Survival and cell culturability of biocontrol *Pseudomonas fluorescens* CHA0 in lysimeter effluent water and utilization of a deleterious genetic modification to study the impact of the strain on numbers of resident culturable bacteria

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Abstract

Little is known on the behavior of soil-inoculated biocontrol pseudomonads once they are transported to deeper soil layers and/or groundwater levels after a heavy rain. This issue was investigated in inoculated microcosms containing lysimeter effluent water, and experimental conditions mimicking a worse-case scenario for potential bacterial dissemination were chosen. First, the survival of the polyketide-producing biocontrol strain *Pseudomonas fluorescens* CHA0-Rif was studied for 175 days at two inoculation levels in unamended and nutrient-amended lysimeter effluent water, and its impact on numbers of resident culturable bacteria was determined. Cell numbers of CHA0-Rif declined to 3–4 log cells ml^{-1} (at high inoculum level) or reached the detection limit or below (at low inoculum level) by day 175, without generating significant numbers of non-culturable cells. At high inoculum level, strain CHA0-Rif resulted durably (from day 50 to 175) in higher numbers of the total resident culturable bacteria when compared with the uninoculated control. This effect, which did not take place at low inoculum level or when nutrients had been added, contrasts with the transient ecological impact of the strain on rhizosphere bacterial populations in previous studies. Neither 2,4-diacetylphloroglucinol nor pyoluteorin were found in the water using HPLC, and inoculation with CHA0-Rif had no effect on the percentages of the total culturable aerobic bacteria sensitive to either antimicrobial polyketide on day 20. Second, the impact of CHA0-Rif on numbers of resident culturable bacteria was compared with that of CHA0-Rif(pME3424). Plasmid pME3424 carries an extra copy of the strain's *rpoD* gene (encoding sigma factor σ^{70}). CHA0-Rif(pME3424) disappeared within 50 days in the water, but had the same impact as CHA0-Rif on the total number of resident culturable bacteria. This suggests that the impact of CHA0-Rif took place at the early stages of the experiment and was probably linked to the release of nutrients by introduced cells during inoculant decline. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Certain bacteria can suppress soil-borne fungal diseases and have received attention as biocontrol agents for crop protection [1–3]. For instance, fluorescent pseudomonads producing antimicrobial polyketides e.g., 2,4-diacetyl-phloroglucinol (Phl) and pyoluteorin (Plt) can protect various crop plants from root diseases caused by phytopathogenic fungi, including *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Gaeumannomyces graminis* var. *tritici*, *Pythium ultimum*, *Rhizoctonia solani* and *Thielaviopsis basicola* [4,5]. Since the commercial use of biocontrol agents in terrestrial ecosystems implies the release of large numbers of cells, biosafety aspects of these inoculations need to be considered [1,6–9]. In the case of pseudomonads that produce antimicrobial polyketides, the fact that Phl and Plt can inhibit various microorganisms [2,10] needs to receive particular attention. So far, the ecological impact of biocontrol pseudomonads has been assessed in the rhizosphere of

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inoculated plants [9,11–14] and subsequent rotation crops [15]. For instance, Natsch et al. [13] showed that the rifampicin-resistant strain *Pseudomonas fluorescens* CHA0-Rif caused a transient modification of the resident community of culturable fluorescent pseudomonads (CFP) in the rhizosphere of cucumber.

Whether biocontrol pseudomonads that produce antimicrobial polyketides can also have an ecological impact at non-target sites, i.e., in habitats where inoculant dissemination can take place, has not yet been assessed. Field experiments have shown that *P. fluorescens* CHA0-Rif released as soil inoculant could be transported vertically to deeper soil layers and potentially to groundwater level when thunderstorm rain was simulated immediately after inoculation [16]. One day after inoculation, the pseudomonad was recovered at levels up to 7 log CFU (g soil)$^{-1}$ at a depth of 1.5 m. In another study, low cell numbers of strain CHA0-Rif (2 to 4 log cells ml^{-1} of lysimeter effluent water at a depth of 2.5 m) were recovered after a significant rainfall that took place several months after soil inoculation [17].

In soils where preferential flow is significant, groundwater contamination can also concern nutrients (e.g., nitrate) derived from manure application [18,19]. Irrigation with wastewater effluent may cause input of dissolved organic carbon (DOC) compounds into aquifers [20,21]. Bacteria naturally present in the groundwater may utilize DOC and nitrate as nutrients for growth [22]. In addition, leaching of nutrients may also influence the ecology of inoculants transported to groundwater level. In microcosm experiments, the addition of diluted Luria Bertani medium to agricultural drainage water influenced maintenance of plasmid RP4 in *P. fluorescens* R2f and survival of the strain [23].

The first objective of the current work was to assess survival and cell culturability of the biocontrol strain *P. fluorescens* CHA0-Rif in lysimeter effluent water, and its impact on numbers of resident culturable bacteria. The pseudomonad was used at low (4 log CFU ml^{-1}) or high inoculum level (7 log CFU ml^{-1}), according to cell numbers of CHA0-Rif recovered in outdoor percolation experiments [16,17]. Laboratory medium was added to some of the microcosms to simulate possible leaching of nutrients applied as e.g., manure. The composition of the laboratory medium added was chosen to promote production of the antimicrobial polyketides Phl and Plt by CHA0-Rif, in an effort to maximize the potential ecological impact of the inoculant (i.e., worse-case scenario).

The second objective was to determine whether or not the impact of CHA0-Rif on numbers of resident bacteria in lysimeter effluent water took place shortly after inoculation, as seen in the rhizosphere. To achieve this, a derivative of CHA0-Rif unable to survive in lysimeter effluent water was developed by altering the balance between sigma factors in the cell, as follows. An extra copy of the strain's own *rpoD* gene (which encodes the housekeeping sigma factor σ^{70}) was introduced into CHA0-Rif using an oligo-copy plasmid as vector [24,25], which resulted in strain CHA0-Rif(pME3424). After having verified that CHA0-Rif(pME3424) failed to persist in lysimeter effluent water, its impact on numbers of resident culturable bacteria was compared with that of CHA0-Rif.

2. Materials and methods

2.1. Bacterial strains and growth conditions

P. fluorescens CHA0-Rif [26] is a spontaneous rifampicin-resistant mutant of the biocontrol agent CHA0 [27] that grows like the wild-type in laboratory media [26]. Plasmid pME3424 was constructed [25] by introducing a copy of the *rpoD* gene of CHA0 into the IncP vector pVK100 [28]. CHA0-Rif(pVK100) was obtained by mobilizing pVK100 into CHA0-Rif, as described [25], and was used in the experiment to distinguish between potential effects on strain survival linked to the presence of the vector or the insert.

Strain CHA0-Rif and its derivatives were routinely grown at 27°C with shaking (150 rev min^{-1}) in King's B broth [29] containing 100 μg rifampicin ml^{-1} (i.e., Rif100; for CHA0-Rif) or both Rif100 and 125 μg tetracycline ml^{-1} (i.e., Tet125; for CHA0-Rif(pME3424) and CHA0-Rif(pVK100)). Cells used to inoculate microcosms were obtained on King's B agar (KBA) containing the appropriate antibiotics. The plates were incubated overnight at 27°C. The cells were harvested from the plates, washed three times with sterile distilled water and the cell suspension was adjusted to 10^7 or 10^{10} cells ml^{-1} based on OD measurements at 600 nm [5].

2.2. Preparation and inoculation of water microcosms

Effluent water was collected at the bottom of a large outdoor lysimeter (2 m diameter; 2.5 m in depth; about 8000 kg of soil) on 8 December 1996. The lysimeter (lysimeter L6; [30]), which was grown with winter barley cv. Trasco (sown on 26 September 1996), had been under crop rotation for 16 years since it was constructed. The lysimeter contains a well-drained cambisol (FAO soil classification) = inceptisol (US soil taxonomy), which consisted of a sandy-loam surface horizon, a sandy-loam subsurface horizon, and parental material (stony alluvium mixed with loamy deposits), above a layer of gravels and stones designed to facilitate water drainage [30,31]. The lysimeter effluent water contained 5.7 log bacterial cells (among which 5.4 log cells with intact membranes) per ml as determined with direct counts using LIVE/DEAD *Bac*-Light™ viability kit (Molecular Probes, Eugene, OR, USA). Chemical properties of the water (pH of 7.6, 1.0 mM DOC, 0.35 mM dissolved oxygen, 0.41 mM NO$_3^-$, 0.45 mM SO$_4^{2-}$, 1.47 mM Cl$^-$, 0.030 mM K$^+$, 0.31 mM

Mg^{2+}, 2.56 mM Ca^{2+}) were comparable to those of groundwater samples [32–34].

Microcosms consisted of 100 ml lysimeter effluent water in previously autoclaved 125-ml serum bottles [31]. Leaching of nutrients to the groundwater was mimicked in one set of microcosms by adding 300 μl of nutrient glycerol broth (NGB). NGB consists of nutrient broth (Difco, Detroit, MI, USA) amended with glycerol (10 ml l^{-1}). This medium was chosen since it promotes production of both Phl and Plt by CHA0 (Christoph Keel, personal communication) and CHA0-Rif (i.e., 44 ng $(10^6$ CFU$)^{-1}$ for Phl and 660 ng $(10^6$ CFU$)^{-1}$ for Plt). Indeed, CHA0 and derivatives synthesize mostly one type of polyketide (Phl or Plt) in the majority of laboratory media tested so far [4,5,25,35]. The quantity of NGB added to the microcosms (i.e., 0.3%) was the same as that of Luria Bertani medium used as water amendment in previous studies [23,36]. All three strains can grow moderately (up to 7.6 log CFU ml^{-1}) in diluted (0.3%) NGB at 27°C with shaking (150 rev min^{-1}). The other set of microcosms (unamended) received 300 μl of sterile distilled water.

For inoculation, 100 μl of the adjusted cell suspension of CHA0-Rif, CHA0-Rif(pME3424) or CHA0-Rif-(pVK100) was used per microcosm, and each strain was added at a final cell concentration of 10^4 or 10^7 CFU ml^{-1} of effluent water. Uninoculated microcosms (controls) received 100 μl of sterile distilled water. The microcosms were closed with sterile cotton stoppers.

2.3. Conditions of incubation of the microcosms

The microcosms were placed at 8°C in the dark in an incubator. Their location in the incubator followed a randomized design. The dissolved oxygen concentration was monitored for 112 days with an oxygen electrode (Rank Brothers, Bottisham, UK), using distilled water as a reference. It amounted to 0.35 mM in the lysimeter effluent water used to prepare the microcosms, and was approximately 0.27 mM from 20 to 112 days, regardless of the treatment considered (data confirmed using the colorimetric test kit *visocolor*® Oxygen SA 10 from Macherey-Nagel, Dueren, Germany).

2.4. Sampling and inoculant monitoring

At each sampling time, three microcosms were studied per treatment (destructive sampling). The cotton stoppers were removed from the bottles, which were tightly closed with rubber caps and agitated on a rotary shaker at 300 rev min^{-1} for 30 min prior to preparing a 10-fold dilution series.

Dilutions were spread plated onto KBA+Rif100 to count culturable cells of the inoculants. Colonies were counted after incubation of plates for 6 days at 20°C. No colonies were found on KBA+Rif100 when samples from uninoculated microcosms were plated (de-

tection limit of about 1 log CFU ml^{-1}). Plasmid maintenance in culturable cells of CHA0-Rif(pME3424) was checked by comparing CFU on KBA+Rif100 and KBA+Rif100+Tet125 at 20 days. The presence of pME3424 in colonies resistant to Tet125 was confirmed by electrophoresis on agarose gels after extraction of plasmids by alkaline lysis [37]. Tet125-sensitive colonies were also studied to confirm that pME3424 was absent.

The possibility that the inoculants persisted in water microcosms as non-culturable cells was assessed by indirect immunofluorescence (IF) microscopy combined with Kogure's viability test [38], as described [31,39,40]. Kogure's viability test identifies substrate-responsive cells after incubation in the presence of nutrients and nalidixic acid, which prevents cell division and results in enlargement of viable cells. The primary antiserum used is specific for CHA0 [41] and no cross-reaction was found when studying water samples from uninoculated microcosms. Under in vitro conditions, the presence of pME3424 or pVK100 in CHA0-Rif had no apparent effect on the reaction of the cells to the primary antiserum. Briefly, samples from the microcosms were incubated for 6 h in the presence of nalidixic acid (20 μg ml^{-1}) and yeast extract (250 μg ml^{-1}) at room temperature in the dark, prior to fixing cells with formaldehyde (20 mg ml^{-1}). Nalidixic acid was effective in preventing inoculants from multiplying, as shown previously [30,31,40]. Presence of pME3424 or pVK100 in CHA0-Rif had no influence on the responsiveness of the cells to yeast extract (i.e., on the percentage of enlarged cells as well as the length of enlarged cells), as indicated using samples from overnight cultures. The cells were immobilized by vacuum filtration on 0.2-μm pore size polycarbonate filters stained with Irgalan black [42] and the filters incubated successively in the presence of the primary antiserum specific for CHA0 (60 min) and a secondary antibody (fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins; 45 min). The filters were treated with 1,4-diazobicyclo-(2,2,2)-octan-glycerol mounting medium, to prevent fading [43], and were studied using a Zeiss Axioskop epifluorescence microscope (filters 450–490 nm). Kogure's viable counts were obtained by counting stained enlarged cells (i.e., > 3 μm in length). Non-responsive cells were found mostly as small, spherical cells (i.e., < 1 μm diameter). The filters were also used to count the total amount of stained cells (i.e., total IF counts). At least 20 fields and/or 150 bacterial cells were counted. The detection limit was 2.0 log cells ml^{-1} for total IF counts (×1000 magnification) and 1.6 log cells ml^{-1} for viable counts (×400 magnification), as enlarged cells were easier to detect.

2.5. Monitoring of culturable resident bacteria

The total numbers of CFP and of culturable aerobic bacteria (CAB) were counted on S1 agar [44] and tryptic soy agar (Oxoid, Basingstoke, UK) at one-tenth strength

(i.e., 10% tryptic soy broth; containing 12 g agar l^{-1}), respectively. Colonies were counted after incubation of plates for 6 days at 20°C. All three inoculants can grow on these media. In addition, the total CAB resistant to Phl at 1 μg ml^{-1} or Plt at 1 μg ml^{-1} were quantified by colony counts on 10% tryptic soy agar amended with the polyketides. This procedure was not done with the total CFP since most fluorescent pseudomonads tend to be resistant to rather elevated levels of Phl and Plt [13]. CHA0-Rif and CHA0-Rif(pME3424) are resistant to levels of Phl or Plt well in excess of 5 μg ml^{-1}. The presence of Phl, its precursor monoacetylphloroglucinol, and Plt in microcosms was assessed on day 20 and 50, after extracting 50 ml from each microcosm studied using ethyl acetate. The extracts were analyzed by reverse-phase HPLC, as described elsewhere [2,45]. The detection limit was 45 ng (ml microcosm)$^{-1}$ for monoacetylphloroglucinol (i.e., 276 nM), 28 ng (ml microcosm)$^{-1}$ for Phl (i.e., 133 nM) and 70 ng (ml microcosm)$^{-1}$ for Plt (i.e., 261 nM).

2.6. Statistics

The experiment was comprised of 14 treatments (three inoculated strains at two inoculum levels as well as one uninoculated control, each with and without added nutrients) when inoculant survival was studied. The impact on numbers of resident culturable bacteria was studied in 10 treatments, as the impact of CHA0-Rif(pVK100) was not included. Each treatment was studied in triplicate at each sampling time (destructive sampling). Bacterial cell numbers and percentages were log- and arcsine-transformed, respectively. Data were processed by analysis of variance followed (when differences were found) with Tukey's HSD test. All statistical analyses were carried out at $P < 0.05$, using SYSTAT 5.05 (SPSS Science, Chicago, IL, USA).

Inoculant persistence was studied by three-way analysis of variance (inoculum level × nutrient addition × cell enumeration method) for each strain at each sampling
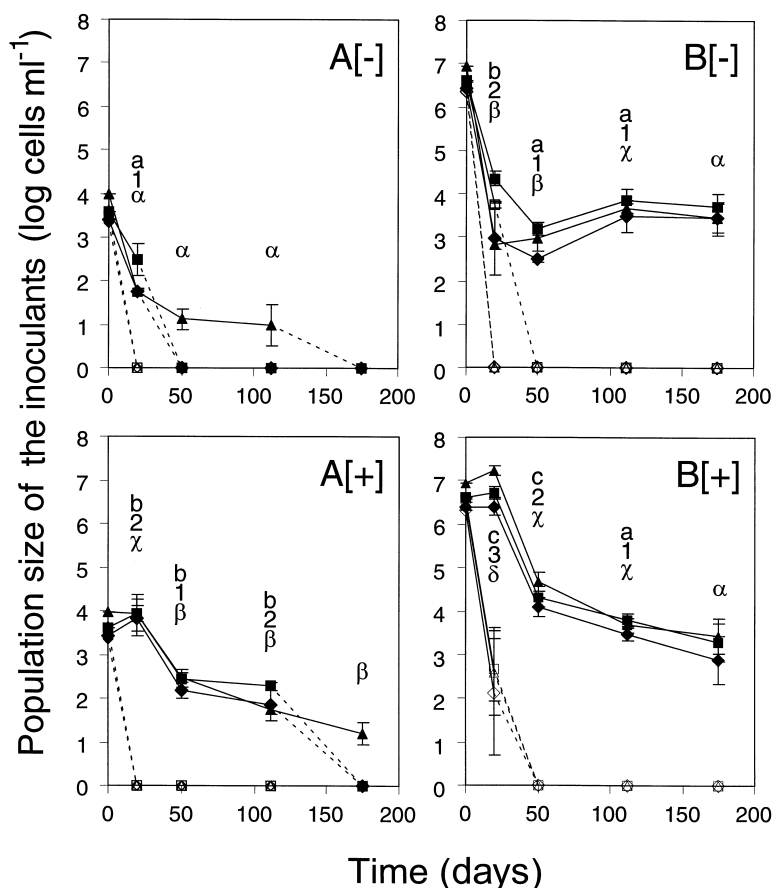

Fig. 1. Effect of nutrient amendment and inoculum level on the survival of *P. fluorescens* CHA0-Rif (filled symbols) and CHA0-Rif(pME3424) (open symbols) in lysimeter effluent water. Microcosms were kept unamended ([−]) or were amended with nutrients ([+]). Inoculation was carried out at 4 (A) and 7 log CFU ml^{-1} (B) and strains were monitored for 175 days (112 days for CHA0-Rif(pME3424) in (A)) using total IF counts (squares), viable counts (diamonds) and CFU (triangles). Cell numbers below the detection limits of 2.0 (total IF counts), 1.6 (viable counts) and 1.0 log cells ml^{-1} (CFU) are arbitrarily shown as zero, and dotted lines were used then. Error bars signify standard deviations. For CHA0-Rif, significant differences between the three cell count methods were found at 20 days, where total IF counts differed from viable counts and CFU (in A[−] and in B[−]), and at 50 days, where total IF counts differed from viable counts in B[−]. At each sampling time, statistical differences between the four CHA0-Rif treatments for each cell count method are indicated using a,b,c (for total IF counts), 1,2,3 (for viable counts) and α, β, χ, δ (for CFU). When CHA0-Rif(pME3424) could be recovered, its population level was always statistically lower than that of CHA0-Rif, regardless of the cell count method.
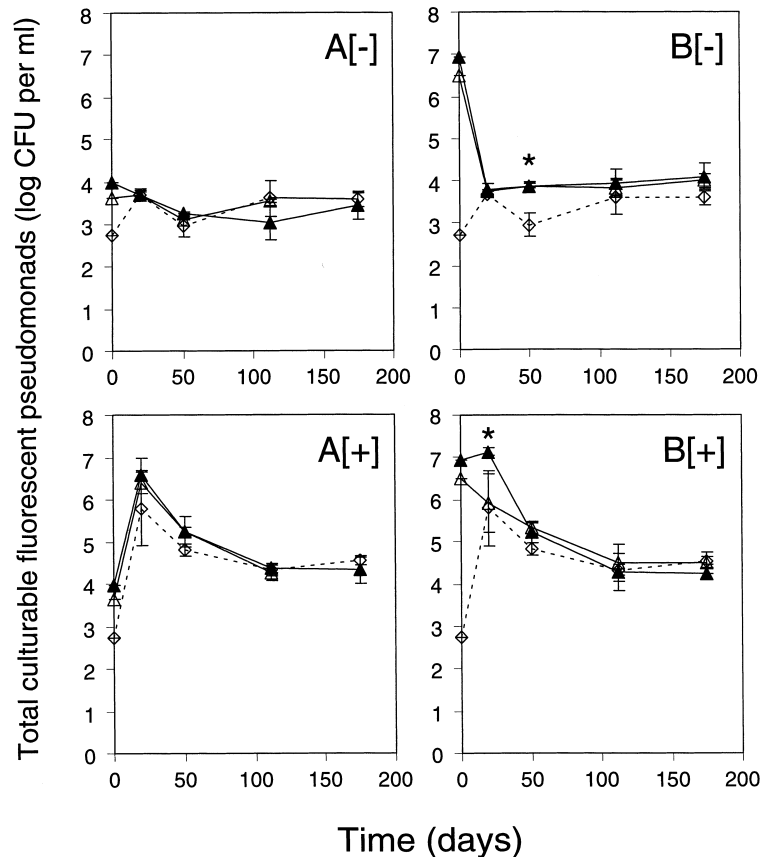
Fig. 2. Effect of nutrient amendment and inoculation with *P. fluorescens* CHA0-Rif (filled triangles) or CHA0-Rif(pME3424) (open triangles) on the total number of CFP in lysimeter effluent water. The uninoculated control is indicated with open diamonds and a dotted line. Microcosms were kept unamended ([−]) or were amended with nutrients ([+]) and inoculation was carried out at 4 (A) or 7 log CFU ml^{-1} (B). Error bars signify standard deviations. CFP were not counted at 175 days in the two CHA0-Rif(pME3424) treatments at 4 log CFU ml^{-1}. Significant differences between the uninoculated control and the inoculated treatments were only found at 20 days in B[+], where the CHA0-Rif treatment differed from the two others (indicated with an asterisk), and at 50 days in B[−], where both inoculated treatments differed from the uninoculated control (indicated with an asterisk).

time. Comparisons between strains were carried out for each type of cell count, at each combination of inoculum level × nutrient addition at each sampling time. For the total CFP, the total CAB, and the percentages of the total CAB sensitive to Phl or to Plt, all 10 treatments were compared together at each sampling time studied.

3. Results

3.1. Effect of nutrient amendment and inoculum level on the survival of P. fluorescens CHA0-Rif and CHA0-Rif(pME3424)

When introduced into lysimeter effluent water without added nutrients, *P. fluorescens* CHA0-Rif declined over time. At low inoculum level, the population level of the strain reached detection limit or fell below detection limit (Fig. 1A[−]), whereas it stabilized at cell numbers of 3–4 log cells ml^{-1} at high inoculum level (Fig. 1B[−]). The addition of nutrients had a positive effect on the survival of CHA0-Rif, but when the strain was inoculated at high

level this effect lasted up to day 50 only (Fig. 1[+]). The three cell count methods yielded similar population levels usually, with the main exception on day 20 in the two CHA0-Rif treatments without added nutrients, where total IF counts of the inoculant exceeded viable counts and colony counts by 0.7 (Fig. 1A[−]) and 1.4 log units (Fig. 1B[−]), respectively.

When used at low inoculum level, CHA0-Rif(pME3424) was below detection limit by 20 days, even when nutrients had been added (Fig. 1A). When introduced at high level, the strain was recovered up to 20 days, by total IF counts only (when no nutrients had been added) or all three cell count methods (in nutrient-amended microcosms) (Fig. 1B). Maintenance of pME3424 was investigated when colonies could be recovered. Colony counts of introduced cells on KBA+Rif100 and KBA+Rif100+Tet125 did not differ statistically, indicating that pME3424 was stable in the pseudomonad (data not shown).

The experiment was also performed with CHA0-Rif(pVK100) to assess whether the poor survival of CHA0-Rif(pME3424) was due to the presence of *rpoD* in the plasmid. Results indicated that at low inoculum

level the strain could be detected by colony counts up to 20 days in unamended water and 50 days in nutrient-amended microcosms, but colony counts were lower than the corresponding ones found for CHA0-Rif by 0.3–0.9 log (data not shown). At high inoculum level, however, CHA0-Rif(pVK100) was recovered throughout the 175-day experiment and cell numbers of the strain were as high as those of CHA0-Rif, regardless of the cell count method used (data not shown).

In summary, CHA0-Rif declined during the 175-day experiment, but cell numbers depended on inoculum level and the addition of nutrients. The pseudomonad failed to survive when it contained pME3424, and the negative effect of the plasmid was mainly due to the presence of *rpoD*.

3.2. Effect of nutrient amendment and inoculation with *P. fluorescens* CHA0-Rif or CHA0-Rif(pME3424) on the total number of CFP

The impact on the resident CFP was studied since the inoculants were likely to interact and compete with fellow pseudomonads naturally present in the lysimeter effluent water. The addition of nutrients resulted in a significantly higher number of total CFP (Fig. 2). This effect was observed for all five treatments at 20 and 50 days, but only for one of five at 112 days (i.e., CHA0-Rif at low inoculum level) and two of four at 175 days (i.e., CHA0-Rif at low inoculum level and the uninoculated control; Fig. 2A).

In contrast, inoculation of lysimeter effluent water with *P. fluorescens* CHA0-Rif or CHA0-Rif(pME3424), at low or high inoculum level, had little or no effect on the number of total CFP when compared with the uninoculated control (Fig. 2). At 20 days, when nutrients were added, the total number of CFP was higher in the CHA0-Rif treatment at high inoculum level than in the control (Fig. 2B[+]), but the increase resulted essentially from the contribution of CHA0-Rif colonies on S1 plates considering CFU of the strain on KBA+Rif100 (Fig. 1B[+]). At 50 days, when no nutrients were added, the total number of CFP in the two inoculated treatments at high inoculum level exceeded that in the control by about 0.8 log units (Fig. 2B[−]), but this time the increase did not result
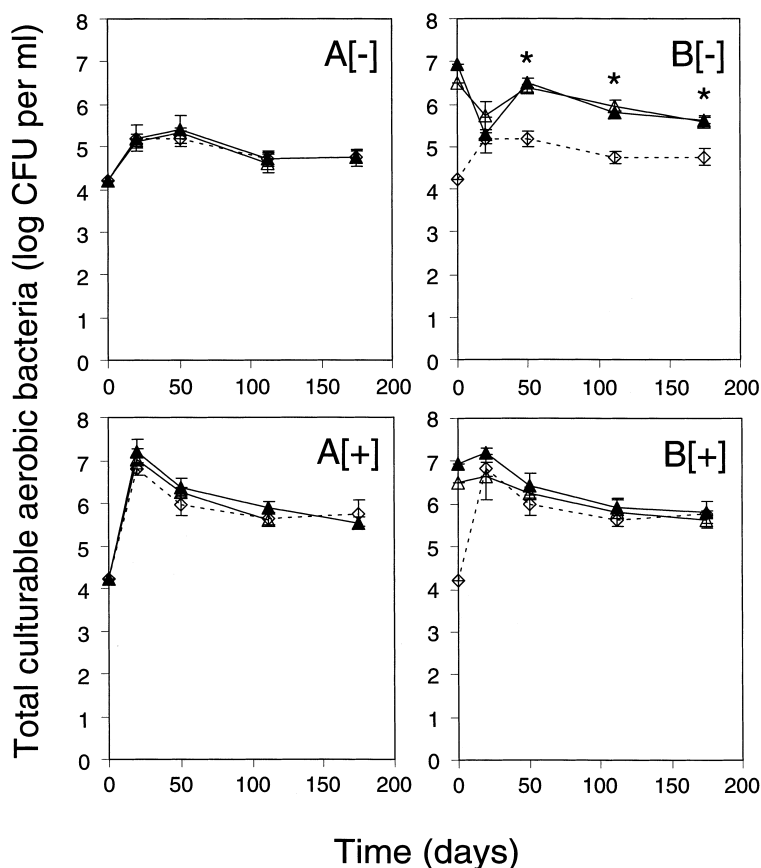

Fig. 3. Effect of nutrient amendment and inoculation with *P. fluorescens* CHA0-Rif (filled triangles) or CHA0-Rif(pME3424) (open triangles) on the total number of CAB in lysimeter effluent water. The uninoculated control is indicated with open diamonds and a dotted line. Microcosms were kept unamended ([−]) or were amended with nutrients ([+]) and inoculation was carried out at 4 (A) or 7 log CFU ml^{-1} (B). Error bars signify standard deviations. CAB were not counted at 175 days in the two CHA0-Rif(pME3424) treatments at 4 log CFU ml^{-1}. Significant differences between the uninoculated control and the inoculated treatments were only found in B[−], where both inoculated treatments differed from the uninoculated control (indicated with an asterisk) from 50 days on.
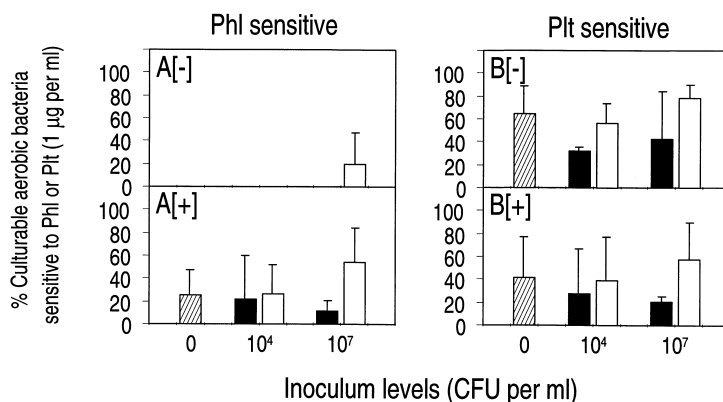
Fig. 4. Effect of nutrient amendment and inoculation with *P. fluorescens* CHA0-Rif (■) or CHA0-Rif(pME3424) (□) on the percentages of the total CAB sensitive to Phl (in A) or Plt (in B) at 1 µg ml^{-1} at 20 days in lysimeter effluent water. Some of the microcosms were not inoculated (▨). Microcosms were kept unamended ([−]) or were amended with nutrients ([+]). Data were not analyzed statistically in A[−] since Phl-sensitive bacteria were seldom found. Statistical analyses were carried out in A[+] and in B, and there was no significant difference between the 5 and 10 treatments, respectively.

from the inclusion of the inoculants in colony counts of S1 plates considering population levels of the introduced strains (Fig. 1B[−]).

Strain CHA0-Rif(pME3424) was not found on plates from 50 days on (Fig. 1), and potential dissemination of pME3424 to resident CFP was studied at 112 days, by spread-plating samples on S1 containing low amounts (25 µg ml^{-1}) of tetracycline. No colonies were found when aliquots from the four treatments previously inoculated with CHA0-Rif(pME3424) were investigated, suggesting that the plasmid had not been transferred to resident CFP.

3.3. Effect of nutrient amendment and inoculation with P. fluorescens CHA0-Rif or CHA0-Rif(pME3424) on the total number of CAB

The inoculants may interact not only with fellow resident pseudomonads but perhaps also with distantly related bacterial taxa naturally present in lysimeter effluent water. Most resident CAB forming a colony on 10% tryptic soy agar could not grow on S1, indicating that they did not correspond to pseudomonads. As for the total CFP, the addition of nutrients had a positive effect on the total number of CAB in the microcosms (Fig. 3). At 20 days, this effect was significant for all five treatments, and was of less magnitude than the effect on the total CFP (Fig. 2). From day 50 on, the positive effect of nutrients on the number of total CAB was significant for the control and the bacterial treatments at low inoculum level (Fig. 3A).

Inoculation of lysimeter effluent water with *P. fluorescens* CHA0-Rif or CHA0-Rif(pME3424), at low or high inoculum level, had no effect on the number of total CAB at 20 days (Fig. 3). However, in the absence of added nutrients, the number of total CAB from day 50 on was higher in microcosms that had been inoculated at high inoculum level compared with uninoculated

microcosms or microcosms inoculated at low level, regardless of the strain used for inoculation (Fig. 3[−]). Interestingly, the increase did not result from the contribution of colonies of the inoculants or of resident pseudomonads on 10% tryptic soy agar considering CFU of the introduced strains (Fig. 1[−]) and the total CFP (Fig. 2[−]).

3.4. Production of antimicrobial polyketides by P. fluorescens CHA0-Rif and CHA0-Rif(pME3424), and effect on the total CAB sensitive to Phl or Plt

Whether or not the inoculants could have an ecological impact on resident culturable bacteria mediated by antimicrobial polyketides was first investigated by HPLC analysis of the polyketides in the microcosms. Results indicated that Phl, its precursor monoacetylphloroglucinol and Plt were below the detection limit at 20 and at 50 days, even in nutrient-amended microcosms.

Second, the ability of the total CAB to grow in the presence of Phl or Plt was investigated at the first sampling (20 days). At that sampling, most of the total CAB corresponded to resident bacteria. The inoculants represented less than 1% (less than 0.1% often) of the total CAB, with the exception of nutrient-amended microcosms that had received high inoculum levels of CHA0-Rif (which represented a large proportion of the CAB). In microcosms without added nutrients, most of the total CAB could grow in the presence of 1 µg Phl ml^{-1} at 20 days, and bacteria sensitive to 1 µg Phl ml^{-1} were found in one of the four inoculated treatments (i.e., about 20% of the total CAB; Fig. 4A[−]). In nutrient-amended microcosms, 25% of the total CAB were sensitive to 1 µg Phl ml^{-1} in the uninoculated control (Fig. 4A[+]). This percentage did not differ statistically from those in the four inoculated treatments. In the uninoculated control, bacteria sensitive to 5 µg Phl ml^{-1} accounted for 31% (nutrient added) and 36% (no nutrient added) of the total CAB.

In contrast to data obtained for 1 μg Phl ml^{-1}, an important proportion of the total CAB recovered at 20 days were sensitive to 1 μg Plt ml^{-1} in unamended microcosms (Fig. 4B[−]). The percentage of the total CAB sensitive to 1 μg Plt ml^{-1} fluctuated to a large extent from one replication to the next for a majority of treatments. When treatment means were considered, this percentage was comprised between 32 and 79% and was not influenced statistically by the inoculation, regardless of the strain and the inoculum level (Fig. 4B[−]). A similar situation was found in nutrient-amended microcosms: a significant percentage of the total CAB was sensitive to 1 μg Plt ml^{-1} (21–60%; Fig. 4B[+]) and treatments had no influence on this percentage. In the uninoculated control, bacteria sensitive to 5 μg Plt ml^{-1} corresponded to 81% (nutrient added) and 94% (no nutrient added) of the total CAB.

4. Discussion

Under field conditions, the biocontrol strain *P. fluorescens* CHA0-Rif can be transported to deeper soil layers and potentially to shallow groundwater [16,17], but still little is known about its capacity to survive at this non-target site [17]. In the current work, experimental conditions were chosen to represent a worse-case scenario regarding potential dissemination of the biocontrol pseudomonad. The results indicate that inoculum level and/or nutrient amendment had a significant influence on the survival of CHA0-Rif in lysimeter effluent water. Similarly, the addition of nutrients to microcosms prepared with agricultural drainage water had a positive effect on persistence of the rhizosphere isolate *P. fluorescens* R2f(RP4) inoculated at 6 log cells ml^{-1} [23]. Strain R2f(RP4) could still be detected in microcosms 1 year after inoculation [36].

Certain Rhizobiaceae can persist as viable but non-culturable cells in tap water depending on water characteristics such as e.g., geographic origin [46] or copper concentration [47]. Similarly, non-culturable cells of CHA0-Rif can be found in lysimeter effluent water depending on (i) the time of the year at which effluent is collected from the lysimeter and soil type in the lysimeter (which both influence water composition), and (ii) conditions of incubation of the water [17,31]. Therefore, colony counts were complemented by viable counts and total IF counts to monitor CHA0-Rif in the current work. Data indicated that the decline of CHA0-Rif did not result in the formation of viable but non-culturable cells, confirming previous results [31], and therefore colony counts were appropriate for strain monitoring.

Since CHA0-Rif persisted at significant population levels in microcosms (at least when introduced at high inoculum level) in the present study, the effect of the inoculant on resident culturable bacteria was assessed. Previous investigations carried out with this strain or other plant-associated pseudomonads have shown that the ecological impact of such bacteria in the target habitat (i.e., the rhizosphere) took place early and was of short duration [13,14,48]. For instance, the application of *P. aureofaciens* SBW25EeZY-6KX onto wheat seeds caused a significant perturbation on resident microbial populations at the seedling stage, on seeds and roots [48]. In soil microcosms, CHA0-Rif affected microbial populations naturally present in the rhizosphere of cucumber, as (i) part of the resident CFP were displaced [13] and (ii) the potential catabolic activity of the microbial community was modified [14]. However, the impact exerted by these two strains was transient, as it disappeared at later stages of plant development. Based on this information, it was anticipated that, in lysimeter effluent water, CHA0-Rif may have an ecological impact on resident culturable bacteria shortly after inoculation (i.e., when cell numbers of the strain were still high), and that potential effects of the inoculant would have disappeared at subsequent samplings. Inoculation with CHA0-Rif had no effect on the number of resident CFP (except at the second sampling), but caused a significant increase in the number of resident CAB when introduced at high inoculum level into unamended microcosms. Unexpectedly, this modification was not apparent until the second sampling, but it was still significant at 175 days, where the inoculant had declined to low cell numbers. Similarly, CHA0-Rif introduced at high inoculum level caused at 112 days (but not at 50 days) a small (0.2 pH units) but statistically significant increase in the pH of unamended lysimeter effluent water (but not in nutrient-amended microcosms) compared with uninoculated microcosms or microcosms inoculated at low level (data not shown). The possibility that the ecological impact of a microbial inoculant could only be detected after amplification in time (perhaps after inoculant decline even) has been raised before [49,50], although certain ecosystems (e.g., the rhizosphere) seem able to buffer the effects of such ecological perturbations [13,14].

Apparently, the impact of CHA0-Rif on the resident CAB was not mediated by antimicrobial polyketides, as the latter could not be detected by HPLC analysis of microcosm water at 20 or at 50 days, even when nutrients promoting production of Phl and Plt had been added. Indeed, significant population levels of resident CAB sensitive to low concentrations (1 μg ml^{-1}) of Phl or Plt were found in a majority of treatments (Fig. 4). Based on this observation, a biocontrol inoculant such as CHA0-Rif would be unlikely to encounter groundwater conditions allowing production of antimicrobial polyketides in situ. This adds to the limitation corresponding to the actual physiological state of CHA0-Rif cells having percolated through the soil profile in situ [17].

One objective of this work was to compare the ecological impact of CHA0-Rif with that of CHA0-Rif-(pME3424). Strain CHA0-Rif(pME3424) is a model genet-

ically modified strain, which overproduces Phl and Plt in vitro, protects plants from soil-borne pathogenic fungi better than what the parental strain can do, and survives well in the rhizosphere [9,13,25]. In lysimeter effluent water, however, CHA0-Rif(pME3424) failed to survive. This is an interesting attribute for a genetically modified inoculant not meant to function outside of the rhizosphere. The poor survival of CHA0-Rif(pME3424) was essentially linked to the presence of an extra copy of the *rpoD* gene, as indicated by population dynamics of the control strain CHA0-Rif(pVK100). The rapid disappearance of CHA0-Rif(pME3424) was probably due to interactions with the resident microbiota since the strain survived well (and at population levels similar to those of CHA0-Rif) for 20 days when incubated in sterile water containing 0.3% NGB (data not shown). Another phenomenon observed only with CHA0-Rif(pME3424) was the transient occurrence, in unamended microcosms inoculated at high level, of large amounts of cells that were neither responsive to Kogure's viability test nor could form a colony on plate (Fig. 1B[−]). Since the modification in CHA0-Rif(pME3424) is likely to concern several aspects of the strain's physiology, one can only speculate on the reasons why survival was poor. Perhaps this strain is better adapted to habitats enabling growth (e.g., the rhizosphere) rather than stress conditions, as could suggest the fact that at high inoculum level culturable cells of the pseudomonad at 20 days were found only when microcosms had been amended with nutrients (Fig. 1B[+]). This hypothesis is strengthened by the observation by Whistler et al. [51] of a link between production of antimicrobial polyketides and stress response in the Phl+Plt+ strain *P. fluorescens* Pf-5.

The ecological impact of CHA0-Rif was mostly apparent once the strain represented a very small percentage of the total CAB. In this context, the rapid disappearance of CHA0-Rif(pME3424) proved useful to analyze the ecological impact of inoculation. Indeed, inoculation with CHA0-Rif(pME3424) caused an ecological impact identical to that of the CHA0-Rif treatment, regardless of the inoculum level × nutrient addition combination studied. This suggests that (i) the culturable cells of CHA0-Rif found from days 20 to 175 (3–4 log CFU ml^{-1}) had a marginal or no ecological impact and (ii) the positive effect of CHA0-Rif on the number of resident CAB, in the treatment where the strain was introduced at high inoculum level in unamended microcosms, was due to the presence of high cell numbers of the inoculant at the early stage of the experiment. It is hypothesized that this positive effect resulted from the availability of nutrients released during the decline of CHA0-Rif. In accordance with this, an effect comparable with the one exerted by CHA0-Rif was observed when laboratory medium was added to the microcosms to mimic leaching of nutrients to the groundwater.

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