of this study were as follows. (i) To measure the impact of CHA0 and CHA0/pME3424 on the size and the structure of the resident pseudomonad population colonizing cucumber roots. (ii) To compare effects of the inoculants to natural changes in the population structure during plant growth. (iii) To apply within the same study both phenotypic and genotypic typing methods for assessing the diversity of resident pseudomonads.

2. Materials and methods

2.1. Organisms and culture conditions

A previously described derivative of *P. fluorescens* strain CHA0 selected for spontaneous resistance to rifampicin (CHA0-Rif) [9,30] was used in this study. The plasmid pME3424 containing an extra copy of the homologous *rpoD* gene on the oligo-copy, broad host range vector pVK100 [6] was introduced into CHA0-Rif and antibiotic overproduction was verified as described by Schnider et al. [6]. Both strains were routinely grown in nutrient yeast broth (NYB) and on King's medium B (KMB) agar containing 100 μg rifampicin per ml. For strain CHA0-Rif/pME3424 125 μg per ml tetracycline hydrochloride was added to the medium.

Cucumber seeds (*Cucumis sativus* L., cv. Chinesische Schlange, Altdorfer Samen, Zürich, Switzerland) were surface-disinfected in 5% (w/v) sodium hypochlorite for 30 min, rinsed with sterile-filtered distilled water and then germinated for 2 days on 0.85% water agar (Difco Laboratories, Detroit, MI) at 24°C.

2.2. Microcosms and plant growth conditions

Soil from Eschikon (Switzerland) used in this study was collected from the upper 20 cm of the soil profile, passed through a 5 mm mesh screen, and stored at 15°C prior to use. The soil is a sandy loam with a pH of 7.7 [9]. For soil inoculation CHA0-Rif and CHA0-Rif/pME3424 were grown on KMB plates overnight at 27°C. Bacteria were removed from the plates, washed once in sterile distilled water and the bacterial concentration was adjusted to 5×10^8 CFU per ml by measuring the optical density of the cell suspension and subsequent dilution with water. Soil was sprayed with the bacterial suspension at a rate of 20 ml kg^{-1} to obtain a final concentration of 10^7 CFU per g soil. An equivalent volume of sterile distilled water was added to soil for the control treatment. The final water content of the soil was then 25%. Soil was thoroughly mixed during inoculation. Quartz sand (50 ml) was added to clay pots (400 ml internal volume) and overlaid with 350 ml of soil. Two pre-germinated cucumber seedlings were planted per pot, and the pots were incubated in a growth chamber with 70% relative humidity with light (160 microeinsteins m^{-2} s^{-1}) for 16 h at 22°C, followed by an 8 h dark period at 18°C. Twice a week the pots were irrigated from below with sterile distilled water. Plants were harvested after 10 and 52 days, and the bacterial populations on the roots were examined as described below. A total of 24 plants was analyzed per treatment, repetition and sampling time.

2.3. Enumeration and isolation of root-associated pseudomonads

The roots were washed gently for 10 s with sterile water to remove loose soil, and then homogenized with a mortar and pestle for 30 s. The homogenate was diluted 100-fold in sterile distilled water and shaken at 300 rpm for 1 h on a rotary shaker. Ten-fold serial dilutions were spread-plated onto S1 selective medium [32] to isolate pseudomonads and on KMB plates amended with 100 μg of rifampicin and 187.5 μg of actidione (inhibiting growth of fungi) per ml to enumerate culturable cells of introduced CHA0-Rif or CHA0-Rif/pME3424. Plates were incubated at 24°C and colonies were counted after 3 days. Plasmid stability was assessed by replica plating on KMB amended with tetracycline hydrochloride (125 μg ml^{-1}). No interfering rifampicin-resistant background population was observed in Eschikon soil. To investigate the population structure of resident pseudomonads, 11–12 isolates from each root system of the control treatment and 48 isolates from each root system of inoculated treatments were picked randomly from final dilutions on S1 medium and transferred to S1 master plates in a 6×8 pattern suitable for replica plating. The isolates from inoculated treatments were checked for rifampicin and tetracycline resistance by transferring them to the appropriate selective medium and 12 rifampicin-sensitive isolates per root system were then transferred to new master plates. The isolates were always subcultured on solid or in liquid S1 medium and liquid cultures were stored at −80°C in microtiter plates (100 μl per well) after adding 50 μl of sterile 60% glycerol to each culture.

To calculate separately the population of either resident or introduced pseudomonads, the counts from S1 plates were multiplied by the proportion of rifampicin-sensitive or rifampicin-resistant isolates, respectively. This calculation was performed for each root system based on the proportion of resistance to rifampicin observed within the 48 isolates tested. To indicate the numbers of introduced strains after 52 days the counts obtained on KMB agar amended with rifampicin were used since the introduced strains were only a minor fraction of the S1 isolates at this sampling date.

2.4. Phenotypic characterization of the isolates

To evaluate the population structure of resident pseudomonads, the 5640 isolates obtained from cucumber roots were tested for growth on 10 substrates as sole carbon source. Carbon sources were chosen which differentiate biovars of *P. fluorescens* [19], and

which are assimilated by a portion of the pseudomonads colonizing cucumber roots in soil from Eschikon as determined in preliminary experiments. A basal mineral medium containing 0.033 M Na/K phosphate buffer pH 6.8; 0.1% NH_4Cl; 0.05% $MgSO_4 \cdot 6H_2O$; 0.005% ferric ammonium citrate; 0.0005% $CaCl_2$ was used [33]. Filter-sterilized stock solutions of the carbon sources were added to the basal medium to obtain a final concentration of 0.1% (w/v), with the exception of glycine, which was made up to a final concentration of 0.4% (w/v). The medium was dispensed in microtiter plates (100 μl in each well) and inoculated with approximately 2.5×10^3 CFU per well by adding 0.5 μl of a 200-fold diluted overnight culture of the isolates in S1 medium. Plates were shaken at 100 rpm for 7 days at 24°C and growth was assessed by measuring the optical density at 570 nm with an ELISA plate reader. Values below 0.05 were considered to indicate no growth of the isolate involved. The isolates were also examined for tryptophan side chain oxidase (TSO) activity [34] and lipase activity [35]. Carbon source utilization and production of enzymes were assessed for 3102 isolates collected at 10 days and 2538 isolates collected at 52 days.

The resistance level of resident pseudomonads to Phl was determined by transferring 0.5 μl of a 2-day-old liquid culture of each isolate to microtiter plates containing 100 μl of 1/10 strength tryptic soy broth (TSB) amended with 25 μg per ml or 100 μg per ml of the antibiotic per well. Synthetic Phl was kindly provided by E. Burger, University of Geneva, Switzerland. Plates were incubated for 3 days and evaluated as described above for the tests for carbon source utilization with the exception that they were read after 3 days.

2.5. Genotypic characterization of the isolates

To distinguish dominant genotypes within the resident *Pseudomonas* population, 1692 isolates obtained after 10 days (188 isolates per treatment from three repetitions) were further analyzed with a modified ARDRA technique. Bacterial cultures were grown in microtiter plates containing 100 μl of 1/10 strength KMB per well and 0.5 μl of the cultures was transferred directly to PCR tubes containing 5 μl of lysis buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.1% Tween 20). Cells were lysed by heating for 10 min to 99°C and the reaction mix was added to give a final reaction volume of 20 μl. The amplification conditions have been described in detail before [16]. Primers were selected which amplify a downstream fraction of the 16S rDNA, the spacer region between the 16S and the 23S rDNA, and a part of the 23S rDNA. The forward primer 926f [36] with the sequence 5′-TAAAACTYAAAKGAATTGACGGGG (where Y is C:T and K is G:T, each 1:1) is located in a conserved region of the 16S rDNA and the reverse primer with the sequence 5′-CCTTTCCCTCACGGTACTGGT [23] is located in the 23S rDNA. Restriction of 10 μl of the amplified product was performed in a total volume of 20 μl of restriction buffer with 2 U of *Taq*I (Boehringer, Mannheim, Germany) for 2 h at 65°C, and restriction fragments were separated on a 2.5% agarose gel. Isolates differing in at least one restriction fragment generated with this method were designated different genotypes.

2.6. Data analysis and statistics

The experiment was repeated at different times. Four repetitions (control treatment without inoculation and treatment with CHA0-Rif) and three repetitions (treatment with CHA0-Rif/pME3424) were performed for the first sampling date after 10 days and three repetitions were performed for the second sampling date at 52 days. Twenty-four plants were analyzed per treatment for each repetition and each sampling date, and 11–12 isolates were selected per root system. The data obtained for bacterial populations approximated a log-normal distribution and they were $\log_{10}$ transformed prior to statistical analysis. For presentation these data were back-transformed. All data were subjected to analysis of variance (ANOVA) (Systat 5.05; SPSS Inc., Evanston, IN), and if significant differences were found, the treatments were compared with the Tukey test. To compare data from the two sampling dates, two-way ANOVA with treatment and time as the two factors was applied. Principal component analysis (PCA) was performed based on the correlation matrix and using varimax rotation (Systat 5.05). Phenotypic similarity between isolates was calculated using the simple matching coefficient in the NTSYS-pc numerical

taxonomy and multivariate analysis system (Applied Biostatistics Inc., Setauket, NY). The Shannon index of diversity [37] was calculated after grouping of the isolates into phenons with 100% similarity for the 12 phenotypic markers that were investigated.

3. Results

3.1. Root colonization by introduced strains and their impact on the abundance of resident pseudomonads

Colonization of cucumber roots by CHA0-Rif was 3.9×10^5 CFU per g root after 10 days, and then declined to about 4.5×10^4 CFU per g root within the next 42 days (Fig. 1). Root colonization by the antibiotic overproducing derivative CHA0-Rif/pME3424 was significantly less with 2.5×10^5 CFU at 10 days and 2.3×10^4 CFU per g root at 52 days (Fig. 1). The plasmid was retained in 89% of the cells of CHA0-Rif/pME3424 after 10 days and in 87% of the cells after 52 days. After 10 days, the resident pseudomonad population was 7.5×10^5 CFU per g root in the control treatment without biocontrol strains. Treatment with CHA0-Rif significantly reduced the population of resident pseudomonads to 4.7×10^5 CFU per g and if CHA0-Rif/pME3424 was present, the population of resident pseudomonads was even more reduced to 3.2×10^5 CFU per g root (Fig. 1). The effect of the inoculants was significant at $P < 0.005$ and the difference between the two strains was significant at $P < 0.05$. After 52 days, no significant difference in the population of the resident pseudomonads was found between the control and the treatment with CHA0-Rif, but the population of resident pseudomonads was still significantly reduced in the treatment with CHA0-Rif/pME3424.

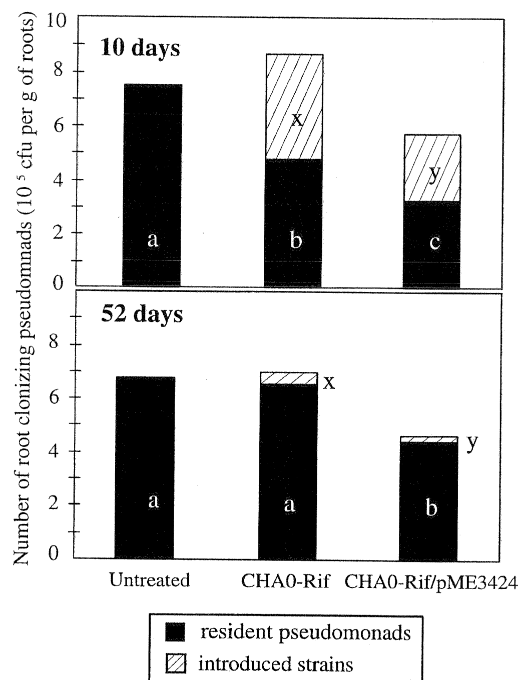

Fig. 1. Effect of *P. fluorescens* CHA0-Rif and CHA0-Rif/pME3424 on the population of resident pseudomonads on cucumber roots, 10 and 52 days after inoculation. Root colonizing pseudomonads (48 isolates per root system) were isolated on S1 medium and assessed for resistance to rifampicin. The proportion of S1 isolates sensitive to rifampicin was regarded as the resident population. The introduced strains were enumerated after 10 days by calculating the proportion of rifampicin-resistant bacteria isolated on S1 medium, and after 52 days they were directly enumerated on KMB agar with rifampicin added. Results of four (10 days) and three (52 days) independent experiments with 24 plants per treatment and repetition are shown. Bars with the same pattern and different letters are significantly different at $P < 0.05$ according to two-way ANOVA and the Tukey test.

3.2. Effects on the phenotypic diversity of resident pseudomonads

Since the numbers of resident pseudomonads were reduced by the inoculants, it was of interest to determine whether the population structure was also affected. A total of 282 isolates of resident pseudomonads per treatment and per repetition were analyzed for utilization of 10 carbon sources and production of two enzymes. Of the 10 carbon sources tested, a significant difference among treatments was detected only for the utilization of L-tryptophan (Table 1). Utilization of this substrate by resident pseudomonads was reduced in inoculated treatments ($P = 0.09$ for CHA0 and $P = 0.03$ for CHA0/pME3424) after 10 days compared with the non-inoculated control but this effect was transient and no longer detectable after 52 days. Substrate utilization between the two sampling dates at 10 and 52 days was compared based on the data of all treatments using two-way ANOVA. Utilization of both ethanol and propanol was significantly ($P = 0.008$ and

Table 1
Growth on 10 carbon sources and production of two enzymes by pseudomonad isolates from cucumber roots inoculated with *P. fluorescens* CHA0-Rif or CHA0-Rif/pME3424 or left untreated[a]

Carbon source (utilization)/ enzyme (production)	10 days[b,c]			52 days[b,c]			10 days[d]	52 days[d]
	Control[b]	CHA0-Rif	CHA0-Rif/pME3424	Control	CHA0-Rif	CHA0-Rif/pME3424	Average of all treatments	Average of all treatments
L-Arabinose	94.8	90.0	91.4	94.3	89.5	89.7	92.1	91.2
Amylamine	53.7	37.6	38.9	56.6	45.7	46.0	43.4	49.4
Glycine	32.8	28.1	32.7	36.9	33.6	31.8	31.2	34.1
D-Xylose	67.6	54.2	57.3	55.1	55.9	57.3	59.7	56.1
myo-Inositol	51.5	49.9	50.6	45.4	55.8	56.1	50.7	52.4
L-Tryptophan	41.4 a	22.1 ab	13.8 b*	17.3	20.1	16.9	25.8	18.1
Sorbitol	52.7	38.3	40.9	37.9	44.4	44.7	44.0	42.4
Propanol	41.1	39.3	44.2	59.0	60.2	61.0	41.6 a	60.1 b**
Ethanol	43.9	38.2	42.6	55.8	56.3	61.9	41.6 a	58.0 b**
Adonitol	34.2	17.1	15.0	10.5	2.5	10.8	22.1 a	7.9 b*
TSO[e]	12.9	15.1	10.4	20.8	40.7	26.1	12.8	29.2
Lipase	35.2	34.6	29.2	22.0	25.6	20.6	33.0	22.7

Data are expressed as percentage of isolates showing positive growth or production of the enzyme.
[a]Cucumber plants were grown for 10 or 52 days in natural soil inoculated with 10^7 CFU g^{-1} of either CHA0-Rif or CHA0-Rif/pME3424 or left untreated. Roots were macerated and spread on S1 medium to isolate pseudomonads. For details see Section 2.
[b]Twelve rifampicin-sensitive isolates from each plant were selected. Data are means from four experiments (10 days) or three experiments (52 days) with 24 plants per treatment and experiment. A total of 5641 isolates were examined.
[c]Values with different letters are significantly different at $P<0.05$ between the treatments according to ANOVA and the Tukey test. Significance levels are $^{*}P<0.05$ and $^{**}P<0.01$.
[d]A separate two-way ANOVA was performed on all data to compare results of the two sampling dates.
[e]TSO = tryptophan side chain oxidase.

$P=0.007$) greater and utilization of adonitol was significantly ($P=0.028$) less after 52 days (Table 1). Thus, natural changes in the pattern of substrate utilization of root-associated pseudomonads during 42 days were more pronounced than effects of inoculation.

The phenotypic data were further evaluated for each individual isolate. Cluster analysis was not possible, since due to the large sample size, the clustering algorithm found many different solutions. Using the 12 phenotypic markers it was possible to group isolates into phenotypes with 100% similarity. About 51% of all isolates belonged to 18 homogeneous types, each including 60–794 isolates in the whole collection of isolates from both sampling dates. One isolate from each of the seven most dominant phenotypes was identified using the Biolog system, to determine whether the S1 medium was indeed appropriate to isolate pseudomonads from cucumber roots. These seven isolates were identified as: *P. fluorescens* type A, *P. fluorescens* type B, *P. fluorescens* type G (two isolates), *P. fuscovaginae*, and *P. putida* type A1 (two isolates).

The 18 dominant phenotypes accounted for about 30% of the isolates in the control treatment and for about 57% of the isolates in both inoculated treatments after 10 days (Fig. 2). The Shannon index of diversity was calculated based on all different phenotypes. A reduced diversity in the presence of either inoculant strain was observed after 10 days (Fig. 2) but not after 52 days (data not shown). The isolates not included in one of the 18 dominant phenotypic groups were quite diverse. With 12 markers, theoretically 4096 different profiles could be obtained. Within all 5640 isolates from both sampling dates, 303 different phenotypic profiles were present once or twice, and the other isolates exhibited one of 227 different profiles.

The frequency of each of the 18 dominant groups was compared between control and inoculated treatments. After 10 days, the frequency of only two groups differed significantly between the control and the treatment inoculated with CHA0-Rif. For only one of the groups a significant difference in presence of CHA0-Rif/pME3424 was detected (data not shown). In contrast, comparing the frequency of

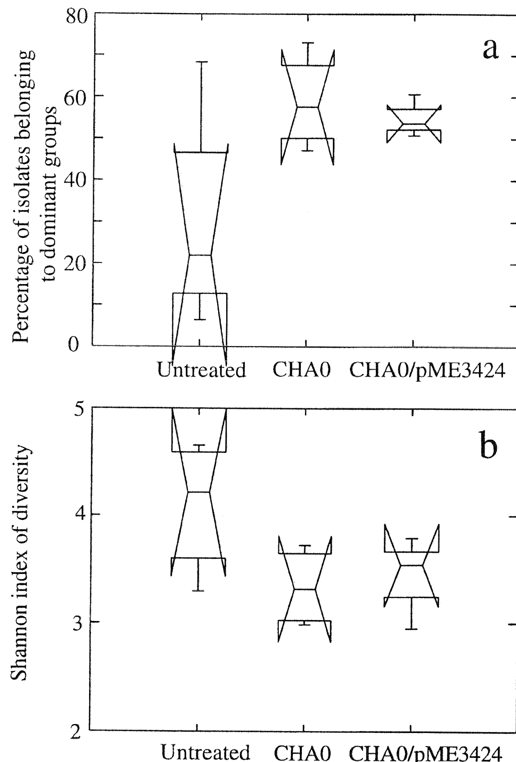

Fig. 2. Phenotypic diversity of resident pseudomonads on cucumber roots expressed by (a) the percentage of isolates belonging to one of the 18 dominant phenotypes and (b) the Shannon index 10 days after inoculation with *P. fluorescens* CHA0 or CHA0/pME3424. Experimental set-up and isolation of the resident pseudomonads are as described in the legend of Fig. 1. No clustering of the isolates was performed. Isolates with 100% similarity based on the 12 investigated phenotypic markers were considered one phenotype to calculate the Shannon index. Data are represented with notched boxplots calculated with the Systat Software.

the 18 groups at the two sampling dates with two-way ANOVA, two significant differences at $P < 0.001$ and four significant differences at $P < 0.05$ were found. Thus, similar to the findings from the evaluation for single phenotypic markers, the differences in the frequency of dominant phenotypes were more pronounced between the two sampling dates than between the different treatments (data not shown).

For the further evaluation of the relationship between samples from different treatments and different sampling dates, the data on the frequency of the 18 dominant phenotypes were subjected to PCA. The resulting ordination plot reduces the multivariate data to two dimensions and represents as much as possible of the total variation present in the data set within two dimensions. The ordination plot obtained by this analysis showed a clear separation of the two sampling dates but no separation of the different treatments along the first principal component axis (Fig. 3), which further underlines the notion that changes in the population structure of root-associated pseudomonads during plant growth were more noticeable than bacterial treatment effects.

To assess whether resident pseudomonads might be affected by Phl, the main antibiotic of CHA0 involved in disease suppression [38], the resistance to Phl of 568 isolates from the control treatment was assessed. About 99% of the isolates were resistant to

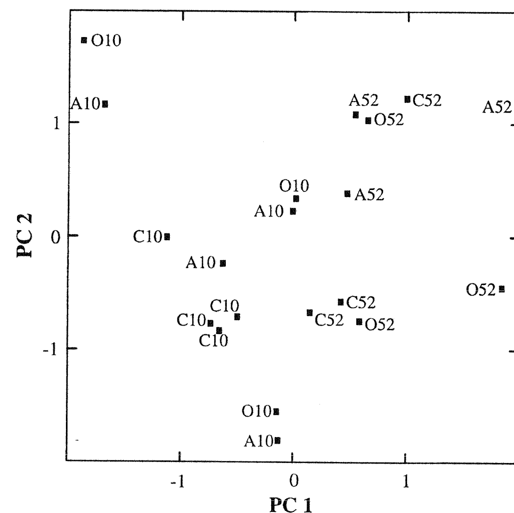

Fig. 3. Impact of plant age and inoculation with *P. fluorescens* CHA0-Rif and CHA0-Rif/pME3424 on the frequency of the 18 dominant phenotypes of resident pseudomonads on cucumber roots. Ordination plot of the first two principal components (PC) resulting from principal component analysis; C10 = control at 10 days, A10 = CHA0-Rif at 10 days, O10 = CHA0-Rif/pME3424 at 10 days; C52, A52, O52: control, CHA0-Rif and CHA0-Rif/pME3424, respectively, after 52 days. The 18-dimensional data-set is reduced by this procedure to two dimensions representing as much as possible of the total variance present in the data of both sampling dates and the three treatments (21% and 16% of the variance are explained by the first two components in this evaluation). Of the 5640 isolates which were investigated, 2896 were included in the dominant phenotypic groups and are represented in this evaluation. For details of the experimental setup see legend to Fig. 1 and Section 2.

Table 2
Shannon index of genotypic diversity and frequency of the 10 most abundant genotypes of resident pseudomonads on cucumber roots 10 days after inoculation with *P. fluorescens* CHA0-Rif or CHA0-Rif/pME3424[a]

	Control[b]	CHA0-Rif[b]	CHA0-Rif/ pME3424[b]
Shannon index H′	2.5	2.3	2.7
Genotypic group			
a	4.4	6.3	6.8
c	12.9	20.0	14.8
d	7.5	7.8	9.9
e	2.4	3.8	5.7
f	2.6	3.5	3.7
i	5.5	3.1	5.1
k	28.7	26.3	19.0
l	2.9	4.5	2.9
y	2.4	2.2	4.3
z	4.8	3.2	2.0
other	25.7	19.3	25.7

[a]For details of the experimental set-up and isolation of bacteria see legend to Table 1 and Section 2. Genotypes were discriminated by restriction of amplified DNA of the ribosomal spacer region with the enzyme *Taq*I.
[b]Twelve rifampicin-sensitive isolates from each plant were selected. Results from three experiments with 16 plants per treatment and experiment. A total of 1692 isolates were investigated. The differences between the treatments are not significant at $P < 0.05$ according to ANOVA and the Tukey test.

25 μg ml^{-1} and 87% were resistant to 100 μg ml^{-1} of Phl (data not shown). Since the incidence of resistance to Phl was that high in the resident population of pseudomonads in the control treatment, effects of the inoculants on this parameter were considered unlikely, and isolates from the other treatments were not assessed for Phl resistance.

3.3. *Effects on the genotypic diversity of resident pseudomonads*

A total of 1692 pseudomonad isolates were subjected to restriction analysis of amplified DNA encompassing the 16S-23S spacer region and flanking sequences of both the 16S and the 23S RNA genes. Amplification and restriction was successful for 1518 isolates. By restriction with *Taq*I, 25 distinct fingerprints differing by one or a few restriction fragments were observed which were common to at least five isolates. Ten of these fingerprints were quite abundant each being shared by at least 3% of all isolates (Table 2). Neither CHA0-Rif nor CHA0-Rif/pME3424 had a significant effect on the frequency of these 10 dominant genotypic groups nor did they significantly affect the genotypic diversity as determined using the Shannon index (Table 2).

3.4. *Comparison of phenotypic and genotypic typing methods*

The fact that phenotypic but not genotypic diversity of the resident pseudomonads differed between the treatments after 10 days raises the question of correspondence of data from genotypic and phenotypic typing methods. To determine the relation between phenotypic and genotypic typing, we first calculated the phenotypic similarity within each homogeneous genotype. The average phenotypic similarity between any two isolates in the collection of isolates obtained after 10 days was 0.57. The similarity within genotypically uniform groups was considerably higher (between 0.75 and 0.82) for four groups, but it was not much above the average similarity of all isolates (between 0.59 and 0.69) for the remaining six genotypic groups (Table 3). We then determined the occurrence of the different genotypes

Table 3
Average phenotypic similarity of resident pseudomonads within genotypic groups discriminated with restriction of amplified ribosomal DNA of the spacer region[a]

Genotypic group	Number of isolates	Average phenotypic similarity[b]
a	85	0.82
c	232	0.64
d	131	0.75
e	59	0.80
f	51	0.62
i	68	0.66
k	389	0.59
l	52	0.77
y	41	0.69
z	55	0.68
All isolates		0.57

[a]For details of the experimental set-up and isolation of bacteria see legend to Tables 1 and 2 and Section 2. Genotypes were discriminated by restriction of amplified DNA of the ribosomal spacer region with the enzyme *Taq*I.
[b]The phenotypic similarity between all isolates within each group was calculated using the simple-matching coefficient, based on the phenotypic markers described in Table 1.

within the nine homogeneous phenotypic groups which were most frequent in the subsample of isolates analyzed with spacer-ARDRA. Within five of these phenotypic groups 70% or more of the isolates exhibited the same restriction pattern. The other four groups included diverse restriction patterns (data not shown). Both of these evaluations indicate that the genotypic and the phenotypic typing of the isolates corresponded only partially.

4. Discussion

In this work effects on the resident pseudomonads caused by the introduction of a large inoculum of a *Pseudomonas* biocontrol strain into the soil ecosystem were studied and compared to the changes resulting from natural succession on plant roots.

Both inoculants CHA0-Rif and CHA0-Rif/pME3424 reduced the population of resident pseudomonads on cucumber roots 10 days after inoculation. In the treatment with CHA0-Rif, this reduction was quite equivalent to the population size of the introduced strain. In the treatment with CHA0-Rif/pME3424, however, the reduction of the resident pseudomonads was more pronounced. A reduction of the population of resident pseudomonads has been reported before by Bolton et al. [24], who introduced *P. fluorescens* strain RC1 into soil and investigated bacterial populations colonizing wheat roots. Moreover, De Leij et al. [10] introduced a large inoculum of a *P. aureofaciens* strain on wheat seeds. After 6 days, the resident *Pseudomonas* population on the seeds was reduced in the presence of the inoculant whereas, similar to our finding for the treatment with CHA0-Rif, the total number of *Pseudomonas* did not change. When introducing a genetically engineered strain from another genus, namely *Erwinia*, Orvos et al. [39] found no effects on the resident pseudomonad population. This specific displacement of resident pseudomonads by *Pseudomonas* inoculants could principally be due either to antibiosis or to competition for nutrients and space. The antibiotic Phl produced by CHA0 contributes not only to its ability to inhibit fungal pathogens [38] but it is also toxic to various soil bacteria [38]. However, the finding that resident pseudomonads even from uninoculated roots were resistant to Phl indicates that they most probably were not inhibited by this antibiotic. Competition for a similar ecological niche therefore seems to be a more likely explanation, and we suggest that the introduced strains occupied part of the ecological niche normally colonized by resident pseudomonads. The observation from this work and from De Leij et al. [10], that the amount of reduction of the resident pseudomonads in inoculated treatments is very similar to the numbers of the introduced strains, hints at competition for a similar ecological niche as the mechanism of antagonism. Moreover, this explanation would be coherent with the data of Fukui et al. [25], who investigated interactions between two co-inoculated strains of *Pseudomonas* in the spermosphere of sugar beet and demonstrated that competition for nutrients was the determining factor of interaction between strains of *Pseudomonas*, whereas there was no evidence for in situ antibiosis between the introduced strains. However, the more pronounced reduction of the population of resident pseudomonads observed in presence of CHA0-Rif/pME3424 compared to the treatment with CHA0-Rif cannot be explained by these arguments. But, as the overproduction of antibiotics in CHA0-Rif/pME3424 is achieved by amplification of the housekeeping sigma factor [6], we cannot exclude pleiotrophic effects in this derivative, which might also lead to recombinant specific interactions of unknown nature with the population of resident pseudomonads.

From the phenotypic characterization of individual isolates it has become evident that the inoculants also induced some changes in the structure of the resident pseudomonad population. The frequency of tryptophan utilization was reduced in both inoculated treatments after 10 days. CHA0-Rif and CHA0-Rif/pME3424 grow efficiently on L-tryptophan as sole carbon source (data not shown), and therefore it seems possible that the inoculants reduced the pool of available tryptophan in the root exudates, thereby diminishing the selective advantage of resident tryptophan utilizers. Further, the phenotypic profiles of each single isolate were evaluated, and 18 homogeneous dominant phenotypes were discerned which included 51% of all isolates. A wide diversity of resident pseudomonads was observed within the remaining 49% of the isolates. After 10 days a higher proportion of the isolates belonged to

these dominant groups and the Shannon index was reduced in the presence of both inoculants. A reduction in the diversity of a bacterial community after disturbance has often been reported for the case of chemical contamination of the soil environment [40,41]. Atlas et al. [40] studied effects of hydrocarbon and 2,4,5-T pollution and found a reduction in genotypic and phenotypic diversity of the microbial populations in polluted soils. Results from our study suggest that the disturbance presented by the introduction of a large inoculum of a *Pseudomonas* sp. may have a similar effect on the phenotypic diversity of resident pseudomonads.

The data from phenotypic typing were further evaluated for differences between the isolates obtained 10 days and 52 days after inoculation, and it was found that the population structure of resident pseudomonads changed significantly during these 42 days. This finding was best illustrated by PCA of the frequencies of dominant phenotypic groups. On the ordination plot of the first two principal components, the populations isolated 10 days and 52 days after inoculation were clearly separated, indicating that in addition to plant species and soil type [12] the plant age may shape the population structure of root-associated pseudomonads. These alterations might be due to changes in the composition of root exudates during plant growth [42]. In addition, it is evident from the ordination plot that changes in the population structure with plant growth were more pronounced than changes due to the inoculants, since for all three treatments a similar shift along the first principal component was observed between the two sampling dates, but the inoculated treatments were not separated from the control treatment. A similar result was obtained by De Leij et al. [11] who investigated the population structure of resident pseudomonads in field experiments by assessing distribution curves for slow and fast growing colonies. While they only occasionally observed significant effects of either a wild-type or a genetically engineered *Pseudomonas* inoculant, they reported a dramatic change in the population structure of resident pseudomonads on the roots of wheat at different growth stages.

The results from comparison of genotypic and phenotypic data are somewhat surprising. Indeed, within some genotypic groups a high phenotypic similarity of the isolates was found, and some phenotypic groups consisted mainly of isolates with the same distinct spacer-ARDRA fingerprint. On the other hand, some phenotypic groups included very different genotypes. Since the spacer-ARDRA fingerprints are representative of only a very small part of the genome and the different fingerprints discriminated with spacer-ARDRA differed only by one or a few restriction fragments, the limited correspondence of the phenotypic and genotypic data might be due to the methodology. On the other hand, it is possible that the specific composition of root exudates selects for pseudomonads with certain phenotypic profiles, and that phylogenetically distinct pseudomonads have independently evolved specific phenotypic profiles which do not correspond to the genotype of the ribosomal RNA locus. In this context it is interesting that, within the isolates which share the most common phenotypic profile with 794 identical isolates, six of the 10 frequent restriction patterns were quite abundant. Thus, it is imaginable that this phenotypic profile represents an adaptation to root exudates present in several phylogenetic groups.

The spacer-ARDRA method with direct lysis of the cells in the PCR tubes prior to DNA amplification has been found to be a rapid and efficient genotypic typing method for *Pseudomonas*. Nevertheless, metabolic profiling with automatic reading of the results with an ELISA plate reader is the faster method to investigate the population structure of resident pseudomonads and in addition to clearly discern different types of root colonizing pseudomonads, it also provides functional information, and therefore seems more appropriate for community studies of pseudomonads.

For biological safety assessment, our data suggest that the main perturbations of the population of resident pseudomonads caused by a biocontrol inoculant strain of *Pseudomonas* were transient and presumably not harmful to the ecosystem function. Essentially similar measurable perturbations occurred if a genetically engineered strain with improved biocontrol capability was applied. Finally, the changes in population structure caused by natural succession were more pronounced than changes due to the inoculant. However, we cannot dismiss the possibility that repeated application of biocontrol strains in the

field might lead to more durable or additive effects on the population of resident pseudomonads in the soil ecosystem.

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