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ECOLOGY OF BIOCONTROL *PSEUDOMONAS FLUORESCENS* CHA0 IN NON-TARGET ENVIRONMENTS: BIOSAFETY IMPLICATIONS

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SUMMARY

New technologies such as bioremediation or biocontrol involve the large-scale release of bacterial inoculants into the environment. Their ability to perform at specific target sites under outdoor conditions is extensively studied and may be enhanced by genetic engineering. Recognising that bacterial inoculants can be transported away from the site of their application, their survival and impact at non-target sites deserve attention. In the current work, non-target environments, such as nonrhizosphere soil and groundwater were chosen to study autecological and synecological aspects of the root-colonising biocontrol inoculant *Pseudomonas fluorescens* CHA0.

Survival patterns of strain CHA0 were investigated in soil microcosms and in groundwater microcosms for 48 d and 175 d, respectively. Introduced into microcosms consisting of non-sterile soil, CHA0 cells persisted at inoculation level (8 log $CFUg^{-1}$ soil) for 14 d. At subsequent samplings, the inoculant was found at lower cell numbers and a majority of these cells had lost their ability to respond in Kogure's direct viability test and to form colony on plates. The survival pattern in soil was not changed for CHA0 cells deprived of a single nutrient (C, S, N, or P) in liquid culture prior to their introduction into soil. Deprivation of CHA0 cells for multiple nutrients prior to their introduction into soil resulted in the early occurrence of inoculant cells, which neither responded in the viability test, nor formed colony on plate. Groundwater microcosms were prepared with effluent water collected from 2.5-m deep outdoor lysimeters at four times during the year and from a well-drained or a poorly-drained lysimeter. After inoculation of groundwater microcosms with CHA0 at 7 log CFU ml^{-1}, inoculant cells declined exponentially. However, the survival of CHA0 in microcosms varied greatly depending on i) the time of the year at which lysimeter effluent water had been collected and ii) the lysimeter type. Correlations were found between population dynamics of CHA0 and characteristics of the effluent water before inoculation, especially the number of resident culturable aerobic bacteria. Nutrient addition to microcosms resulted in a delay of inoculant decline, whereas no decline took place in microcosms consisting of filter-sterilised effluent water. This suggests that inoculant decline in non-sterile groundwater microcosms was largely due to the presence of the resident microbiota. Introduced at two inoculum levels (i.e. 7 and 4 log CFU ml^{-1}) into microcosms prepared with effluent water collected from a well-drained lysimeter in winter, strain CHA0 was compared with its derivatives CHA0(pVK100) and CHA0(pME3424). The plasmid pME3424 consists of the plasmid vector pVK100 and a copy of CHA0's *rpoD* gene (encoding sigma factor σ^{70}). Strain CHA0(pME3424) displays enhanced production of the antimicrobial polyketides 2,4-diacetylphloroglucinol (Phl) and pyoluteorin (Plt) *in vitro* and improved biocontrol efficacy in soil microcosms. In groundwater microcosms, CHA0(pME3424) disappeared within 50 d, regardless of the inoculum level studied. In contrast, cell numbers of CHA0 and CHA0(pVK100) were similar and significantly higher than those of CHA0(pME3424). CHA0(pVK100) cells displayed a progressive decrease in the expression of a plasmid-vector marker (i.e. tetracycline-resistance) during their persistence in groundwater microcosms. The chromosomal marker (i.e. rifampicin-resistance) of CHA0 was appropriate for monitoring inoculant cells in groundwater microcosms. Only at early samplings, inoculant cells were found, which did not form colony on plates with rifampicin and failed to respond in Kogure's viability test. These cells were probably dead, as they did not persist in the microcosms. Inoculant cells,

which responded in the viability test, but failed to form colony on plates (i.e. viable but non-culturable cells) were essentially not found in soil and groundwater microcosms.

It was investigated whether inoculation with CHA0 or CHA0(pME3424) had an impact in groundwater microcosms. Inoculation of microcosms with CHA0 at 7 log CFU ml^{-1}, but not at 4 log CFU ml^{-1} resulted in a significant increase of i) the pH (but only 0.2 unit; at 112 d), ii) the numbers of resident culturable fluorescent pseudomonads (0.8 log unit; at 50 d) and iii) the numbers of resident culturable aerobic bacteria (about one log unit; from 50 to 175 d) compared with uninoculated groundwater microcosms. Apparently, the impact was not due to the antimicrobial polyketides Phl and Plt, since i) these compounds were not found by HPLC in microcosm water (even when nutrients promoting the production of Phl and Plt had been added) and ii) there was no increase in the percentage of resident culturable aerobic bacteria resistant to Phl or Plt. An identical impact was caused by the inoculation with a high cell number of CHA0(pME3424), which shows characteristics of a suicide strain in groundwater microcosms. This suggests that the impact, which was observed after inoculation of groundwater microcosms with a high number of CHA0 cells was caused by the high number of inoculant cells present at an early stage of the experiment rather than the 3-4 log CFU ml^{-1} of CHA0 found from days 20 to 175. A similar impact resulted from the addition of nutrients to uninoculated microcosms.

In conclusion, strain CHA0 persisted in soil partly as cells, which were not detectable with methods addressing the ability of bacteria to form colony on plate. The occurrence of these cells in soil was influenced by the availability of nutrients during inoculum preparation. In groundwater microcosms, inoculant cells retained their colony-forming ability, but CHA0(pVK100) cells were not correctly monitored considering their tetracycline-resistant phenotype. Survival of inoculants in groundwater microcosms was restricted due to interactions with the resident microbiota and could be described taking into account particular water characteristics. A lasting effect on the number of resident culturable aerobic bacteria was caused by the inoculation of groundwater microcosms under particular conditions (i.e. when microcosms were prepared with water collected in winter and inoculated at high inoculum level). That impact was not due to the production of the antimicrobial polyketides Phl or Plt, and a comparable effect was observed when nutrients were added to uninoculated microcosms. In the rhizosphere, the presence of the inoculants causes only a transient impact. For biosafety considerations, it is emphasised that bacterial inoculants interact differently with the environment at their target site and at non-target sites.

ZUSAMMENFASSUNG

Bei der Anwendung von Mikroorganismen für den Abbau von Schadstoffen oder für die biologische Bekämpfung von Pflanzenschädlingen wird eine grosse Anzahl von Bakterien in die Umwelt freigesetzt. Die Funktionen solcher Bakterien am Ort ihres Einsatzes werden intensiv erforscht. Da freigesetzte Bakterien vom Ort ihres Einsatzes in der Umwelt verbreitet werden können, gilt es abzuklären, wie sie an anderen Orten, als denen ihres Einsatzes, überleben und welche Wirkungen sie dort erzielen können. Das in dieser Arbeit betrachtete Bakterium *Pseudomonas fluorescens* Stamm CHA0 besiedelt Pflanzenwurzeln und unterdrückt mehrere bodenbürtige Pflanzenkrankheiten. Gegenstand dieser Studie ist die Ökologie von CHA0 in wurzelfreiem Boden und im Grundwasser.

Überlebensmuster von CHA0 wurden während 48 Tagen in Mikrokosmen aus natürlicher, wurzelfreier Erde und während 175 Tagen in Grundwasser-Mikrokosmen festgehalten. In Erd-Mikrokosmen wies der Stamm während 14 Tagen eine zu der inokulierten, unveränderte Zellzahl auf. Diese ging in der Folge zurück und ein Grossteil der in der Erde überdauernden CHA0-Zellen sprach weder auf 'Kogures Viability-Test' an, noch bildete er Kolonien auf Selektivmedien. Identisch verhielten sich CHA0-Zellen, die in Flüssigkultur ohne die einzelnen Nährstoffe C, S, N oder P gehalten worden waren, bevor sie der Erde zugegeben wurden. Inkubieren von CHA0-Zellen in physiologischer Kochsalzlösung hatte zur Folge, dass ein Grossteil der anschliessend der Erde zugegebenen Zellen schon nach wenigen Stunden weder in 'Kogures Viability-Test' reagieren noch Kolonien bilden konnte. Da die Anzahl solcher CHA0-Zellen in den Mikrokosmen weitgehend konstant blieb, wird vermutet, dass es sich dabei um inaktive ("schlafende") Zellen handelte. Grundwasser-Mikrokosmen wurden mit Wasser erstellt, das in 2,5 m Tiefe am Ausfluss von Lysimetern entnommen worden war. Die Entnahme geschah zu 4 Zeitpunkten im Jahr und Wasser wurde sowohl von gut wie von schlecht drainierten Lysimetern gesammelt. Stamm CHA0 zeigte bei einer Startkeimzahl von 7 log KbE/ml einen exponentiellen Rückgang seiner Populationsdichte in Grundwasser-Mikrokosmen. Die Populationsdynamik des Inokulanten variierte innerhalb von Mikrokosmen aus Wasser, das zu verschiedener Jahreszeit und das verschiedenen Lysimetertypen, entnommen worden war. Es wurden Korrelationen zwischen der Populationsdynamik von CHA0 und Eigenschaften des Wassers zum Zeitpunkt der Entnahme aus den Lysimetern gefunden. Bei den korrelierenden Wassereigenschaften gilt es, die Keimzahl residenter, aerober Bakterien hervorzuheben. Wurden den Mikrokosmen Nährstoffe zugefügt, so verzögerte sich der Rückgang inokulierter CHA0-Zellen. Der Rückgang blieb in Mikrokosmen aus sterilfiltriertem Grundwasser aus, was vermuten lässt, dass residenten Mikroorganismen eine Schlüsselrolle beim Rückgang des Inokulanten zukam. In Mikrokosmen aus Wasser, das im Winter einem schlecht drainierten Lysimeter entnommen worden war, wurde ein Vergleich zwischen CHA0 und der Mutante CHA0(pME3424) bei Startkeimzahlen von 7 oder 4 log KbE/ml erstellt. Das Plasmid pME3424 besteht aus dem Plasmidvektor pVK100 sowie einer Kopie von CHA0s *rpoD* Gen, welches für den Sigmafaktor σ^{70} kodiert. Stamm CHA0(pME3424) vermag die antimikrobiellen Polyketide 2,4-Diacetylphloroglucinol (Phl) und Pyoluteorin (Plt) *in vitro* vermehrt zu produzieren und Pflanzen besser zu schützen als CHA0. In Grundwasser-Mikrokosmen konnte CHA0(pME3424) nach 50 Tagen, unabhängig von der betrachteten Startkeimzahl, nicht mehr nachgewiesen werden. Die Zellzahlen von CHA0 und

CHA0(pVK100) waren vergleichsweise höher und voneinander nicht verschieden. Bei CHA0(pVK100)-Zellen wurde eine kontinuierliche Abnahme der Expression der Tetrazyclinresistenz festgestellt, welche auf pVK100 lokalisiert ist. Bei Probenentnahmen zu einem frühen Stadium des Experimentes wurden inokulierte Bakterien gefunden, die die Fähigkeit verloren hatten, in 'Kogures Viability-Test' zu reagieren und Kolonien zu bilden. Da diese Zellen in Grundwasser-Mikrokosmen nicht überdauerten, waren sie vermutlich tot. Weder in Erd- noch in Grundwasser-Mikrokosmen traten relevante Mengen inokulierter Zellen auf, die in 'Kogures Viability-Test' reagierten, obgleich sie keine Kolonien zu bilden vermochten, d.h. sogenannte VBNC-Zellen.

Inokulation der Grundwasser-Mikrokosmen bei hoher, nicht jedoch bei niedriger Startkeimzahl, der Inokulanten hatte folgende Auswirkungen: der pH Wert nach 112 Tagen war um 0,2 Einheiten, die Keimzahl residenter, fluoreszierender Pseudomonaden nach 50 Tagen um 0,8 log-Einheiten und die Keimzahl residenter, aerober Bakterien ab 50 Tage um ca. eine log-Einheit höher als in nicht inokulierten Mikrokosmen. Dabei ging offensichtlich kein Einfluss von den antimikrobiellen Polyketiden Phl und Plt aus. Diese Verbindungen konnten durch HPLC-Analyse in den Mikrokosmen nicht nachgewiesen werden, auch dann nicht, wenn den Mikrokosmen Nährstoffe zugefügt worden waren, die die Produktion von Phl und Plt fördern. Zudem nahm die relative Keimzahl residenter, aerober Bakterien, die gegenüber Phl oder Plt resistent sind, in den Mikrokosmen nicht zu. Überraschend erzielte die Inokulation mit CHA0 die gleiche Wirkung wie jene mit CHA0(pME3424), welcher in Grundwasser-Mikrokosmen Eigenschaften eines Suizid-stammes aufwies. Die Auswirkungen der Inokulation mit CHA0 waren demnach eher auf die hohe Startkeimzahl zurückzuführen, als auf die 3 bis 4 log-Einheiten KbE/ml von CHA0, die zwischen 20 und 175 Tagen in den Mikrokosmen gefunden wurden. Eine der Inokulation mit hoher Startkeimzahl ähnliche Wirkung erzielte eine Nährstoffzugabe zu nicht inokulierten Mikrokosmen.

Folgende Schlüsse können aus den Resultaten dieser Arbeit gezogen werden: Stamm CHA0 kann in Erde in einer Form von Zellen überdauern, die anhand der Keimzahl auf Selektivmedien nicht nachgewiesen werden kann. Das Auftreten dieser Form von CHA0-Zellen kann über die Nährstoffverfügbarkeit während der Zubereitung des Inokulums beeinflusst werden. In Grundwasser-Mikrokosmen ist die Keimzahl auf Selektivmedien ein geeignetes Mass zur Quantifizierung überdauernder Zellen von CHA0 oder seinen Abkömmlingen. Allerdings ist eine korrekte Quantifizierung anhand der Resistenz gegenüber Tetrazyclin in Zellen nicht gewährleistet, die den Plasmidvektor pVK100 bergen. Die Überlebensfähigkeit der Inokulanten in Grundwasser-Mikrokosmen ist unter dem Einfluss residenter Mikroorganismen eingeschränkt. Die Populationsdynamik der inokulierten Bakterien kann unter Berücksichtigung bestimmter Grundwassereigen-schaften beschrieben werden. In Mikrokosmen, die aus im Winter gesammeltem Wasser bestanden und mit einer hohen Startkeimzahl inokuliert wurden, hatten die Inokulanten einen nachhaltigen Einfluss auf die Keimzahl residenter, aerober Bakterien. Dies war nicht auf die Produktion der antimikrobiellen Polyketide Phl oder Plt zurückzuführen und konnte auch durch Zugabe von Nährstoffen zu nicht inokulierten Mikrokosmen bewirkt werden. Auswirkungen auf 'Nicht-Ziel-Organismen' der Inokulanten im Wurzelraum sind von kurzer Dauer. Daraus muss geschlossen werden, dass von bakteriellen Inokulanten, die den Ort ihres Einsatzes verlassen, Auswirkungen zu erwarten sind, die sich von jenen am Ort ihres Einsatzes beträchtlich unterscheiden können.

INTRODUCTION

1. Microbial Releases into the Environment

Release of microbes into the environment can take place for several reasons. In contained systems like fermenters, large numbers of wildtype and genetically modified microorganisms are produced for medical and food industry (e.g. for the production of pharmaceutical polypeptides and commercially important enzymes). Such indoors-produced microbes may be released unintentionally into the environment through accidental escape, but safety precaution may restrict the frequency of such scenarios. Little concern is allocated on the release of microbes used for brewing, vaccine production or some agricultural applications (e.g. of rhizobia), which have been performed since a long time without recognised harm to the environment.

During the last decades, the spectrum of environmental applications involving the release of large numbers of indoors-produced microorganisms has widened, as their ability to fulfil a broad range of functions is recognised. Specific pollutants at contaminated sites can be degraded by certain bacteria (Chaudry, 1994; Ensley and De Flaun, 1995; Kumar et al., 1996; Bonaventura and Johnson, 1997) and the turnover of biomass in composts can be accelerated by a variety of bacterial formulations (Chen and Yang, 1998). Protection of plants against damage due to surface freezing can be accomplished by the displacement of ice nucleation-positive wildtype bacteria after application of ice nucleation-negative mutants of *Pseudomonas syringae* (Lindow, 1995). With *Bacillus thuringiensis* spores, plant damaging insects and mosquitoes carrying causative agents of human diseases are controlled (Entwistle et al., 1993; Seyoum and Abate, 1997). Due to their ability to fix nitrogen and/or produce phytohormones, strains from the genera *Rhizobium, Bradyrhizobium* and *Azospirillum* are utilised for active crop plant fertilisation (Antoun et al., 1998; Döbereiner and Pedrosa, 1987; Laeremans and Vanderleyden, 1998). Other bacteria are able to enhance nutrient uptake or stimulate the growth of plants (Amara and Dahdoh, 1997). Beneficial bacteria particularly from the genera *Pseudomonas* and *Bacillus* are capable to suppress a broad variety of crop plant diseases (Cook et al., 1995; Keel and Défago, 1996). Whereas some of the above mentioned environmental applications of microbes have

been performed successfully for many years, other products have been commercialised only recently (Reddy et al., 1996; Tengerdy and Szakacs, 1998).

2. Improvement of Inoculants by Genetic Engineering

Whereas several microbial agents have been applied successfully for a long time (e.g. the inoculation of legumes with rhizobia and the spraying of plants with inactivated *B. thuringiensis* spores), other inoculants often do not perform consistently in the environment. The effects of microorganisms after their release into the field are sometimes smaller than those observed under controlled conditions in the laboratory, and they vary to a large extent with changing environmental conditions (Goldstein et al., 1985; Weller, 1988). Therefore, many promising microbial applications have not been commercialised yet. The reasons for unsatisfactory performance of microbial applications under outdoor conditions include that expression of genes whose products fulfil important functions for a particular application may be to low, or that the inoculant is not able to survive at the target site.

Biotechnology may provide ways to increase the performance of microbial applications under field conditions. Low expression of functional genes can be genetically enhanced due to constitutive expression or overexpression of those genes (Baek et al., 1999; Ensley and De Flaun, 1995; Gutterson, 1990). Insufficient performance caused by decreased fitness of the host strain carrying the functional genes may be improved by insertion of those genes into a host, which survives better at the target site (Crowley et al., 1996; Ensley and De Flaun, 1995; Gelernter and Schwab, 1993). Theoretically, an inoculant's ecological fitness could also be genetically increased (Chand et al., 1999), but intraspecific competition with the parental strain in most cases leads to impaired fitness of the engineered strain (Recorbet et al., 1992; van Elsas et al., 1994). However, mutants capable to grow on a specific substrate may exhibit increased fitness compared with their parental strains when this substrate is available. For example, increased population densities due to the presence of the plasmid NAH7 providing the ability to degrade salicylate in *P. putida* strains were reported in soil amended with salicylate (Colbert et al., 1993). Finally, genetic engineering could be used to combine several functions in one microorganism to

enhance or broaden its spectrum of activity (Ensley and De Flaun, 1995; Hara et al., 1994).

However, altered ecological fitness and improved capacity to fulfil a certain function of genetically modified organisms (GMOs) may not only be of interest for the application of a genetically modified inoculant at the target site. It has been recognised that microbial inoculants after being applied at the target site can reach other habitats (Natsch et al., 1996; Troxler et al., 1998). Whether a genetic modification in an inoculant can affect ecological fitness or gene expression of its genetically modified derivative, once transported to a non-target site, is a question to be addressed when constructing GMOs for environmental release.

Genetic modification may involve a changed ecological fitness of GMOs, and the requirement of investigations with model GMOs has been raised for regulation (Stewart-Tull and Sussman, 1992). However, such model GMOs should be functionally improved and have potential practical applications in the environment (Natsch et al., 1998b).

3. Risk Assessment

Some environmental applications of microbes have been experienced for a long time and negative consequences of their application have not been recognised. In contrast, exotic animal or plant species introduced unintentionally or by purpose into ecosystems in which they had not been present before can result in the elimination and thereby in a loss of the diversity of native species (McKnight, 1991; Ledig 1992; Pearce et al., 1997; Hobbs and Mooney, 1998). For decades, intentional release of animals or plants has been practised, for example in classical biological control. Here, exotic natural enemies are introduced to control previously introduced, nonindigenous insect pests and weeds (Knutson and Coulson, 1997). There are few documented instances of damage to non-target organisms or the environment from nonnative species released for biological pest control, relative to the number of such releases (Simberloff and Stiling, 1996). However, monitoring of non-target species is minimal, particularly in sites and habitats far from the point of release. In fact, the discovery of such impacts usually rests on a remarkable concatenation of events.

Enhanced ecological fitness can be a characteristic of an exotic species when introduced into a habitat where its natural enemies are lacking or its competitors are inferior. An increase of the ecological fitness of an organism targeted for release theoretically can be achieved by genetic engineering. However, genetically enhanced fitness can be an undesired trait of a GMO when leading to outcompetition of non-target organisms, once the GMO is released into the environment. With respect to this property potentially shared by wild-type organisms and GMOs it becomes evident that environmental releases at commercial scale of both wild-type organisms and GMOs should deserve attention. It has also been recommended that the full set of characteristics of an organism targeted for environmental release (i.e. those of the host and those of the introduced gene in the case of a GMO) should be evaluated, rather than on how the organism was obtained (Nat. Acad. Sci., 1987; Van Elsas, 1995).

However, there is much more concern on potential releases of GMOs than of wild-type organisms into the environment. The negative side effects of new technologies such as nuclear power and the use of pesticides were discussed only after negative effects had become obvious. In the case of genetic engineering it has been recognised that safety of this new technology should be ensured before an application at a large scale. The possible risks coming from the application of this new technology should be evaluated before its commercial products are ready for release, as otherwise the application of such products may be restricted by the following (e.g. economic) considerations: i) the pressure may become too high to apply those products despite of possible risks, ii) the costs resulting from a possible ban of the technology may also be high (Woodhouse, 1992) and iii) monitoring (and possible treatment) of the environment and the at-risk population is difficult and expensive (Stephenson and Warnes, 1996). Therefore, it is comprehensible that large-scale environmental applications of GMOs in most developed countries are cautiously driven forward by commercial and scientific communities. A consequence of this cautious approach is that for regulation, risk assessment studies are conducted with genetically engineered model organisms, which are not ready for commercialisation. However, this has been impeded for a long time due to the lack of such model GMOs (Stewart-Tull and Sussman, 1992) and authorities in most countries pursue a case-by-case practice when evaluating the risks associated with the release of GMOs into the environment.

When considering the release of bacteria into the environment, several characteristics distinguishing them from eukaryots should be stressed. Some bacteria have the potential to multiply within short time and mutate at high rate, which may contribute to their adaptation to changing (e.g. environmental) conditions (Taddei et al., 1997; Rainey and Travisano, 1998). Adaptation to environmental conditions can also entail the loss of certain characteristics and/or phenotypes of bacteria. The ability of bacteria to form colony on plate and/or to respond to viability tests may be addressed when monitoring bacterial populations after their release into the environment (Brayton and Colwell, 1987; Xu et al., 1988; Troxler et al., 1997b; McDougald et al., 1998). Due to the widespread presence of extrachromosomal, mobilizable genetic elements in bacteria, horizontal gene transfer could represent a frequently occurring event (Droge et al., 1998; Sørensen and Jensen, 1998). To ensure satisfactory performance, many environmental applications of bacterial agents require the release of high numbers of the microorganisms. Finally, there is still little knowledge on the non-target species of bacteria targeted for release into the environment. Thus, even long-term environmental applications of microorganisms may have unrecognised, negative consequences on non-target organisms and there is a need for more information on those non-target organisms and their ecology.

However, for evaluation of the risks associated with microbial releases, approaches similar to those used for assessment of the ecotoxicology of pesticides and chemical contaminants are applied. Analyses of the ecotoxicology of chemicals include two main aspects (Bacci, 1994): First, in an exposure study the persistence and the transport away from the site of application of a chemical substance (and its degradation products) is investigated. In a second step, possible effects adverse to non-target organisms or ecosystem processes are evaluated in an effects study. The results from these two studies serve to assess i) the hazard of a substance (i.e. the concentration of a substance in an environmental compartment that can produce adverse consequences to some organisms or ecosystem parameters) and ii) the risk of the application of a substance (i.e. the probability that a hazard might be of significance e.g. to humans or the whole ecosystem function) (Bacci, 1994; OECD, 1989).

There are several homologies between assessments of the ecotoxicology of chemicals and the biological safety of microbial releases (Levin, 1991). As for chemicals, it is important to know i) how long and in which form a microbial population

persists at the site of release, ii) to what extent it is transported away from the site of release and iii) what are the effects of the release at both target sites and non-target sites. However, there are some differences between microorganisms and chemicals that should be considered when assessing the risks coming from microbial releases: Whereas chemicals tend to be diluted in the environment, microorganisms have the inherent potential to proliferate and, thereby, to extend the time of their persistence (Krimsky et al., 1995). Degradation of chemicals may result in more stable or more toxic compounds, but their number is limited (Krimsky, 1991). In contrast, microorganisms can change their genotype by mutation and gene exchange resulting in new phenotypes of these microorganisms. Transferred genes can i) persist, ii) multiply and iii) change the phenotype in the host organism. Therefore, not only the released organism itself, but also the hazard of new genes in a foreign host has to be considered (Miller and Levy, 1989). Moreover, microorganisms can actively migrate to a certain extent (Heijnen and van Veen, 1991), although active movement is considered to be limited in many environments (van Elsas and Trevors, 1991). Although microorganisms released into the environment do not necessarily display these characteristics distinguishing microbes from chemicals, they extend the aspects to be considered when assessing the risks of microbial releases compared with the ecotoxicology of chemicals.

In biological safety assessment, organisms instead of chemicals are considered. Here, information on the fate and the effects can be obtained from studies on the autecology and the synecology of the organism to be released (Natsch et al., 1998b). Somehow homologous to exposure studies for chemicals, autecology considers a specific organism's survival strategies, its ability to colonise ecological niches and its dissemination potential. The subjects of synecology studies are interactions between a specific organism and other organisms as well as possible adverse effects, as they are addressed in the effects study of chemicals. To assess the potential hazard and risk of large-scale microbial releases into the environment, results from both autecological and synecological studies should be combined. Such studies should also be conducted under outdoor conditions, since results obtained in microcosms or mesocosms are often not completely predictive of the field (Van Elsas, 1995; Natsch et al., 1998b). Therefore, a step-by-step approach has been recommended to assess the risks associated with the environmental release of microbes (Elliot et al., 1986). This approach means that studies are performed gradually from highly contained and controlled situations to open

and less controlled ones, and decisions of further work are based on the safety data obtained from previous more confined systems (Gillett, 1989). A step-by-step procedure of biosafety assessment may include autecology and synecology studies performed in laboratory microcosms, in mesocosms and in the field. This allows that i) conditions, which are expected to entail the occurrence of adverse effects in the environment, can be tested in enclosed systems already at an early stage of the assessment and ii) familiarity with the microorganism and the application is produced.

4. Aspects of the Biosafety Assessment of Microbial Releases

Aspects of the autecology and the synecology at the target site

a) Persistence

The target site of large-scale microbial applications, such as bioremediation, biocontrol, plant growth stimulation or nitrogen fixation is represented in many cases by the soil habitat. Field release of bacterial inoculants revealed that their populations undergo a progressive decline, once the inoculants had been introduced into soil (Van Elsas et al., 1986; Troxler et al., 1997b; Molina et al., 1998). The rate of such declines can be species specific, given by the intrinsic physiological characteristics of the inoculated strains used (Thompson et al., 1992). Altering the characteristics of an inoculant strain by genetic engineering can lead to enhanced survival in soil due to specific functions mediated to the GMO by the modification. For example, the *P. putida* strain PpG7, which utilises salicylate owing to the plasmid NAH7 displayed increased population densities in soil and rhizosphere amended with salicylate (Colbert et al., 1993). However, the presence of a specific substrate, degradable by a GMO, but not by its parental strain does not always lead to enhanced survival in soil of the derivative compared with its non-engineered parental strain (Short et al., 1991; Brazil et al., 1995). Additional siderophore receptor genes when cloned into *Pseudomonas* can enhance the ecological fitness of the engineered strain (Raaijmakers et al., 1995), but this is not necessarily the case (Moënne-Loccoz et al., 1996). Genetic modification resulting in enhanced production of antimicrobial compounds in biocontrol inoculants has not

turned out to positively influence the ecological fitness of the modified strains in soil habitats (Natsch et al., 1998a). Apart from inoculant specific characteristics, other factors are discussed to be influential on inoculant survival in soil. Those include the soil type, the temperature and the content of soil in moisture and available nutrients (Van Elsas et al., 1986; Wessendorf and Lingens, 1989; Natsch et al., 1994; Van Veen et al., 1997; Hase et al., submitted). The soil apart from the rhizosphere can be regarded as an oligotrophic environment (Williams, 1985) exhibiting a growth/survival-inhibitory effect on introduced microbes (Van Veen et al., 1997). From plant roots and particularly from old and decaying root tissue, nutrients are excreted, which may support growth of inoculants (Thompson et al., 1992; Troxler et al., 1997c).

Inoculant monitoring in soil under outdoor conditions has revealed that methods addressing the culturability of bacterial cells (i.e. their ability to form visible colonies after culturing on plates) may not detect the total, persisting inoculant population (Troxler et al., 1997b). Inoculant cells, which are not detectable with that method can still exhibit metabolic activity and/or substrate-responsiveness, as shown when applying certain viability tests (Heijnen et al. 1995, Troxler et al., 1997b). Such cells are termed viable-but-nonculturable (i.e. VBNC) cells and they were found on several occasions in soil or root-associated environments (Binnerup et al., 1993; Turpin et al., 1993; Troxler et al., 1997c; Hase et al., submitted). Application of the viability tests in combination with immunofluorescence microscopy to samples from inoculated soil revealed that inoculant cells can persist in soil without exhibiting metabolic activity and/or substrate-responsiveness (Heijnen et al. 1995, Troxler et al., 1997b; Hase et al., submitted). Such cells are termed inactive/dormant cells. For inoculants such as *Pseudomonas* spp., the ecological significance of the VBNC cells and the dormant/inactive cells is not known and there is no clear evidence for resuscitation from these physiological states (Natsch et al., 1998b).

However, the overall physiological status and activity of inoculants (e.g. as biopesticides or as detoxifying agents for contaminated soils) is of prime interest for the efficacy of the introduction (Van Veen et al., 1997). If a potential hazard is associated with the expression of (heterologous) genes and this gene expression is linked to overall cellular physiology, the physiological status of inoculant cells is of interest for biosafety considerations in releases of genetically modified microorganisms into soil. The mechanisms leading to the occurrence of nonculturable inoculant cells in soil are not

understood so far. Stress conditions, such as a scarcity of easily available nutrients, noxious compounds and physical factors like temperature and soil water content, as well as fluctuations in these conditions, which are typical for the soil environment are discussed to be involved in the occurrence of nonculturable inoculant cells in soil (Kennedy and Wollum II, 1988; Pedersen and Jacobsen, 1993; Kragelund and Nybroe, 1996; Troxler et al., 1997b; Van Veen et al., 1997; Hase et al., submitted).

For resident microorganisms in soil, it is suggested that VBNC cells and the dormant/inactive cells may play a role in processes like nutrient turnover, the breakdown of xenobiotics or infection (Akkermans, 1994), and that a majority of those cells are not dead (Bohlool and Schmidt, 1980).

b) Horizontal gene transfer

Transfer of genes present on plasmids between released donor and released recipient strains, or between released donor and resident recipient strains is amply documented (Miller and Levy, 1989). Generally it was observed that plasmids can readily be transferred in water, bulk soil, in the rhizosphere and in the spermosphere (Van Elsas and Smit, 1994; Lilley et al., 1994; Sørensen and Jensen, 1998). It has been suggested that introducing the gene(s) into the chromosome would offer a better containment. However, it was found that that in the presence of fertility plasmids even chromosomal markers can be transferred in the rhizosphere (Troxler et al., 1997a). There is also evidence from population genetic studies that chromosomal gene transfer occurs in the environment, e.g. between released and indigenous rhizobia (Sullivan et al., 1995). Hence, considering the large number of bacterial cells to be released for applications such as biocontrol (i.e. about 10^{18} cells/ha), gene transfer events may happen at a certain frequency.

c) Effects on the ecosystem

There are different ways how released microorganisms may be expected to interact with the resident microbiota. First, they may compete for e.g. nutrients and space with an indigenous population colonising a similar ecological niche, and they could thus displace at least part of this resident population. Second, toxic compounds (e.g. antibiotics, bacteriocins or degradation products of xenobiotics) produced by the introduced strains could affect sensitive microorganisms. Many ecological processes such as nitrogen or carbon turnover in soil are dependent on specific groups of microorganisms. If some functional groups of microorganisms are affected as a whole, also certain ecological processes may be influenced. Apart from quantifying total resident microbial populations, methods for the evaluation of effects on microbial populations and processes may include i) measurement of gross metabolic activity and carbon mineralization, ii) quantification of enzymatic activities, iii) evaluation of indicators of the indigenous microbiota, iv) measurement of the rates of nitrogen fixation and v) analysis of soil nutrients (Doyle and Stotzky, 1993; Moënne-Loccoz et al., 1998a; Naseby et al., 1998 and 1999).

The introduction of bacteria into soil can affect resident microbial populations although the inoculants may not exhibit a specific practical application (de Leij et al., 1994 and 1995; Naseby and Lynch, 1997; Kozdrój, 1999). However, with regards to the biosafety of the application of microbial agents, effects of wild-type or genetically modified inoculants, which have a potential practical application, are most interesting. Effects from the introduction of such strains have not always been observed (England et al., 1995; Villadas et al., 1996; Mahaffee and Kloepper, 1997; Moënne-Loccoz et al., 1998b; Naseby et al., 1998). However, introduction of functional inoculants has been shown to cause adverse non-target effects in several cases (Table 1). Introduction of a biocontrol pseudomonad, which had been genetically modified to enhance performance (i.e. by overexpressing genes encoding antimicrobial polyketides) did not produce non-target effects, which were different from those produced by the introduction of its parental strain (Natsch et al., 1997 and 1998a). When a genetic modification provides a function to the GMO that is not exhibited by the parental strain, introduction of the GMO can promote undesired non-target effects, which are not observed with the parental strain.

Table 1. Non-target effects caused by the introduction of functional, microbial inoculants into soil systems.

Strain	Application	Affected parameters	Site	Source
P. fluorescens CHA0	Biocontrol	- Resident fluorescent pseudomonads [1]	Rhizosphere (cucumber)	Natsch et al. (1997)
		- Metabolic activity of the bacterial community [1]		Natsch et al. (1998a)
P. fluorescens F113	Biocontrol	- Chlorine content [1]	Foliage (red clover)	Moënne-Loccoz et al. (1998a)
		- Total *Pseudomonas* population [2]	Rhizosphere (pea)	Naseby and Lynch (1998)
		- Bacterial population structure [2]		
		- Activity of certain soil enzymes [2]		
		- Plant nitrogen uptake [2]		Brimecombe et al. (1998)
		- Microbial activity [2]		
		- Total aerobic bacteria [2]		Naseby and Lynch (1999)
		- Acid phosphatase activity [2]		
		- Carbon fractions [2]		Naseby et al. (1999)
		- Organic acid content [2]		
		- Content in certain ions [2]		
P. putida PPO(pRO103)	Biodegradation	- Fungal propagules [3]	Soil	Short et al. (1991)
		- CO_2 evolution [1]		
Bacillus cereus UW85	Biocontrol	- Bacterial population structure	Rhizosphere (Soybean)	Gilbert et al. (1993)
Streptomyces lividans TK23.1	Lignin degradation	- Populations of certain groups of microorganisms [1]	Soil	Crawford et al. (1993)
		- Activity of certain soil enzymes [1]		
		- Nitrogen cycling [1]		
		- Soil pH [1]		

[1] Transiently affected.
[2] Only once investigated.
[3] Lastingly affected.

This was shown for a herbicide degrading GMO, which after introduction into soil caused a reduction in the number of fungal propagules and in CO_2 evolution (Short et al., 1991). Moreover, this effect was attributed to a toxic intermediate of herbicide degradation in soil and the non-target effect was not predictable from the phenotype of this GMO. The potential occurrence of unexpected non-target effects associated with the release of genetically modified as well as wild-type inoculants may point to the importance of monitoring both introduced populations and potentially affected parameters for extended periods (Doyle and Stotzky, 1993). However, most of the effects observed after the introduction of inoculants into soil systems turned out to be transient and of smaller magnitude than the effects resulting from standard agricultural practices.

Aspects of the autecology and the synecology at non-target sites

A central issue of biosafety assessment of microbial releases is the question how released microorganisms survive at non-target sites of their application, and which effects are to be expected from their occurrence at such sites. For bacterial soil inoculants, emphasis is given to the possibility that after application, inoculant cells are transported through soil to groundwater. Indeed, vertical transport of inoculant cells through soil has been detected in several studies performed in microcosms (Frederickson et al., 1989; van Elsas et al., 1991) and in the field (de Leij et al., 1995; Troxler et al., 1998). Moreover, disadvantageous interaction between the time of release, weather conditions (i.e. the occurrence of heavy rainfalls shortly after release) and the soil type at the site of release (i.e. soil types, which promote the occurrence of preferential flow) can even lead to vertical transport of large numbers of inoculant cells to groundwater level (Natsch et al., 1996).

Whereas there are several reports on the survival of bacteria introduced into surface freshwater (Awong et al., 1990; Brettar et al., 1994; Leung et al., 1995; Oliver et al., 1995; Leff et al., 1998), there is little information on the persistence of soil inoculants in subsurface aquatic environments. The chlorobenzoate-metabolizing *Pseudomonas* sp. strain B13 could be detected more than a year after injection into an aquifer and molecular methods indicated that gene exchange with resident bacteria had taken place

(Thiem et al., 1994). In contrast, the trichloroethylene-degrading *Burkholderia cepacia* strain G4 5223-PR1 decreased to low population levels within 10 d after introduction of the cells into groundwater microcosms (Winkler et al., 1995). Long-term persistence was also shown for a biocontrol pseudomonad introduced into groundwater microcosms (Troxler et al., 1998). This strain persisted for more than a year and mostly as VBNC cells in the microcosms. In any case, there is virtually nothing known on the potential effects of the introduction of soil inoculants on resident groundwater microorganisms.

5. Pathogenicity

A prerequisite for the development of microbial agents to be released into soil is the identification of any pathogenicity to humans and animals during the screening program. Nevertheless, certain clinical isolates and opportunistic human pathogens of *P. aeruginosa* are known to proliferate in the rhizosphere (Morales et al., 1996) and to have biocontrol activities against phytopathogenic fungi (Buysens et al., 1996; Duffy and Défago, 1996). The antifungal and degradative properties of *Burkholderia cepacia* have created interest in its potential use as a biological control agent and for bioremediation of contaminated soils (Govan et al., 1996). Since certain *B. cepacia* spp. are human pathogens, the clarification of the taxonomy of environmental isolates of *B. cepacia* deserves a high priority.

Background and Objective of the Current Study

Pseudomonas fluorescens strain CHA0 is a biocontrol inoculant isolated from a field in Morens (Switzerland), which was suppressive to black root rot of tobacco caused by *Thielaviopsis basicola* (Stutz et al., 1985). This strain was found to be capable of protecting wheat against take-all caused by *Gaeumanomyces graminis* under field conditions and to suppress various other plant diseases such as *Pythium* induced damping-off of cucumber and cress, black-root rot of tobacco and *Fusarium*-wilt of tomato under greenhouse conditions (Défago et al., 1990). The mechanisms of disease suppression were thoroughly studied in the last ten years, and strain CHA0 was also

subject of many molecular biological studies. It was found that the production of the antimicrobial polyketide 2,4-diacetylphloroglucinol (Phl) is crucial for bacterial disease suppression (Keel et al., 1992). However, the bacterial metabolites hydrogen-cyanide (HCN) and the antibiotic pyoluteorin (Plt) do also contribute to the disease suppressive capacity of CHA0 (Voisard et al., 1989; Maurhofer et al., 1994). The *rpoD* gene, encoding the essential housekeeping sigma factor σ^{70} of CHA0, has been cloned and sequenced (Schnider et al., 1995). When this gene is introduced into CHA0 on a multicopy plasmid, this leads to manifold increase of Phl and Plt production *in vitro* and in the rhizosphere. In artificial soil, this derivative has a significantly enhanced disease suppressive capacity, but it can be toxic to some plant species (Maurhofer et al., 1992).

Autecology studies performed with CHA0 under outdoor conditions and in microcosms revealed that the inoculant can persist partly as nonculturable (e.g. VBNC) cells in soil, rhizosphere, root tissue and groundwater (Troxler et al., 1997b, 1997c, 1998; Hase et al., submitted; Mascher et al., submitted). Transfer of chromosomal markers of CHA0 has been found to take place in the rhizosphere (Troxler et al., 1997a). After application, inoculant cells can be translocated to groundwater level (Troxler et al., 1998). Transport of CHA0 cells from the surface to deeper layers in the field can even occur at a cell number of CHA0 as high as 7 log CFU g^{-1} of soil (Natsch et al., 1996). In groundwater microcosms the inoculant persisted for more than one year mostly as nonculturable cells (Troxler et al., 1998).

The synecology of CHA0 and its derivative harbouring the plasmid pME3424, which contains an extra copy of the strain's rpoD gene (i.e. CHA0 (pME3424), was studied in the rhizosphere of cucumber. Introduction of either of the strains transiently displaced a part of the resident culturable fluorescent pseudomonads and changed the potential catabolic activity of the microbial community (Natsch et al., 1997 and 1998a).

In the current work, emphasis is given to the ecology of CHA0 and CHA0(pME3424) at non-target sites, such as nonrhizosphere soil and groundwater. It is investigated whether nutrient deprivation could explain the persistence patterns of CHA0 observed by Troxler et al. (1997b) in soil under outdoor conditions (e.g. the occurrence of nonculturable CHA0 cells). To achieve this goal, first CHA0 cells are deprived of single nutrients (C, S, N, or P) or multiple nutrients in laboratory media. Second, these cells are introduced into microcosms prepared with nonrhizosphere soil

similar to the soil studied by Troxler et al. (1997b). Third, the persistence of these CHA0 cells in the microcosms is compared with the persistence of log-phase cells of the strain introduced into another set of soil microcosms.

An extensive study is conducted addressing the survival and the impact of biocontrol strains potentially transported through soil to groundwater. These experiments are performed in groundwater microcosms prepared with effluent water collected from 2.5-m deep outdoor lysimeters. The persistence displayed by CHA0 is investigated in groundwater when the bacteria are applied and subsequently transported i) through different types of soil at ii) different times of the year to groundwater level. To obtain this, for preparation of groundwater microcosms, lysimeter effluent water is collected i) from different types of lysimeters (i.e. containing either a well-drained or a poorly-drained cambisol) and ii) at different times of the year.

A further aspect of the study includes the possibility that biocontrol bacteria when introduced into groundwater produce toxic compounds such as antimicrobial secondary metabolites. To assess this possibility, strain CHA0 and its derivative CHA0(pME3424) are introduced into groundwater microcosms at two inoculum levels representing the numbers of CHA0 cells found to be transported through soil in previous studies (i.e. 4 log cells ml^{-1} (Troxler et al., 1998) and 7 log cells ml^{-1} (Natsch et al., 1996)). To mimic a worse-case scenario of nutrient leaching to groundwater one set of microcosms is amended with a small amount of a laboratory medium, which promotes the production of the antimicrobial polyketides Phl and Plt by CHA0 and CHA0(pME3424).

Finally, the impact of biocontrol inoculants in groundwater is addressed. Therefore, the culturable fluorescent pseudomonads and the culturable aerobic bacteria are monitored and their numbers are compared between inoculated and uninoculated groundwater microcosms.

Monitoring of inoculant survival includes total immunofluorescence counts, direct viable counts and colony counts on selective media. This is done in order to record potentially occurring nonculturable cells of the inoculants as it was observed by Troxler et al. (1997b and 1998).

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CHAPTER 1

Effect of Nutrient Deprivation on Subsequent Survival of Biocontrol *Pseudomonas fluorescens* CHA0 in Soil

Abstract

The effect of deprivation of multiple nutrients or of selected single nutrients (C, S, N, or P) for 7 d *in vitro* on the subsequent persistence of the biocontrol agent *Pseudomonas fluorescens* CHA0 in natural soil was investigated. Experiments were carried out with the spontaneous rifampicin-resistant mutant CHA0-Rif and the strain was monitored *in vitro* and in soil using colony counts (on plates containing rifampicin), Kogure's direct viable counts and total cell counts (by immunofluorescence microscopy). Single nutrient or multiple nutrient deprivation *in vitro* did not affect the colony-forming ability of CHA0-Rif cells. However, cell length of the strain was smaller in all nutrient deprivation treatments but one (P deprivation) when compared with cells from log-phase cultures. Once introduced into soil, CHA0-Rif cells from log-phase cultures persisted up to 14 d as culturable cells, and their population level (10^8 CFU (g soil)$^{-1}$) was comparable to inoculation level. The strain was recovered at lower cell numbers at subsequent samplings, regardless of the method used for cell counts, and at 48 d about 90% of the cells had lost both their ability to respond to Kogure's viability test and to form a colony on plate. Cells of CHA0-Rif deprived of a single nutrient persisted similarly to log-phase cells once introduced into soil. In contrast, deprivation of CHA0-Rif cells for multiple nutrients prior to their introduction into soil resulted in the early occurrence (i.e. within hours of soil inoculation) of cells that had lost their colony-forming ability and that did not respond to Kogure's viability test. This suggests that the lack of a single nutrient (other than C, S, N, or P) or deprivation of a combination of several nutrients, under *in vitro* conditions, had a negative impact on the subsequent survival of CHA0-Rif in soil.

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Introduction

Bacterial inoculants are used in soil systems for a variety of purposes e.g. biofertilisation, bioremediation, or biocontrol of pathogens and pests. It is well recognised that the ability of introduced bacteria to survive in soil and/or colonise the rhizosphere represents a prerequisite for efficient implementation of their beneficial effect (Weller, 1988). Unfortunately, bacterial inoculants undergo a progressive reduction in the size of their culturable population once in soil, whose rate is influenced by soil characteristics and environmental conditions (Wessendorf and Lingens, 1989; van Elsas and van Overbeek, 1993; Natsch *et al.*, 1994).

However, a decline in the number of colony-forming units (CFUs) is not always synonymous with disappearance of the cells, as several bacteria e.g. *Salmonella typhimurium* (Turpin *et al.*, 1993), *Flavobacterium* sp. (Heijnen *et al.*, 1995), *Alcaligenes eutrophus* (Pedersen and Jacobsen, 1993), or *Pseudomonas fluorescens* (Binnerup *et al.*, 1993) were found to persist as mixed populations of culturable and non-culturable cells in soil. Furthermore, a fraction of the non-culturable cells responded positively to a viability test in some of the experiments, indicating that they were not dead (Binnerup *et al.*, 1993; Turpin *et al.*, 1993). Virtually nothing is known about the mechanisms implicated in the transition from culturable to viable but non-culturable (VBNC) cells, and the ecological significance of the latter physiological state(s) remains to be determined (McDougald *et al.*, 1998). The biocontrol strain *P. fluorescens* CHA0 controls various soil-borne fungal diseases of plants (Keel and Défago, 1997). A spontaneous rifampicin-resistant mutant of the strain i.e. CHA0-Rif was released into soil under outdoor conditions (in lysimeters), and its persistence was studied by comparing total immunofluorescence (IF) counts, Kogure's direct viable counts (DVCs; Kogure *et al.*, 1979) and colony counts of the pseudomonad (Troxler *et al.*, 1997b). Kogure's DVCs enable the identification of substrate-responsive cells by incubating samples in the presence of nutrients and nalidixic acid, which prevents cell division and results in enlargement of viable cells. The results of Troxler *et al.* (1997b) indicated that a fraction of the cells of CHA0-Rif persisted as VBNC cells in the surface soil horizon. Based on the comparison of population dynamics of CHA0-Rif in different cropping systems, water availability, soil temperature and nutrient limitation were

identified as environmental factors potentially implicated in the development of non-culturable cells of CHA0-Rif (Troxler *et al.*, 1997b).

The soil outside of the rhizosphere can be regarded as an oligotrophic environment (Williams, 1985). Therefore, nutrient limitation is probably a common situation for bacteria in non-rhizosphere soil. Incubation of *P. fluorescens* R2f Rp^r in soil or under C-deprived conditions *in vitro* resulted in both cases in the development of resistance to abiotic stress and a change in cellular morphology (van Overbeek *et al.*, 1995). Nutrient deprivation of pseudomonads under *in vitro* conditions may cause a decrease in the culturability of the cells (Kragelund and Nybroe, 1994; Clegg *et al.*, 1996).

The objective of this investigation was to assess whether nutrient deprivation could explain the persistence patterns of *P. fluorescens* CHA0-Rif observed by Troxler *et al.* (1997b) in soil under outdoor conditions. To achieve this goal, log-phase cells of CHA0-Rif and cells previously deprived of multiple nutrients or of a selected single nutrient (C, S, N, or P) during a 7-d incubation *in vitro* were introduced into non-sterile soil microcosms and their persistence in soil was studied using colony counts, viable counts (DVCs) and total IF counts of the strain.

Materials and Methods

Strain and growth conditions

P. fluorescens CHA0 was isolated from the rhizosphere of tobacco grown in a soil suppressive to black root rot disease and located near Morens, Switzerland (Stutz *et al.*, 1986). In the current work, a spontaneous rifampicin-resistant mutant of CHA0 (named CHA0-Rif; Natsch *et al.*, 1994) was used. Strain CHA0-Rif was kept at -80°C in 44% glycerol and cells from stock were first grown in King's B broth (King *et al.*, 1954) containing 100 µg rifampicin ml^{-1}. Other media used to grow CHA0-Rif included M9 medium (Miller, 1972) i.e. (l^{-1}) 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 500 mg NaCl, 246 mg $MgSO_4.7H_2O$, 15 mg $CaCl_2.2H_2O$, pH adjusted to 6.8, in which glucose was present at 1 g l^{-1} (as used by van Overbeek *et al.*, 1995) and MOPS medium (Neidhardt *et al.*, 1974) i.e. (l^{-1}) 8.37 g 3-morpholino-propanesulfonic acid, 717 mg Tricine, 2.78 µg $FeSO_4.7H_2O$, 509 mg NH_4Cl, 48 mg K_2SO_4, 74 mg $CaCl_2.2H_2O$, 106

mg $MgCl_2.6H_2O$, 2.92 g NaCl, 3.7 µg $(NH_4)_6(Mo_7O_{24}).4H_2O$, 25 µg H_3BO_3, 7 µg $CoCl_2.6H_2O$, 1.6 µg $CuSO_4$, 16 µg $MnCl_2.3H_2O$, 3 µg $ZnSO_4.7H_2O$, 301 mg $K_2HPO_4.3H_2O$, pH adjusted to 7.4 with KOH, in which glucose was present at 2 g l^{-1} (as used by Givskov *et al.*, 1994a). All liquid cultures were incubated at 27°C with shaking (at 150 rev min^{-1}). All cells serving as inoculum during the experiment were washed three times with sterile distilled water prior to use.

Nutrient deprivation conditions

Nutrient deprivation in CHA0-Rif was implemented by incubating cells of the strain for 7 d in liquid media lacking a single nutrient (C, S, N, or P) or in a NaCl solution (9 g NaCl l^{-1} sterile distilled water; i.e. multiple nutrient deprivation). Carbon deprivation was achieved using M9 lacking glucose (as done by van Overbeek *et al.*, 1995) and S deprivation with M9 containing glucose but in which $MgSO_4.7H_2O$ was replaced with $MgCl_2.6H_2O$ (203 mg l^{-1}). These media are hereafter referred to as C-free M9 and S-free M9, respectively. The cells subjected to C or S deprivation were obtained from mid log-phase M9 cultures. M9 could not be used to achieve P deprivation conditions since the medium is buffered by phosphate salts. Phosphorus deprivation conditions were obtained using MOPS medium lacking $K_2HPO_4.3H_2O$ (hereafter referred to as P-free MOPS), as done by Givskov *et al.* (1994a) and Eberl *et al.* (1996). Givskov *et al.* (1994a) chose again a medium derived from MOPS to subject a pseudomonad to N deprivation. Similarly, N deprivation of strain CHA0-Rif in the current work was achieved using MOPS in which NH_4Cl was omitted (hereafter referred to as N-free MOPS). The cells subjected to P or N deprivation were obtained from log-phase MOPS cultures. The use of a NaCl solution to achieve multiple nutrient deprivation was proposed by Givskov *et al.* (1994a, 1994b). The cells subjected to multiple nutrient deprivation were obtained from log-phase cultures in M9.

In all treatments, the cells subjected to nutrient deprivation were obtained from log-phase CHA0-Rif cultures (MOPS or M9) containing 10^8 CFU ml^{-1} (based on optical density determinations and confirmed by colony counts). All nutrient-deprived media (100 ml in 300-ml Erlenmeyer flasks; three flasks per treatment) were inoculated with washed cells of CHA0-Rif at the rate of 10^8 CFU ml^{-1}. The flasks were incubated at

27°C with shaking (at 150 rev min^{-1}) during the 7-d treatment. The cells from each flask were then harvested by centrifugation, washed (as described above), and resuspended in 1 ml sterile distilled water.

Preparation of soil microcosms and inoculation of soil with P. fluorescens CHA0-Rif

The persistence in non-sterile soil of cells of *P. fluorescens* CHA0-Rif previously deprived of single or multiple nutrients was compared with that of log-phase cells of the strain prepared in M9 or MOPS (i.e. two control treatments). The log-phase cells of CHA0-Rif were obtained as described above, and cell suspensions were adjusted to 10^{10} CFU ml^{-1}.

Loamy soil (15% clay, 42% silt, 43% sand; pH 7.0, 3.5% organic matter) was collected in the spring from the surface horizon of a cambisol (fallow) located near Eschikon, Switzerland (Natsch *et al.*, 1994). The soil was air-dried at room temperature until friable and passed through a 5-mm mesh screen. Plant roots were removed. The soil was stored for 14 d at 8°C (in the dark) prior to use.

The day before inoculation, 10-g soil samples were placed into sterile 25-ml glass vials. Inoculation was carried out by adding 100 µl of cell suspension to the soil in the vials. The same volume of sterile distilled water was added to soil when preparing un-inoculated control samples. The vials were placed in loosely-capped 250-ml plastic containers (three vials per container). The plastic containers were put in an incubator at 12°C and 70% relative humidity. To minimise water loss from evaporation but still allow air circulation, the containers were placed together under a loosely-closed plastic cover (eight containers under each cover). The water content of the soil was 22% w/w after inoculation, which corresponds to a water potential of about Ψw = -0.03 MPa (determined by the filter paper method; McInnes *et al.*, 1994). A 30 µl volume of sterile distilled water was added per vial on day 36 to maintain a water content of 22 (± 0.1)%.

Sampling, cell length determination and cell counts of P. fluorescens CHA0-Rif

The length of CHA0-Rif cells in log-phase cultures and after a 7-d incubation under nutrient deprivation conditions was determined by immunofluorescence microscopy, as described by Troxler *et al.* (1997c). A total of 43 randomly-selected cells were studied per treatment.

Cell counts were done at the start and the end of the 7-d incubation *in vitro* (for the five nutrient deprivation treatments) as well as in soil. When soil was studied, the entire 10 g of soil from a vial was transferred into a 300-ml Erlenmeyer flask containing 100 ml of sterile distilled water and the flasks were agitated for 15 min on a rotary shaker (300 rev min^{-1}) to obtain a soil extract. A dilution series was prepared from each sample.

Culturable cells of CHA0-Rif *in vitro* or in soil were quantified by colony counts on King's B agar with 100 µg rifampicin ml^{-1}. Counts were made after incubating the plates for 48 h at 27°C. No colony was found when un-inoculated soil samples were studied (detection limit in soil 10^2 CFU g^{-1}).

The number of viable cells of CHA0-Rif *in vitro* or in soil was ascertained by using the DVC technique of Kogure *et al.* (1979) and counting enlarged cells by immunofluorescence microscopy, as described by Troxler *et al.* (1997b). The primary antiserum is specific for CHA0 (Troxler *et al.*, 1997a) and no cross-reaction was found when studying un-inoculated soil samples in the current work. Briefly, yeast extract (250 µg (ml sample)$^{-1}$) and nalidixic acid (20 µg (ml sample)$^{-1}$) were added to the samples, which were fixed with formaldehyde (20 mg (ml sample)$^{-1}$) 6 h later. Nalidixic acid effectively prevents population growth of CHA0-Rif (Troxler *et al.*, 1997b, 1997c), which was confirmed in the current work. The samples were passed through 0.2-µm pore size polycarbonate filters stained with Irgalan Black (Hobbie *et al.*, 1977). Two filters were prepared per sample. The filters were incubated successively in presence of the primary antiserum (60 min) and the secondary antibody (fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins; 45 min) prior to treatment with 1,4-diazobicyclo-(2,2,2)-octan-glycerol mounting medium to prevent fading (Johnson *et al.*, 1982). Cells of CHA0-Rif were enumerated using a Zeiss Axioskop epifluorescence microscope (filters 450-490 nm; at least 20 fields and/or 150 bacterial cells). Elongated

cells (length > 3 μm) were counted as viable cells. The filters were also used to count the total number of cells of CHA0-Rif.

Statistical analyses

Cell length of CHA0-Rif in log-phase cultures (two treatments) or at the end of the 7-d nutrient deprivation (five treatments) was investigated using 43 cells per treatment. Data were studied by analysis of variance followed with Tukey's HSD test to compare the seven treatments.

In the first part of the microcosm experiment, the effect of nutrient deprivation *in vitro* on CFUs, DVCs and total IF counts of CHA0-Rif was determined. The experiment followed a randomised design and each of the five treatments was studied in triplicate. Two additional treatments were investigated in soil i.e. log-phase cells of CHA0-Rif grown in M9 or in MOPS. Each of the seven treatments was replicated three times at each sampling time, along a randomised block design.

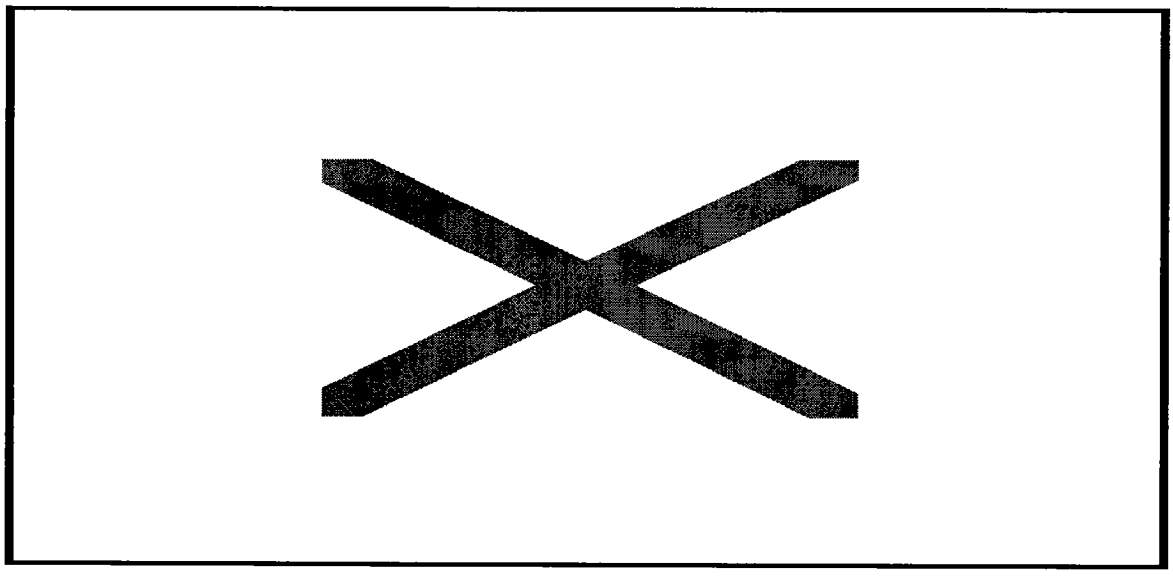
All cell counts were log-transformed before statistical analysis. First, analyses were carried out to compare the influence of cell enumeration method (i.e. total IF counts, DVCs and CFUs) within each treatment at each sampling time (*in vitro* and in soil). Second, within each type of cell count the influence of treatments was studied at each sampling time in soil. Since all nutrient deprivation conditions could not be achieved by modification of a single growth medium, the latter type of analysis was performed separately to compare (1) MOPS and M9 log-phase controls, (2) M9 log-phase control, deprivation of C and deprivation of S, (3) MOPS log-phase control, deprivation of N and deprivation of P, and (4) M9 log-phase control and multiple nutrient deprivation.

Data were processed by ANOVA followed (when appropriate) with Tukey's HSD test. Regression analyses were used to study trends over several samplings (especially from days 14 to 48 in soil microcosms). All statistical analyses were carried out at P=0.05 using version 5 of SYSTAT for Windows (SYSTAT Inc., Evanston, IL).

Results and Discussion

Effect of nutrient deprivation on cell number of P. fluorescens CHA0-Rif in vitro

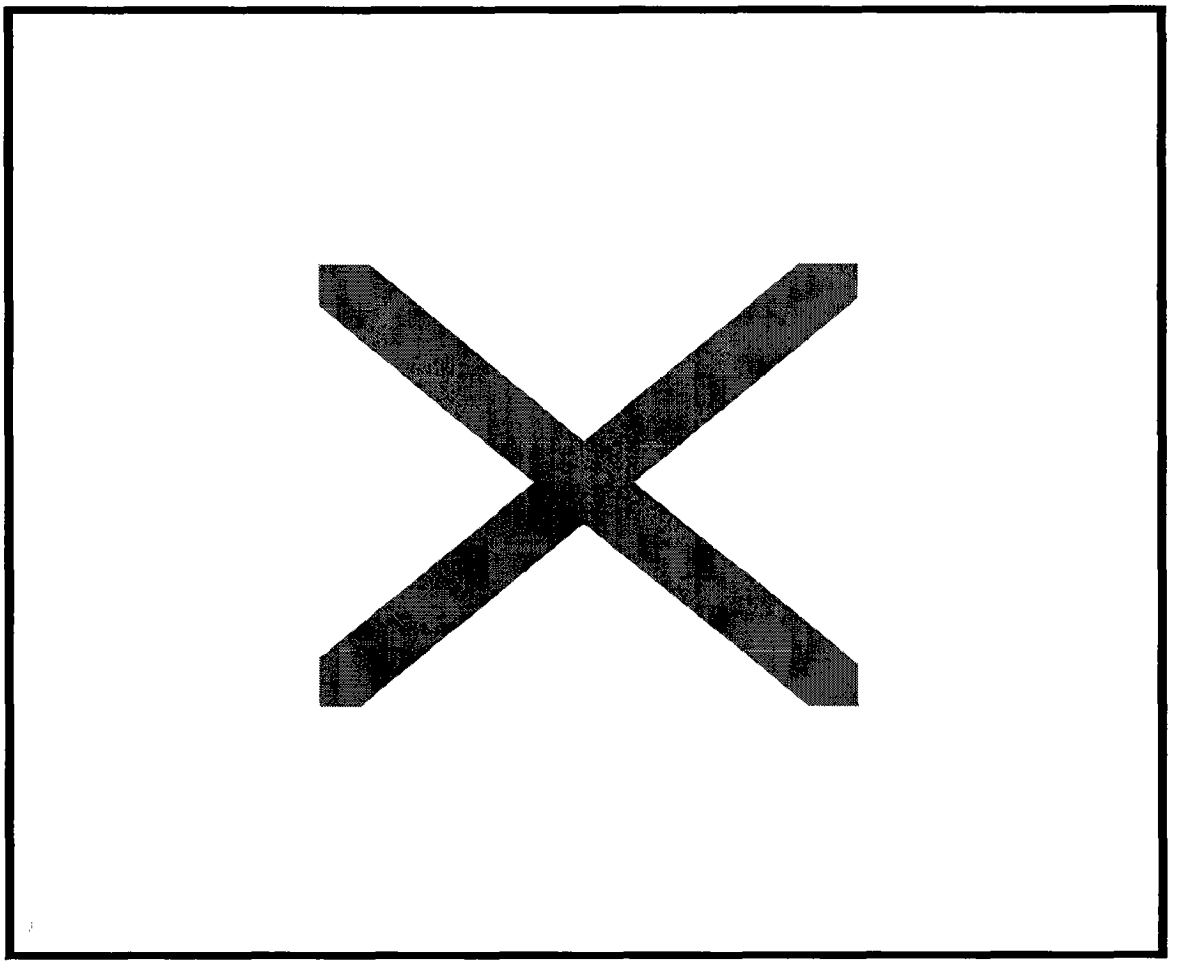
Total IF counts, DVCs and colony counts of *P. fluorescens* CHA0-Rif were essentially similar at the end of the 7 d incubation *in vitro*, regardless of the nutrient deprivation condition studied (Table 1). Similarly, deprivation of *P. fluorescens* DF57 for C or N did not affect the colony-forming ability of the cells (i.e. their culturability) during a 4 d experiment (Kragelund and Nybroe, 1994). The latter strain also maintained its colony-forming ability when subjected to P deprivation, in contrast to *P. fluorescens* ON13 or certain *P. putida*, which lead these authors to the hypothesis that the ability of pseudomonads to retain cell culturability under P deprivation was strain specific rather than species specific (Kragelund and Nybroe, 1994). The effect of S deprivation on pseudomonads was studied by Givskov *et al.* (1994b) but without considering consequences on the colony-forming ability of the cells. Here, subjection of CHA0-Rif to multiple nutrient deprivation (in a NaCl solution) for 7 d did not result in diminished culturability of the cells (Table 1), which is in accordance with the results obtained with *P. fluorescens* R2f Rp^r (Clegg *et al.*, 1996).



Effect of nutrient deprivation on cell length of P. fluorescens CHA0-Rif in vitro

The mean length of log-phase cells of CHA0-Rif in M9 and in MOPS were statistically identical (Table 2). However, cells deprived of C (in C-free M9), S (in S-free M9), or multiple nutrients (in a NaCl solution) for 7 d were smaller than those in log-phase M9 cultures by about 0.8 μm. Cells of CHA0-Rif studied after a 7 d incubation in N-free MOPS were smaller than those in log-phase MOPS cultures, but cells subjected to P deprivation (in P-free MOPS) were not. Changes in cell morphology (towards smaller, rounder cells) have been reported for pseudomonads deprived of C (Jørgensen *et al.*, 1994; van Overbeek *et al.*, 1995), N (Givskov *et al.*, 1994b), S (Givskov *et al.*, 1994b), or multiple nutrients (Jørgensen *et al.*, 1994; Clegg *et al.*, 1996). The decrease in the length of CHA0-Rif cells that occured during C or multiple nutrient deprivation in the current work was of lower magnitude compared with that observed elsewhere (Jørgensen *et al.*, 1994; van Overbeek *et al.*, 1995), which may be explained by differences between strains of *P. fluorescens* and/or experimental conditions. Only P deprivation had no effect on cell length of CHA0-Rif (Table 2). Similar findings were reported with *P. putida* KT2442 after 4 d of P deprivation (Eberl *et al.*, 1996), but strain KT2442 was found as small round cells when subjected to P deprivation for 90 d (Givskov *et al.*, 1994b).

Changes in cell length and/or cell shape have been observed with pseudomonads not only when exposed to nutrient deprivation *in vitro* but also after introduction of the cells into natural soil (van Overbeek *et al.*, 1995). In the surface horizon of large outdoor lysimeters, strain CHA0-Rif was found as small spherical cells 1.5 μm in diameter (Troxler *et al.*, 1997c). However, whether the same mechanism was responsible for the reduction in the cell dimensions of CHA0-Rif during nutrient deprivation *in vitro* (in this work) and in soil (as seen by Troxler *et al.*, 1997c) remains to be shown.



Persistence of log-phase cells of P. fluorescens CHA0-Rif introduced into soil

Cells of CHA0-Rif harvested from log-phase M9 cultures persisted in soil at population levels of about 8 log cells (g soil)$^{-1}$ for 14 d after their introduction into soil, regardless of whether cells were enumerated using total IF counts, viable counts or colony counts (Fig. 1A). Similarly, there was no difference between total IF counts and colony counts of log-phase cells of *P. fluorescens* R2f Rp^r for 20 d after their introduction into Flevo silt loam (van Overbeek *et al.*, 1995). However, a decrease in the colony-forming ability of cells of the same strain was observed when the experiment was carried out in Ede loamy sand, indicating that the persistence of a pseudomonad may vary from one soil to the next. In the current work, the numbers of total cells, viable cells and culturable cells of CHA0-Rif obtained at 30 and 48 d were lower than those found at 14 d, and total counts exceeded viable counts and colony counts of the strain by about one log unit at the last two samplings. Similar population dynamics were

observed with cells of CHA0-Rif harvested from log-phase MOPS cultures (Fig. 2A). Statistical differences between the two treatments were found on days 30 (a 0.4 log difference in CFUs) and 48 (0.3 log differences in total IF counts and in CFUs), but apparently without ecological significance.

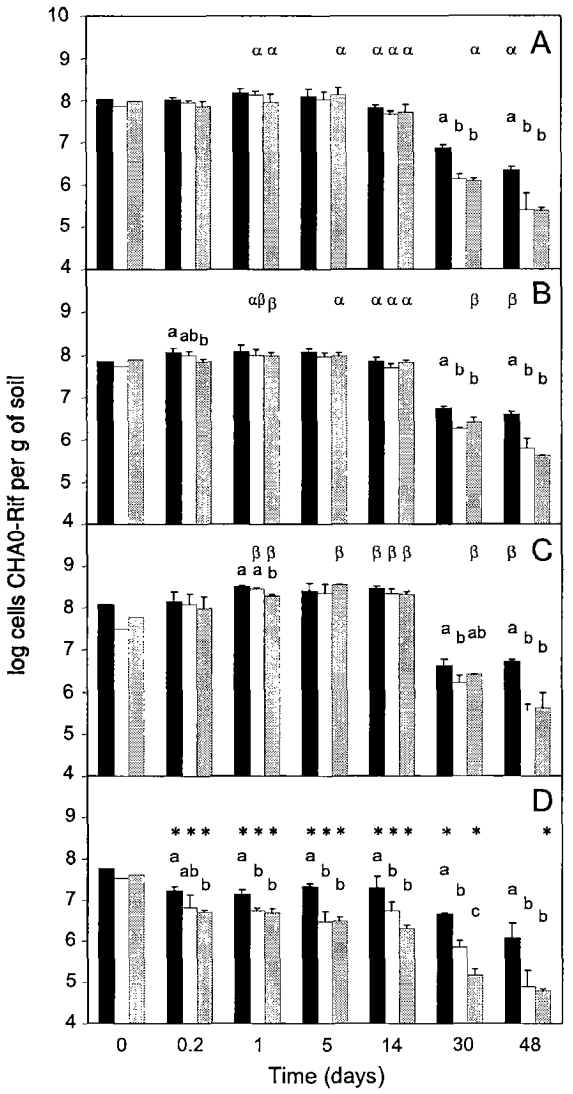


Fig. 1. Persistence in soil of log-phase cells of *P. fluorescens* CHA0-Rif (A) and CHA0-Rif cells previously deprived of carbon (B), sulfur (C) or multiple nutrients (D). Cells were prepared in M9 with 1 g glucose l^{-1}, C-free M9, S-free M9 and a NaCl solution, respectively. Monitoring included total IF counts (black bars), viable counts (white bars) and colony counts (grey bars) of the strain. Error bars signify standard deviations. Data were processed by analysis of variance followed (when differences were found) with Tukey's HSD test (P =0.05), as follows. First, statistical analyses were performed within each treatment at each sampling time to compare the different types of counts (i.e. total IF counts, viable counts and colony counts of CHA0-Rif), and statistical differences between those counts are shown with letters a, b and c. Second, the control (log-phase cells) and the two single-nutrient deprivation treatments

were compared within each type of cell count at each sampling time, and statistical differences are shown with letters α and β. Third, the control and the multiple-nutrient deprivation treatment were compared within each type of cell count at each sampling time, and statistical differences are shown with asterisks in D.

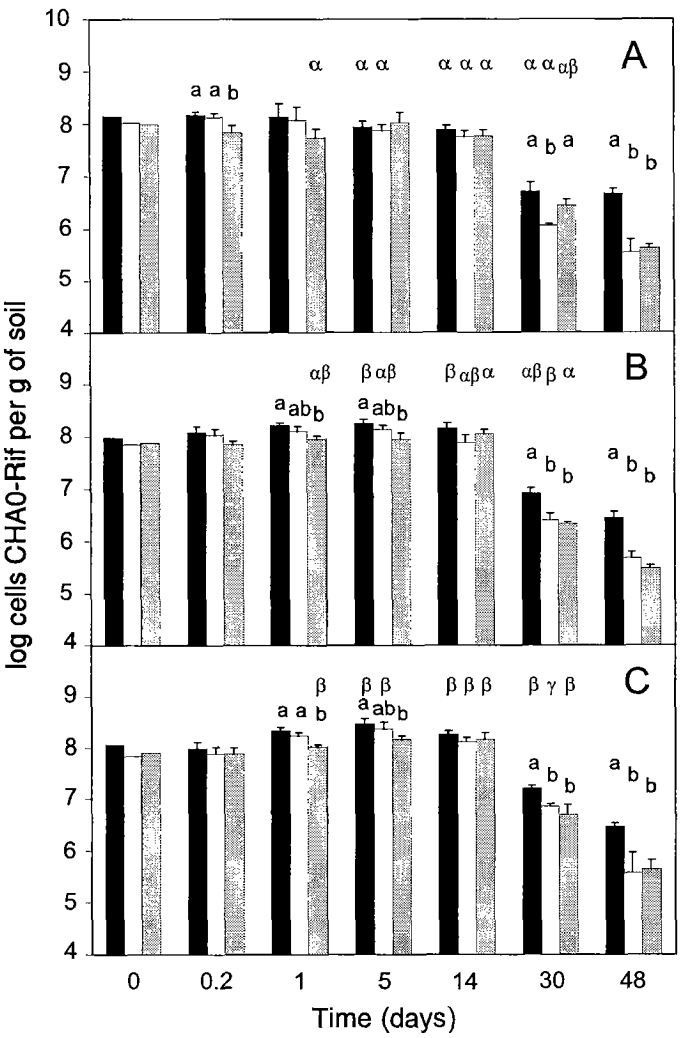


Fig. 2. Persistence in soil of log-phase cells of *P. fluorescens* CHA0-Rif (A) and CHA0-Rif cells previously deprived of nitrogen (B) or phosphorus (C). Cells were prepared in MOPS with 2 g glucose l^{-1}, N-free MOPS and P-free MOPS, respectively. Monitoring included total IF counts (black bars), viable counts (white bars) and colony counts (grey bars) of the strain. Error bars signify standard deviations. Data were processed by analysis of variance followed (when differences were found) with Tukey's HSD test (P =0.05), as follows. First, statistical analyses were performed within each treatment at each sampling time to compare the different types of counts (i.e. total IF counts, viable counts and colony counts of CHA0-Rif), and statistical differences between those counts are shown with letters a and b. Second, the three treatments were compared within each type of cell count at each sampling time, and statistical differences are shown with letters α, β and γ.

Persistence of single nutrient-deprived cells of P. fluorescens CHA0-Rif introduced into soil

Little information is available on the effect of single nutrient deprivation on subsequent survival of pseudomonads in soil. In Ede loamy sand, introduced log-phase cells of *P. fluorescens* R2f Rp^r were found as a mixed population of culturable and non-culturable cells (van Overbeek *et al.*, 1995). However, subjection of R2f Rp^r to carbon deprivation conditions (*in vitro*) for 5 days prior to introduction of the cells into soil had apparently no influence on their persistence in soil up to 10 d. In the current investigation, cells of CHA0-Rif deprived of a single nutrient (C, S, N, or P) displayed population dynamics similar to those of log-phase cells after introduction into natural soil, as follows (Fig. 1 and 2). During the first 14 d, total IF counts, viable counts and colony counts of the strain remained at high levels and there was essentially no difference between the three types of counts. Cell numbers recorded at subsequent samplings were lower by one log unit or more, and on day 48 total IF counts of CHA0-Rif were statistically higher than viable counts and colony counts of the strain.

Persistence of multiple nutrient-deprived cells of P. fluorescens CHA0-Rif introduced into soil

There was no statistical difference between total IF counts, viable counts and colony counts of CHA0-Rif at the end of the 7-d multiple nutrient deprivation *in vitro* (Table 1). However, as early as a few hours after introduction of those cells into soil, total IF counts were found to be statistically higher than colony counts (Fig. 1D). Colony counts of CHA0-Rif decreased to less than 5 log CFU $(g\ soil)^{-1}$ by day 48. In contrast, van Elsas *et al.* (1994) found that subjection of *P. fluorescens* R2f Rp^r to multiple nutrient deprivation *in vitro* for 31 d resulted in higher colony counts of the strain in soil compared with those of introduced log-phase cells. This discrepancy may result from the fact that R2f Rp^r was subjected to multiple nutrient deprivation for a longer period (31 d) than CHA0-Rif before introduction of the cells into soil. However, physiological modifications resulting from multiple nutrient deprivation can take place rapidly, sometimes within hours (Givskov *et al.*, 1994a; Jørgensen *et al.*, 1994), and in

the current work the reduction in cell length undergone by CHA0-Rif indicated that by day 7 cell characteristics had already changed in response to multiple nutrient deprivation (Table 2). The negative effect of multiple nutrient deprivation on survival of CHA0-Rif may be due to the combination of several nutrient deficiencies, and/or was linked to the absence of a nutrient other than C, S, N, or P (e.g. a micronutrient).

In soil microcosms, the difference found at the first sampling between total IF counts and colony counts for cells of CHA0-Rif previously deprived of multiple nutrients increased during the course of the experiment (Fig. 1D). Viable counts and colony counts were comparable at all samplings but one (on day 30), where viable counts exceeded colony counts by approximately 0.7 log unit. Therefore, the early loss of colony-forming ability exhibited by cells of CHA0-Rif in soil after multiple nutrient deprivation resulted essentially in the presence of cells that were not substrate-responsive in Kogure's DVC test (Fig. 1D). One possibility is that those cells were dead. However, dead bacterial cells introduced into soil do not remain as long as non-responsive cells of CHA0-Rif did in the current work (Cleyet-Marel and Crozat, 1982; Turpin *et al.*, 1993). Another possibility is that cells of CHA0-Rif not responding to the viability test were not dead, but Kogure's DVC technique had become inappropriate to identify them. Such inactive/dormant cells have been found with the same pseudomonad in the surface horizon of field lysimeters (Troxler *et al.*, 1997b). The significance of this physiological state is unknown.

Adaptation of bacteria to unfavorable environmental conditions may entail the formation of cells in a viable but non-culturable (VBNC) state (Pedersen and Jacobsen, 1993; Troxler *et al.*, 1997b; McDougald *et al.*, 1998). In the current work, such VBNC cells were only found on day 30 in soil, in the multiple nutrient deprivation treatment, where they represented as many as 5.7 log cells $(g\ soil)^{-1}$ (Fig. 1D). Comparison of cell counts on days 30 and 48 indicated that the VBNC status did not promote cell persistence in soil, as no subpopulation of VBNC cells was detected on day 48 and viable counts decreased more than total IF counts or colony counts between the two samplings. This finding suggests that the VBNC state observed here corresponded more to a moribund condition than a survival strategy (McDougald *et al.*, 1998). Therefore the transient occurrence of VBNC cells in this study appears of limited ecological significance.

Significance of nutrient deprivation in the persistence of P. fluorescens CHA0-Rif in soil

In a previous investigation, *P. fluorescens* CHA0-Rif persisted as mixed populations of culturable, VBNC cells and inactive/dormant cells in the surface horizon of field lysimeters (Troxler *et al.*, 1997b). Introduction of log-phase cells of CHA0-Rif into soil microcosms, however, did not result in the formation of VBNC cells of the strain in this work (Fig. 1A and 2A), although VBNC cells of *P. fluorescens* can occur in soil microcosms following the introduction of cells from overnight cultures (Binnerup *et al.*, 1993). The soil in the lysimeters and in Eschikon correspond both to cambisols and they display similar organic matter contents (2.9% and 3.5%, respectively), clay contents (17% and 15%, respectively) and pH (7.4 and 7.0, respectively). Therefore, it is unlikely that nutrient supply was lower in the lysimeter soil than in Eschikon soil. This suggests that nutrient limitation was not a major factor involved in the appearance of VBNC cells of CHA0-Rif in the lysimeters. This hypothesis is strengthened by the observation that exposure of CHA0-Rif cells to multiple nutrient deprivation for 7 d prior to soil inoculation accelerated the loss of colony-forming ability of CHA0-Rif cells without generating significant amounts of VBNC cells (Fig. 1D). Therefore, it is likely that the occurrence of VBNC cells of CHA0-Rif in the field was caused by factors other than nutrient deprivation. The evaluation of those factors, therefore, needs to be the aim of further studies.

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CHAPTER 2

Survival of Biocontrol *Pseudomonas fluorescens* CHA0 in Lysimeter Effluent Water Depends on Time of the Year at which Water is Collected and Soil Type in the Lysimeter

Abstract

Survival of the biocontrol agent *Pseudomonas fluorescens* CHA0 at a non-target site (i.e. in groundwater) was studied in microcosms prepared with effluent water collected at four times during the year from large outdoor lysimeters (2.5 m deep) containing a well-drained or a poorly-drained cambisol. The spontaneous rifampicin-resistant mutant CHA0-Rif was inoculated at 7 log cells/ml and microcosms were incubated for 175 d at 8°C under aerobic conditions. The inoculant was monitored by colony counts (on King's B agar with rifampicin), total cell counts (by immunofluorescence microscopy) and viable cell counts (using Kogure's direct viable counts). Cell numbers of CHA0-Rif obtained with the three cell count methods used were essentially similar. In filter-sterilized water, the inoculant persisted at inoculation levels during the 175 d. In non-sterile water, cell numbers of CHA0-Rif decreased exponentially in time, according to the Vandenhove model. Population levels of the inoculant in the microcosms depended on the time of the year at which effluent water had been collected from the lysimeter and the soil type in the lysimeter. However, correlations were found between physical, chemical and biological characteristics of lysimeter effluent water (especially the number of resident culturable aerobic bacteria) and cell numbers (or Vandenhove model's daily reduction factors) of CHA0-Rif in microcosms. These results suggest that survival of biocontrol pseudomonads transported through soil to groundwater level may be predicted by taking into account particular water characteristics.

Submitted, 1999.

Introduction

The use of bacterial inoculants for biofertilization, bioremediation or biocontrol of pathogens requires their release into the soil ecosystem in high cell numbers. Transport of released bacteria through soil is thought to be limited, but can nevertheless occur (Smith et al., 1985; Frederickson et al., 1989; van Elsas et al., 1991; Lovins et al., 1993; Natsch et al., 1994). A few field experiments have addressed whether *Pseudomonas* inoculants can be transported from the surface horizon to deeper soil layers. De Leij et al. (1995) recovered *Pseudomonas fluorescens* SBW25 from a depth of 45 cm at 65 d after application, but deeper soil horizons were not studied. Rapid vertical transport (i.e. within 24 h) of *P. fluorescens* CHA0-Rif can take place if a heavy rainfall follows immediately the release of the bacterium to the soil surface (Natsch et al., 1996). In the latter study, the pseudomonad was found at population levels as high as 7 log colony-forming units (CFU)/g soil at a depth of 150 cm. It is likely that movement of water through cracks and biopores (i.e. by preferential flow; Gish and Shirmohammadi, 1991) was responsible for transport of CHA0-Rif through soil.

Preferential flow is often assisted by high amounts of precipitation (i.e. > 40 mm/d) and occurs typically in well-structured soil (Flury et al., 1994). Since bacteria have active mechanisms to adhere to soil particles (Robertson et al., 1993) and can migrate to physically-protected microsites in soil (Stotzky, 1985; Lindquist and Bengtsson, 1997), thereby reducing the possibility of being removed by free water, the time between bacterial release and the onset of preferential flow may influence the extent of vertical transport of an inoculant. Indeed, *P. fluorescens* CHA0-Rif was recovered at low cell numbers in the effluent water (about 3 log CFU/l) of large outdoor lysimeters, at a depth of 2.5 m (Troxler et al., 1998). In this experiment, the inoculant remained in the soil for extended periods (i.e. several months) and was detected in lysimeter effluent water one day after a heavy rainfall (about 70 mm). One implication of these results is that the time at which a *Pseudomonas* soil inoculant may reach groundwater level cannot be foreseen since it will depend on the soil type, the date of inoculation and rainfall patterns. In fact, biocontrol pseudomonads are likely to be released into different types of soil and, depending on the crop to be protected, at different times during the year.

In terms of biosafety, the possibility that *Pseudomonas* inoculants may be transported to deeper soil layers needs to be considered with regards to potential

contamination of groundwater. It is therefore important to understand how such bacteria can survive once they reach the groundwater, regardless of whether wild-type or genetically-modified strains are used. In the case of *P. fluorescens* CHA0 for instance, genetically-modified derivatives overproducing antifungal secondary metabolites have been developed to enhance protection of certain plants against soil-borne fungal pathogens (Maurhofer et al., 1992; Schnider et al., 1995). The persistence of non-native bacteria introduced into groundwater is thought to depend on physical, chemical and biological characteristics of the groundwater (Matthess and Pekdeger, 1981). Indeed, survival of *P. fluorescens* CHA0-Rif in groundwater microcosms was different when incubation conditions were changed (aerobic versus anaerobic conditions) or the resident microbiota removed by filtration (Troxler et al., 1998). Groundwater characteristics can be very different from one site to the next or at different times during the year (Vrba and Pekný, 1991; Tindall et al., 1994; Clay et al., 1996; Rasmussen, 1996; Banks, 1997; Miettinen et al., 1997), which could perhaps lead to different survival patterns of *Pseudomonas* inoculants. However, this possibility has not been assessed so far.

The objective of this study was to assess predictability of the survival of *P. fluorescens* CHA0-Rif in groundwater microcosms. To achieve this goal, an experimental approach was followed in which microcosms prepared with effluent water collected from two types of large outdoor lysimeters and/or at different times during the year were used. The microcosms were inoculated with *P. fluorescens* CHA0-Rif and survival of the introduced pseudomonad was monitored by colony counts, total immunofluorescence (IF) cell counts and Kogure's direct viable counts (DVCs).

Materials and Methods

Sampling of lysimeter effluent water

Groundwater samples consisted of effluent water collected at the bottom of large outdoor lysimeters (diameter 2 m, depth 2.5 m; about 10.000 kg of soil in each) located at the Eidgenösische Forschungsanstalt für Agrarökologie und Landbau (FAL) in Zürich Reckenholz, Switzerland. The lysimeters contained either a well-drained (in lysimeters L1 to L6) or poorly-drained cambisol (pseudogleyic; in lysimeters L7 to L12) and have been under crop rotation (without irrigation) for 16 years (mainly grass-based pasture, maize, rapeseed, sugarbeet and wheat) (Troxler et al., 1997b). Effluent water was collected only from lysimeters that had not been inoculated with *P. fluorescens* CHA0-Rif in previous experiments (i.e. from lysimeters L3, L6, L9 and L12; Troxler et al., 1997b and 1998).

The lysimeters were described in detail by Nievergelt (1991) and Troxler et al. (1997b and 1998) and their main characteristics are given in Fig. 1. Whereas the surface horizons (A_P) of both types of cambisols (= inceptisols) were essentially similar, the texture of horizon B was finer in the poorly-drained cambisol (loam) than in the well-drained cambisol (sandy loam). The main difference between the two cambisols was in the composition of the parental material (C horizon), which had implications for water permeability. The parental material for the well-drained cambisol was a *Schotter* (i.e. a stony alluvium mixed with later loamy deposits) and that for the poorly-drained cambisol was a *Moränelehm* (i.e. loamy moraine deposits). Both cambisols had been repacked above layers of gravels and stones (Fig. 1).

To assess the influence of temporal fluctuations of groundwater characteristics, survival of the inoculant *P. fluorescens* CHA0-Rif was studied using effluent water collected from a single lysimeter (i.e. L6; well-drained cambisol), at four different times during the year (on 8 December 1996, 22 May 1997, 4 July 1997 and 22 October 1997; i.e. four treatments). In addition, a portion of the effluent water sampled on 8 December 1996 and 4 July 1997 from lysimeter L6 was filter-sterilized (0.22-µm pore size) to investigate the effect of the resident microbiota on inoculant persistence. Effluent water was also sampled from a lysimeter containing a poorly-drained cambisol (i.e. L9), on 4 July 1997, to assess if differences in groundwater characteristics resulting from water

percolation through different soil types could lead to different survival patterns for strain CHA0-Rif. Finally, inoculant survival was monitored in groundwater microcosms prepared by pooling effluent water sampled on 22 October 1997 from lysimeters L3, L6, L9 and L12 (in a 1:1:1:1 ratio), an approach similar to that used by Troxler et al. (1998).

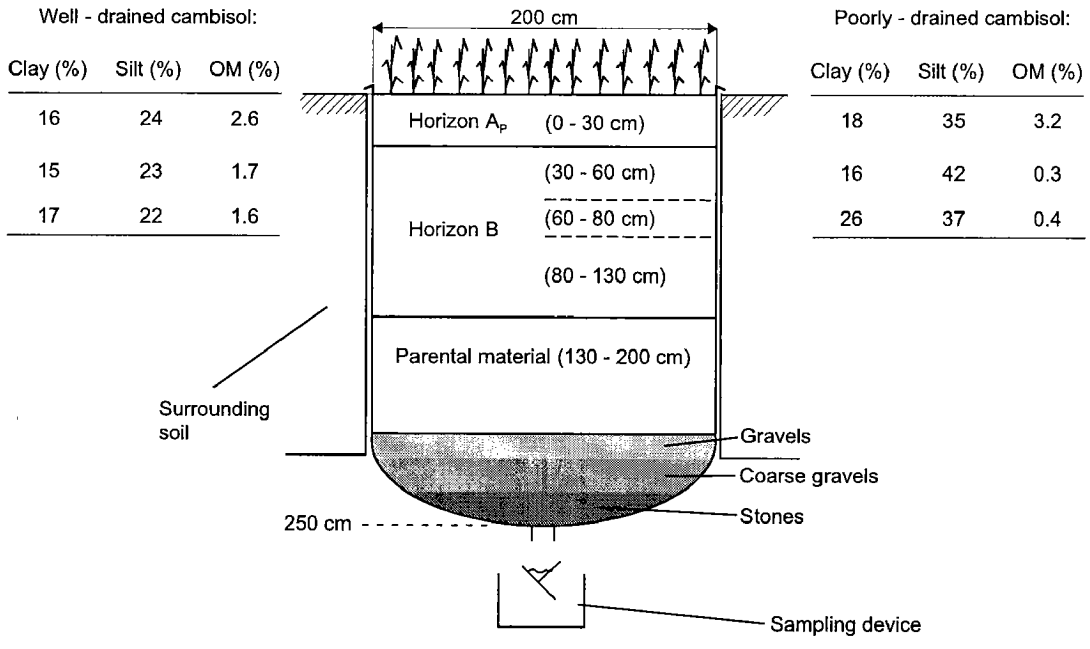


Fig. 1. Profile of a lysimeter and composition of well-drained and poorly-drained cambisols. Soil analysis was performed with horizon A_p and sub-horizons B_1 and B_2 of horizon B. The soil $pH_{(water)}$ was comprised between 7.3 and 7.7 for the well-drained cambisol and between 7.5 and 8.3 for the poorly-drained cambisol. OM, organic matter.

Analysis of lysimeter effluent water before preparing microcosms

Chemical analysis of lysimeter effluent water was carried out before starting the microcosm experiments. The content in dissolved organic carbon (DOC) was determined using a Shimadzu 500 high temperature (690 °C) catalytic (0.5% Pt on Al_2O_3) combustion instrument. CO_2 was detected in the reaction gas stream by a non-dispersed infra red detector (Shimadzu Corporation, Kyoto, Japan). Values reported are

means of three 20-µl injections. Calibration was obtained using potassium phthalate. The contents in NO_3^-, SO_4^{2-} and Cl^- were determined by anion exchange separation using a hydroxide gradient (1-25 mM) on a AG11/AS11 column. A DX-500 ion chromatography system (Dionex, Olten, Switzerland) with suppressed (ASRS) background conductivity detection was used. Cations (Na^+, K^+, Mg^{2+} and Ca^{2+}) were determined by flame atomic absorption spectrometry. For Mg^{2+} and Ca^{2+}, the samples were diluted 20-fold in 0.5% lanthanum chloride. K^+ was measured after the addition of 1 M barium chloride (0.2 ml per 10-ml sample). The pH was measured in 500-µl volumes with a Knick Digital pH-meter (Auer Bittmann Soulié AG, Zürich, Switzerland), after readings had stabilized (i.e. about one min). Dissolved oxygen was measured in 500-µl volumes with an oxygen electrode (Rank Brothers, Bottisham, UK), using distilled water as a reference. The results obtained with the oxygen electrode were confirmed by determinations done with the colorimetric test kit *visocolor*™ Oxygen SA 10 (Macherey-Nagel, Dueren, Germany).

Biological determinations were done as well. The numbers of total resident bacteria and viable resident bacteria in each water sample were determined with the LIVE/DEAD *Bac*Light™ bacterial viability kit (Molecular Probes, Eugene, OR, USA). After treatment with the kit (done following manufacturer's recommendations), water samples were passed through 0.2-µm pore size polycarbonate filters stained with Irgalan Black (Hobbie et al., 1977) and filters were observed using a Zeiss Axioskop epifluorescence microscope (filters 450-490 nm). The total number of bacterial cells was determined (at least 20 fields and/or 150 bacterial cells). Among the latter, cells that were green (indicative of membrane integrity) were counted as viable bacterial cells. Culturable resident aerobic bacteria and culturable resident fluorescent pseudomonads were recovered by spread-plating of samples on 1/10 strength tryptic soy agar (Difco) and S1 agar (Gould et al., 1985), respectively. Colonies were counted after a 6-d incubation at 20°C.

Inoculation and incubation of groundwater microcosms

The biocontrol agent *P. fluorescens* CHA0 was isolated from a tobacco field located near Morens, Switzerland (Stutz et al., 1986). In the current experiments, a spontaneous rifampicin-resistant mutant (i.e. CHA0-Rif) of the strain was used (Natsch et al., 1994; Troxler et al., 1998; Hase et al., 1999). CHA0-Rif was first grown overnight at 27°C with shaking (at 150 rev/min) in King's B broth (King et al., 1954) containing 100 µg rifampicin/ml (i.e. Rif100). The cells were washed three times with sterile distilled water and used to inoculate King's B agar (i.e. KBA) containing Rif100. 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 log cells/ml based on OD measurements (600 nm).

Groundwater microcosms were prepared from freshly-collected lysimeter effluent water, as described by Troxler et al. (1998). They consisted of 100 ml of water in 125-ml serum bottles (previously sterilized). Microcosms were inoculated with 100-µl cell suspensions of CHA0-Rif, which resulted in about 7 log cells of CHA0-Rif added per ml (as confirmed both by total IF counts and colony counts; methods outlined below).

Each serum bottle was closed with a sterile cotton stopper, which enabled aeration of samples during incubation. The microcosms were incubated at 8°C in the dark (without shaking) for 175 d. Some microcosms were left uninoculated to serve as controls.

Sampling and monitoring of bacterial populations in groundwater microcosms

At each sampling, three microcosms were studied per treatment (destructive sampling). The cotton stoppers were removed and the serum bottles were tightly closed with sterile rubber caps. The bottles were agitated on a rotary shaker (300 rev/min) for 30 min (Troxler et al., 1998) and a 10-fold serial dilution of each microcosm was prepared in sterile distilled water. The dilutions were spread-plated onto KBA + Rif100, S1 agar and 1/10 strength tryptic soy agar to count culturable cells of CHA0-Rif, the total culturable fluorescent pseudomonads and the total culturable aerobic bacteria, respectively. Colony counts were done after incubating the plates for 6 d at 20°C. No

colony was found when uninoculated water was plated onto KBA + Rif100 (detection limit of about 1 log CFU/ml).

Additionally, viable cells and total cells of CHA0-Rif in the samples were quantified using the DVC technique (Kogure et al., 1979) in combination with IF microscopy, as described in detail by Troxler et al. (1997b). Groundwater samples were treated with nalidixic acid (20 µg/ml) and yeast extract (250 µg/ml) and incubated at room temperature in the dark for 6 h prior to fixing with formaldehyde (final concentration 20 mg/ml). Nalidixic acid effectively inhibited population growth of CHA0-Rif during the reaction, as shown also in previous studies (Troxler et al., 1997b and 1997c; Hase et al., 1999). Bacteria present in the samples were immobilized by vacuum filtration on 0.2-µm pore size polycarbonate filters stained with Irgalan Black (Hobbie et al., 1977), which were incubated successively in the presence of a primary antiserum (60 min) specific to CHA0 (Troxler et al., 1997a) 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 (Johnson et al., 1982) and studied using a Zeiss Axioskop epifluorescence microscope (filters 450-490 nm). The total amount of CHA0-Rif cells was determined (magnification × 1000; at least 20 fields and/or 150 bacterial cells). Enlarged CHA0-Rif cells (length > 3 µm) were counted as viable cells. CHA0-Rif cells that were not substrate-responsive were often found as spherical cells (they were not counted). In contrast to the latter cells, viable CHA0-Rif cells could still be detected at the × 400 magnification. Since a larger area on the filters could be studied at × 400 than at × 1000 magnification, the detection limit was lower for DVCs (1.6 log cells/ml) than for total IF counts (2.0 log cells/ml). Stained bacterial cells were not found when studying samples from uninoculated microcosms.

Statistical analyses and modeling

When comparing survival of CHA0-Rif in lysimeter effluent water collected at different times of the year, microcosms were prepared and inoculated shortly after each collection rather than storing the water and running all treatments simultaneously.

Groundwater microcosms prepared with water collected at a same time were placed in a randomized design during incubation. Each treatment was studied in triplicate.

Bacterial cell numbers were log-transformed (when expressed per ml) or arcsine-transformed (when expressed as a percentage of a larger bacterial population). Data were then processed by analysis of variance followed (when differences were found) with Tukey's HSD test. First, at each sampling of inoculated microcosms, cell numbers of CHA0-Rif obtained using a given cell count method (i.e. total IF count, DVC or colony count) were compared across treatments. This was done also for colony counts of CHA0-Rif expressed as a percentage of the total culturable aerobic bacteria. Second, within each treatment, analyses were carried out to compare cell counts of CHA0-Rif obtained by different methods (i.e. total IF count, DVC and colony count) at each sampling of inoculated microcosms.

The function $Y = A + B \times (1 - R_f)^t$ (Vandenhove et al., 1991) was used to describe population dynamics of the inoculant in each treatment. Y is the log of cell numbers at time *t*; A represents the log of the final cell numbers; B is the difference between the log of the initial cell numbers and A; R_f is the daily reduction factor and *t* the time (d). The equation is equivalent to the Gompertz equation, as used by Corman et al. (1987). Model fit was tested for total IF counts, for DVCs and for CFUs using data from all samplings where cell number was higher than the limit of detection. This was achieved by non-linear regression using the Quasi-Newton algorithm for iteration (up to 100 steps). The goodness of the fit was checked by comparing the variation in Y explained by the sum of squares due to the regression with that explained by the total sum of squares.

The relationship between survival of CHA0-Rif (total IF counts, DVCs and CFUs of CHA0-Rif at each sampling of inoculated microcosm, as well as daily reduction factors) and properties of lysimeter effluent water (physical, chemical and biological parameters) was analyzed using Pierson correlation coefficient.

All statistical analyses were carried out at P=0.05 level. They were done using SYSTAT for Windows (version 5; SYSTAT Inc., Evanston, IL, USA).

Results

Survival of CHA0-Rif in microcosms prepared with lysimeter effluent water collected at different times during the year

In microcosms consisting of non-sterile effluent water collected from lysimeter L6 on 8 December 1996 (Fig. 1), total IF counts of the inoculant decreased from 6.7 to about 4 log cells/ml within 20 d (Fig. 2B). DVCs and CFUs dropped from about 6.6 to 3 log cells/ml during the same time, and consequently at 20 d a large fraction of the CHA0-Rif population was neither substrate-responsive nor able to form colonies on agar. At subsequent samplings, cell numbers of the strain ranged between 2.5 and 3.9 log cells/ml and stabilized at about 3.5 log cells/ml, regardless of the counting method used.

Decreases in cell numbers of CHA0-Rif were also observed in microcosms prepared with L6 effluent water from 22 May, 4 July or 22 October 1997 (Fig. 2C,E,G). At 20 d, similar total IF counts of CHA0-Rif were recorded in all four treatments, but DVCs (in E and G) and CFUs were statistically higher when L6 effluent water was collected in 1997 rather than on 8 December 1996 (i.e. in B). However, unlike in the latter treatment, cell numbers of CHA0-Rif continued to decrease with time, regardless of the counting method used, and at 175 d total IF counts and DVCs of the strain were below detection limit in all groundwater microcosms prepared with L6 effluent water collected in 1997 (CFUs also when collected on 4 July 1997). Among those 1997 treatments, differences between cell counts were found mostly between total IF counts and DVCs in microcosms containing L6 effluent water sampled on 22 May 1997 (i.e. in C).

Survival of CHA0-Rif introduced into microcosms with lysimeter effluent water from well- and poorly-drained cambisols

On days 20, 50 and 112, total IF counts of CHA0-Rif were higher when groundwater microcosms consisted of effluent water sampled on 4 July 1997 from a poorly-drained lysimeter (i.e. L9) rather than a well-drained lysimeter (i.e. L6) (Fig. 2E,F). At 175 d after inoculation, total IF counts of the strain amounted to 3.6 log cells/ml in water from L9 but were below detection limit in water from L6. As for total IF counts, DVCs and CFUs of CHA0-Rif remained higher in microcosms containing water from L9 than in those prepared with water from L6 (Fig. 2E,F). Cell numbers of the inoculant in L9 effluent water determined with the different counting methods were identical at each sampling but at 50 d, where total IF counts exceeded CFUs (Fig. 2F).

In a previous study, Troxler et al. (1998) investigated the survival of strain CHA0-Rif in groundwater microcosms prepared by pooling effluent water obtained from different lysimeters, which contained a well-drained or a poorly-drained cambisol. This approach was also followed in the current study, using water collected on 22 October 1997 from lysimeters L3, L6, L9, and L12 (in a 1:1:1:1 ratio; Fig. 2H). Survival of the strain in pooled water was compared with that in effluent water obtained from L6 (well-drained cambisol) on 22 October 1997. Differences in cell numbers between the two treatments were found only on days 20 and 50. At 20 d, total IF counts, DVCs and CFUs of CHA0-Rif were higher in pooled water (Fig. 2H) than in water from L6 (Fig. 2G), but at 50 d total IF counts of the strain were lower in pooled water than in water from L6. At those two samplings, total IF counts were higher than DVCs and CFUs in pooled effluent water.

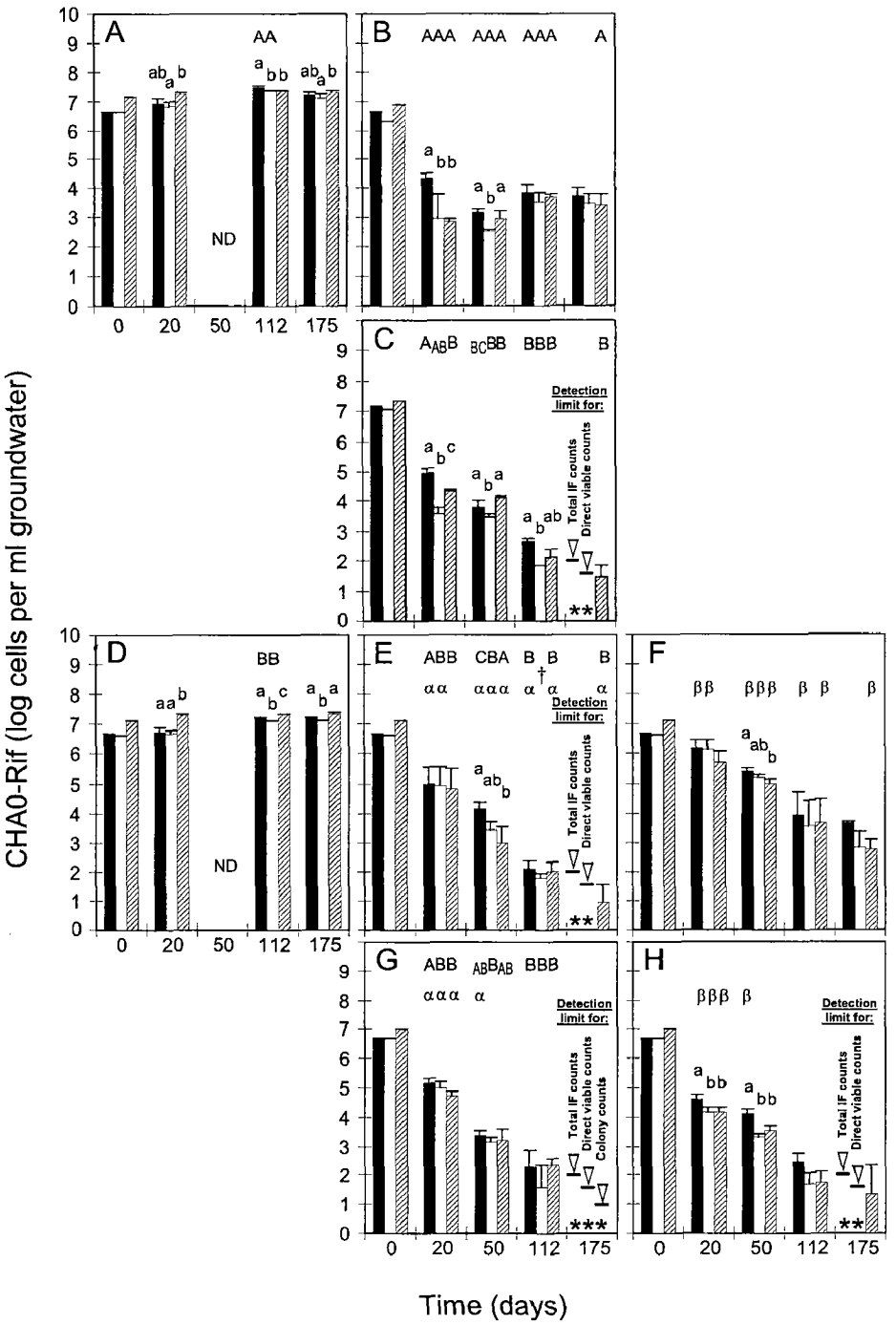


Fig. 2. Survival of *P. fluorescens* CHA0-Rif in groundwater microcosms prepared with effluent water collected at the bottom of large outdoor lysimeters. Microcosms contained water collected on 8 December 1996 (A,B), 22 May 1997 (C), 4 July 1997 (D-F) and 22 October 1997 (G,H). Water originated from a lysimeter containing either a well-drained (A-E,G) or a poorly-drained (F) cambisol. Certain microcosms were prepared by pooling water from four different lysimeters (H). In A and D, the water was sterilized by filtration before inoculating with CHA0-Rif. Monitoring of the strain included total IF counts (black bars), DVCs (white bars) and CFUs (hatched bars). Error bars signify standard deviations. Data were processed by analysis of variance followed (when differences were found) with Tukey's HSD test ($P < 0.05$), as follows: First, treatments were compared at each sampling time based on total IF counts, DVCs

and CFUs. For each cell count method, comparisons were done vertically (i.e. among A and D and among B, C, E and G; statistical differences shown with letters A, B, and C) as well as horizontally for non-sterile microcosms (i.e. among E and F and among G and H; statistical differences shown with letters α and β). Since one replicate for DVCs was missing at 112 d in E (indicated by †), this particular treatment was not included when comparing DVCs at 112 d. Asterisks at 175 d indicate cell numbers below the detection limit (i.e. 2.0 log cells/ml for total IF counts, 1.6 log cells/ml for DVCs and 1.0 log CFU/ml). For each cell count method, significant differences were found between non-sterile and the corresponding filter-sterilized microcosms at each sampling (they are not shown on the Figure). Second, total IF counts, DVCs and CFUs were compared within each treatment at each sampling time, and statistical differences are indicated with letters a, b and c.

Interaction between resident bacteria and CHA0-Rif in groundwater microcosms

In the absence of inoculation, the number of resident culturable aerobic bacteria in L6 effluent water collected on 8 December 1996 ranged from 4.2 to 5.2 log CFU/ml during the 175-d experiment (Fig. 3A). Similar population levels were observed in the other uninoculated microcosms, and they fluctuated more from one sampling to the next within a given treatment than from one treatment to the next at a given sampling (data not shown). Surprisingly, in inoculated microcosms prepared with L6 effluent water from December 1996, the total number of culturable aerobic bacteria decreased from 6.9 to 5.3 log CFU/ml during the first 20 d but increased to 6.5 log CFU/ml by 50 d, and those counts remained higher in inoculated microcosms than in uninoculated microcosms from 50 to 175 d (Fig. 3A). In contrast, in other microcosms, the increase in the number of total culturable aerobic bacteria resulting from the introduction of CHA0-Rif at high cell numbers was observed essentially at the first sampling, i.e. immediately after introduction of CHA0-Rif (data not shown). In addition, the number of total culturable aerobic bacteria was higher from 50 d onwards in inoculated L6 water from December 1996 than in inoculated microcosms prepared with L6 effluent water collected on 22 May, 4 July or 22 October 1997 (Fig. 3A). At each sampling, the total number of culturable aerobic bacteria in inoculated effluent water was statistically identical in microcosms prepared with water collected on 4 July 1997 from L9 (poorly-drained lysimeter) or L6 (well-drained lysimeter) (Fig. 3A).

Since the size of the community of total culturable aerobic bacteria fluctuated in time and from one inoculated treatment to the next, survival of CHA0-Rif was also assessed in relation with population dynamics at the scale of this community. When expressed as a percentage of the total culturable aerobic bacteria, cell numbers of CHA0-Rif in effluent water from L6 declined from almost 100% to less than 0.1% in 175 d, except that the inoculant stabilized at about 1% of the total culturable aerobic bacteria when effluent water was collected in December 1996 (Fig. 3B). When the experiment was carried out with effluent water sampled in July 1997, CHA0-Rif represented, at each sampling, a higher proportion of the total number of culturable aerobic bacteria in effluent water from L9 (poorly-drained cambisol) than in L6 (well-drained cambisol).

To assess the influence of the resident microbiota on survival of CHA0-Rif, the pseudomonad was also introduced into microcosms prepared with water collected from L6, in December 1996 or July 1997, and that had been filter-sterilized before inoculation. In both cases, CHA0-Rif was found at about 7 log cells/ml during the 175-d experiment (Fig. 2A,D), regardless of the method used to count the cells. These population levels of CHA0-Rif were higher than those in the corresponding microcosms consisting of non-sterile water (Fig. 2A,B,D,E).

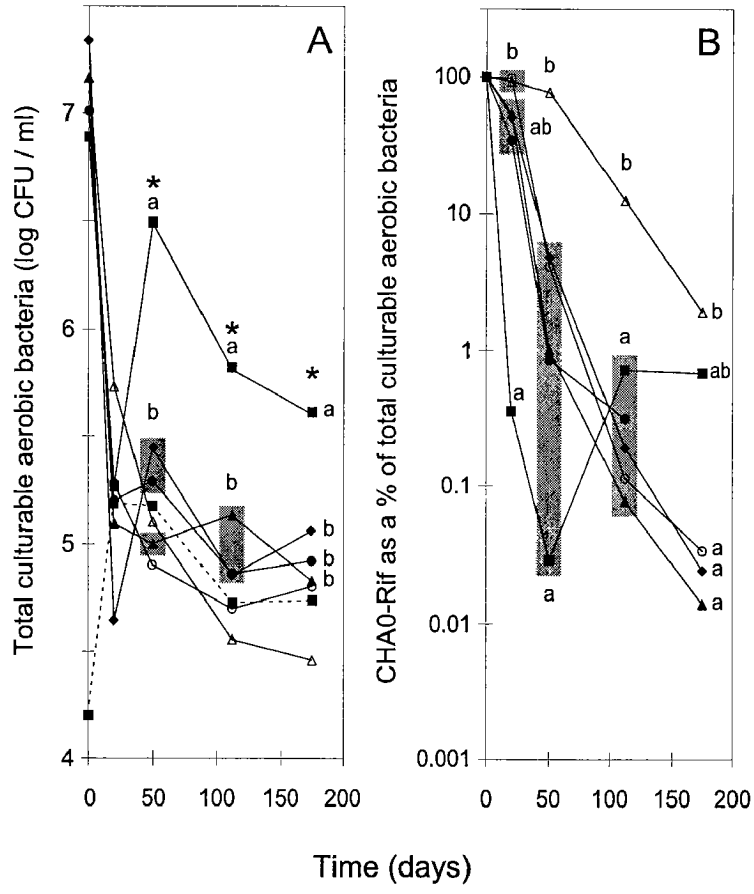
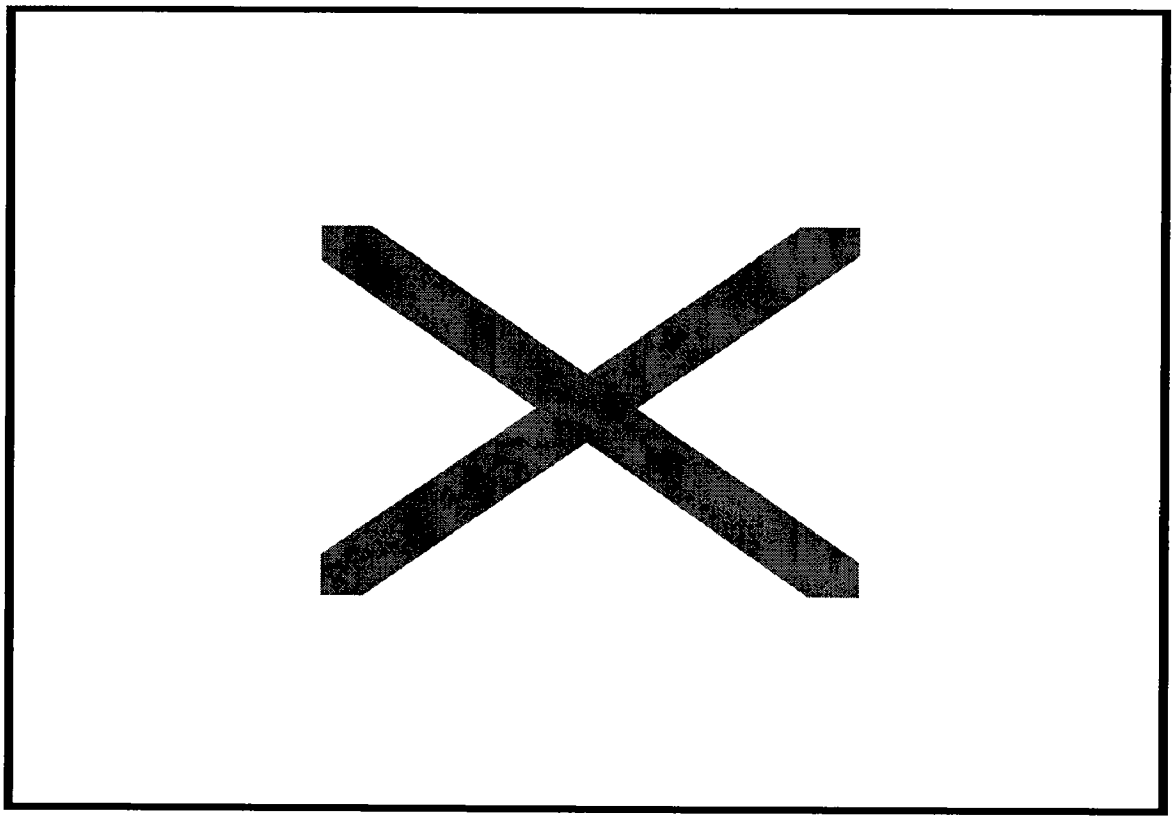


Fig. 3. Numbers of total culturable aerobic bacteria (TCAB) in inoculated and uninoculated microcosms prepared with effluent water collected from different types of lysimeters at different times during the year (A) and survival of inoculated *P. fluorescens* CHA0-Rif expressed as a percentage of the TCAB (B). Filled symbols denote treatments where effluent water was collected from lysimeter L6 on 8 December 1996 (■), 22 May 1997 (◆), 4 July 1997 (▲), or 22 October 1997 (●). Open symbols represent treatments with water sampled from L9 on 4 July 1997 (Δ) or from L3, L6, L9 and L12 on 22 October 1997 (O). The dotted line corresponds to uninoculated microcosms containing water sampled from L6 on 8 December 1996. At each sampling time, data were processed by analysis of variance followed (when differences were found) with Tukey's HSD test ($P < 0.05$). In A, statistical differences between inoculated and uninoculated microcosms were found mainly for L6 effluent water collected on 8 December 1996, and they are shown with asterisks (small but statistically significant differences were also found at 20 and 50 d for water from L9; data not shown). For visual clarity, the numbers of TCAB in the other uninoculated microcosms are not shown. The numbers of TCAB were also compared across inoculated treatments where microcosms consisted of L6 effluent water, and statistical differences are indicated with letters a and b. There was no difference between inoculated treatments when water was collected on 4 July 1997 or on 22 October 1997. In B, comparisons were done across all treatments at each sampling time, and significant differences are indicated with letters a and b. The inoculant was below detection limit at 175 d when L6 effluent water collected in October 1997 was used.

Modeling of the survival of CHA0-Rif in groundwater microcosms

The population dynamics of CHA0-Rif introduced into non-sterile groundwater was well described with the equation $Y = A + B \times (1 - R_f)^t$ proposed by Vandenhove et al. (1991), regardless of the treatment considered, since R^2 values ranged from 0.983 to 0.999 (Table 1). The fit to the model was good, regardless of whether total IF counts (R^2 of 0.994 or more), DVCs (R^2 of 0.984 or more) or CFUs (R^2 of 0.983 or more) were used. In contrast, the model failed to describe adequately the population dynamics of CHA0-Rif in filter-sterilized water, where cell numbers of the strain did not decrease in time (Fig. 2A,D).

In non-sterile groundwater, the daily reduction factor R_f, which describes the rate at which final cell numbers were reached, ranged from 0.006 to 0.74 depending on lysimeter type, the time at which effluent water had been collected, and the cell count method used (Table 1). Daily reduction factors obtained for DVCs or CFUs of CHA0-Rif in microcosms prepared with L6 effluent water collected on 8 December 1996 exceeded 0.6, whereas all the others were below 0.08 (Table 1).

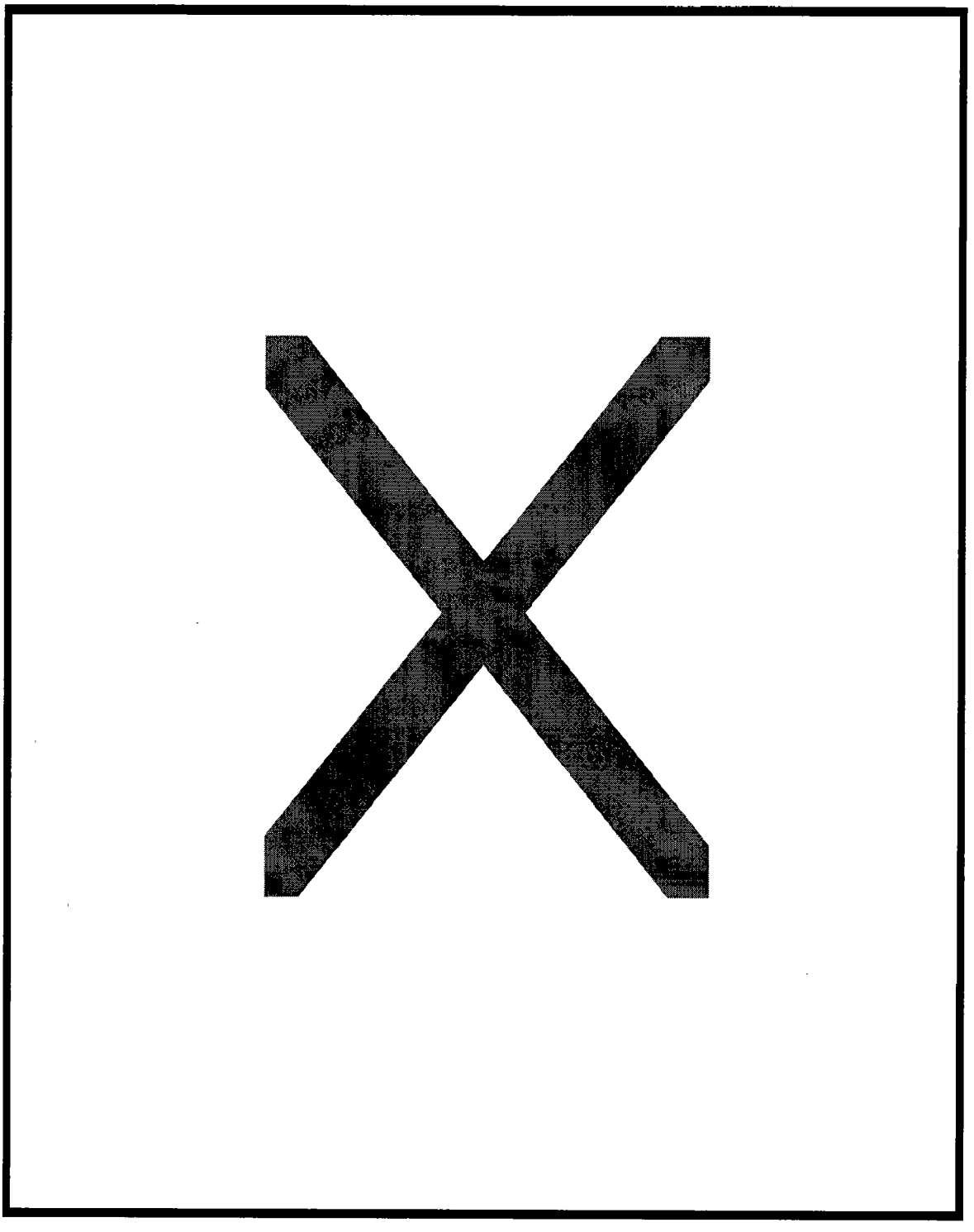


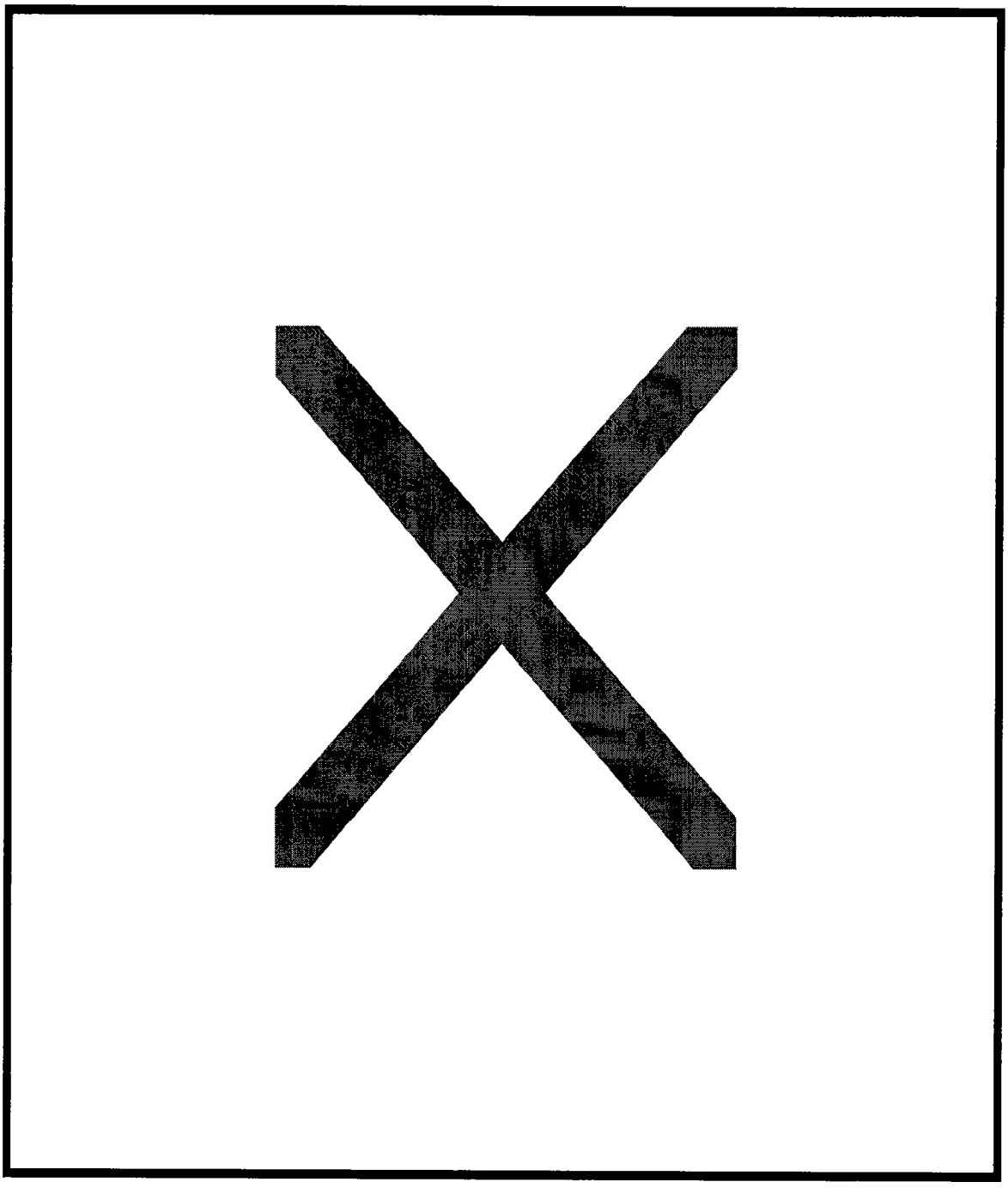
Relationship between survival of CHA0-Rif and groundwater characteristics

Water samples were analyzed each time non-sterile groundwater microcosms were set up (Table 2). Pearson correlation indicated that the calcium content of the uninoculated lysimeter effluent water was positively correlated with those in chloride ($r = 0.96$; $P = 0.002$) and nitrate ($r = 0.85$; $P = 0.034$). Additionally, correlations were found between chemical or biological parameters and the amount of lysimeter effluent water recorded during the day (i.e. sulfate), the week (i.e. potassium, log number of total resident bacteria) or the month (i.e. potassium, log number of total resident bacteria) before the water was collected (see footnote of Table 3).

Correlations were found between chemical water parameters (nitrate, magnesium, calcium) and cell numbers of CHA0-Rif (total IF counts, DVCs, CFUs), but they were never significant at more than one sampling (Table 3). However, inoculant survival at the first two samplings (i.e. total IF counts and DVCs at 20 d and at 50 d, CFUs at 20 d) correlated well (and positively) with the number of resident culturable aerobic bacteria determined prior to inoculating the microcosms.

The daily reduction factor R_f (for DVCs and CFUs) was positively correlated with water flow measured during either the week or the month prior to the collection of water at the lysimeters (Table 3). R_f (regardless of whether derived from total IF counts, DVCs or CFUs) was also correlated positively with the concentrations in nitrate and calcium, and negatively with the log number of resident culturable aerobic bacteria prior to inoculation with CHA0-Rif.





Discussion

In each non-sterile groundwater microcosm, the population size of *P. fluorescens* CHA0-Rif decreased in time, regardless of the cell count method used to monitor the inoculant (Fig. 2). Such declines have been observed before with other bacteria e.g. *Burkholderia cepacia* (Winkler et al., 1995) or *Aeromonas hydrophila* (Kersters et al., 1996) introduced into groundwater microcosms. Interestingly, population levels of CHA0-Rif at the end of the 175-d incubation could differ by several orders of magnitude from one type of non-sterile microcosm to the next. Population dynamics of CHA0-Rif cells in non-sterile microcosms was influenced by the time of the year at which effluent water had been collected from lysimeter L6 (Fig. 2B,C,E,G). Similarly, Winstanley et al. (1991) noted differences in the survival of *P. putida* PaW8 in lake water microcosms, with higher CFUs when the water had been sampled in November than in September. Furthermore, at a given sampling time, persistence of CHA0-Rif was different when water effluent was collected from lysimeters containing different types of soil (a poorly-drained or a well-drained cambisol; Fig. 2E,F), although the two soils were not very different in terms of soil composition (Fig. 1). Comparable studies have not been done before with groundwater microcosms. However, in aquatic microcosms consisting of lake water placed above a layer of sandy or loamy lake sediment, CFUs of the fish pathogen *A. salmonicida* in the water were higher when microcosms were prepared with sandy sediment (Deere et al., 1996). Overall, the results of the current study suggest that survival of the soil inoculant CHA0-Rif, once transported to the groundwater by percolating water after a heavy rainfall (Troxler et al., 1998), may be difficult to predict since it will depend not only on field site characteristics but also on the time of the year at which the strain will reach deeper soil layers.

Interestingly, population dynamics of CHA0-Rif in each non-sterile microcosm was well described by the model of exponential decline (Table 1) used by Vandenhove et al. (1991) to model the decline of *P. fluorescens* 88W1 in non-sterile soil. Since the survival of CHA0-Rif could vary to a large extent from one treatment to the next, the daily reduction factor R_f used in the model fluctuated a lot depending on lysimeter type and the time at which effluent water had been collected (Table 1). R_f corresponds to the rate at which the final population level was reached (but gives no information about the

value of this final population level). In filter-sterilized microcosms, cell numbers of CHA0-Rif remained at high levels throughout the 175-d experiment, indicating that the decline of the inoculant in non-sterile groundwater microcosms was largely due to the presence of the resident microbiota, as shown with *P. fluorescens* R2f in microcosms consisting of agricultural drainage water (Trevors et al., 1989).

In the current work, colony counts were statistically identical to total IF counts and DVCs in most cases (Fig. 2). The occurrence of non-culturable cells of the strain in non-sterile effluent water took place at a few samplings only (e.g. at 20 d with water collected on 8 December 1996). Most of these non-culturable cells were not substrate-responsive in Kogure's DVC test, as found when exponentially-grown cells of *P. putida* KT2442 were subjected to a temperature downshift to 0°C (Eberl et al., 1998). This observation made with CHA0-Rif contrasts with the results of a previous experiment, in which large amounts of viable but non-culturable (VBNC) cells of the strain were evidenced (Troxler et al., 1998).

In the latter investigation, effluent water had been collected once (on 19 October 1993) from all 12 FAL lysimeters (i.e. L1 to L12) and was pooled before preparing the microcosms. A somewhat similar treatment was included in the current study, by pooling effluent water collected from well-drained (i.e. L3 and L6) and poorly-drained lysimeters (i.e. L9 and L12) on 22 October 1997, but VBNC cells of CHA0-Rif were not found even in this treatment. The discrepancy between these results and those of Troxler et al. (1998) is in accordance with the fact that CHA0-Rif persistence in groundwater microcosms can be strongly influenced by water characteristics. Likewise, the prevalence of VBNC cells of CHA0-Rif in soil under field conditions could vary with field location, even for a same soil type (our unpublished results). In a similar way, VBNC cells of *Agrobacterium tumefaciens* and *Sinorhizobium meliloti* were found following the inoculation of the bacteria into tap water obtained at one location but not at another (Manahan and Steck, 1997). These authors speculated that an as yet unidentified compound present in one of the tap water samples was responsible for the occurrence of VBNC cells of *A. tumefaciens*. This hypothesis could also explain why the occurrence of VBNC cells of CHA0-Rif in filter-sterilized groundwater microcosms depended on the lysimeter effluent water sampled (Troxler et al., 1998; this study). Overall, VBNC cells of CHA0-Rif in lysimeter effluent water were found with water collected on one occasion but not with water obtained on four other occasions (Troxler

et al., 1998; this study), which suggests that water conditions promoting the formation of VBNC cells occur infrequently at this site.

The characteristics of the lysimeter effluent water used in this study were comparable with those of the groundwater in situ, especially regarding pH, the contents in calcium, chloride and nitrate, and the numbers of resident bacteria (Pedersen and Ekendahl, 1990; Claus *et. al.*, 1992; Rasmussen, 1996; Banks, 1997). One of the correlations (between calcium and chloride) found here has already been reported (Schot and van der Wal, 1992). However, whether correlations exist between groundwater characteristics (measured prior to inoculation) and the survival of an introduced bacterium has, to our knowledge, never been investigated before. The positive correlations found between the log numbers of CHA0-Rif and the resident culturable aerobic bacteria (Table 3) are interesting since (1) they were found at several samplings, with the three methods used to monitor CHA0-Rif and (2) such correlations were not found with other biological characteristics of the effluent water (listed in Table 2). In accordance with this, negative correlations were found between the numbers of resident culturable aerobic bacteria and daily reduction factors for total IF counts, DVCs and CFUs of CHA0-Rif. Thus, it appears that water batches whose characteristics had promoted higher numbers of resident culturable aerobic bacteria favoured also survival of the inoculant. Arguably, it could be that the number of CHA0-Rif cells persisting in the groundwater microcosms was determined by the amount of certain compounds released during the turnover of the resident culturable aerobic bacteria and that were used as substrate(s) by the inoculant.

Among chemical water characteristics, the contents in nitrate and calcium were both positively correlated with the daily reduction factors for total IF counts, DVCs and colony counts of CHA0-Rif. Certain pseudomonads (but not CHA0-Rif; Voisard et al., 1988) can use nitrate as terminal electron acceptor under anaerobic conditions and the addition of nitrate to oxygen-limited aquifers can result in the selection of *Pseudomonas* spp. without modifying the number of total culturable aerobic bacteria (Vermace et al., 1996). However, nitrate respiration was probably negligible in the current study since the levels of dissolved oxygen remained higher than 0.26 mM during the 175 d (data not shown). Differences in the chemical composition of the effluent water may be linked to the amount of time spent by rain water inside the soil before exiting the lysimeters at groundwater level, as percolating water interacts with the soil and contributes to

leaching of nutrient and transport of resident bacteria through the soil profile. Such differences can be expected at any time of the year when comparing effluent water from lysimeters L6 (well-drained cambisol) and L9 (poorly-drained cambisol) (Table 2). They may occur also between effluent water collected at different times of the year from a same lysimeter, as a result of contrasted rainfall patterns. Indeed, correlations were found between water flow and certain chemical and biological characteristics of the effluent water (see footnote of Table 3). Therefore, it was not surprising to also find correlations between water flow and daily reduction factors of CHA0-Rif (Table 3).

In conclusion, interactions with the resident microbiota resulted in the exponential decline of the biocontrol agent *P. fluorescens* CHA0-Rif introduced into groundwater microcosms. Inoculant survival varied greatly depending on the time of the year at which effluent water had been collected from the lysimeter and soil type in the lysimeter, but correlations were found between population dynamics of CHA0-Rif and certain characteristics of effluent water before inoculation (especially the number of resident culturable aerobic bacteria). This raises the possibility of developing predictive models for describing survival of biocontrol pseudomonads transported through soil to groundwater, and further work will address this possibility.

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CHAPTER 3

Impact of Biocontrol *Pseudomonas fluorescens* CHA0 and a Genetically-Modified Derivative on Resident Culturable Bacteria in Groundwater Microcosms

Abstract

In field experiments, the rifampicin-resistant biocontrol strain *Pseudomonas fluorescens* CHA0-Rif inoculated to surface soil was recovered at high cell numbers from deeper soil layers after a heavy rain, raising the possibility of groundwater contamination. In the current work, the ecological impact of strain CHA0-Rif and its derivative CHA0-Rif(pME3424) on resident culturable bacteria was assessed for 175 days in groundwater microcosms prepared with lysimeter effluent water. pME3424 carries an extra copy of CHA0's *rpoD* gene (encoding sigma factor σ^{70}). Strain CHA0-Rif(pME3424) displays enhanced production of the antimicrobial polyketides 2,4-diacetylphloroglucinol (Phl) and pyoluteorin (Plt) *in vitro* and improved biocontrol efficacy in soil microcosms. Cell numbers of CHA0-Rif in groundwater microcosms 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. In contrast, the pseudomonad disappeared within 50 days when it contained pME3424. Inoculation with either strain had little or no effect on the number of resident culturable fluorescent pseudomonads when compared with the uninoculated control. However, unexpectedly, the addition of CHA0-Rif or CHA0-Rif(pME3424) at high inoculum level resulted in significantly higher numbers of resident culturable aerobic bacteria at each sampling from day 50 on compared with uninoculated microcosms. This increase did not take place at low inoculum level, or when nutrients had been added before inoculation. Neither Phl nor Plt were found in the microcosms by HPLC, and inoculation with CHA0-Rif or CHA0-Rif(pME3424) had no effect on the percentages of the total culturable aerobic bacteria sensitive to Phl or to Plt (1 $\mu g\ ml^{-1}$) on day 20. Inoculation caused a small but statistically significant increase of pH at 112 days when either strain was added at high inoculum level in unamended microcosms. Overall, CHA0-Rif and CHA0-Rif(pME3424) had a lasting impact on resident

culturable bacteria in groundwater microcosms at high inoculum level, and this effect was probably linked to the release of nutrients by introduced cells during inoculant decline. This contrasts with the transient ecological impact of biocontrol pseudomonads on rhizosphere bacterial populations in previous studies.

Submitted, 1999.

Introduction

Certain bacteria can suppress soil-borne fungal diseases and have received attention as biocontrol agents for crop protection (Cook, 1996; Keel and Défago, 1997; Sarniguet et al., 1995). For instance, fluorescent pseudomonads producing antimicrobial polyketides e.g. 2,4-diacetylphloroglucinol (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* (Keel et al., 1996; Sharifi-Tehrani et al., 1998). Interestingly, the efficacy of such biocontrol agents may be improved by genetic modifications causing enhanced synthesis of antimicrobial polyketides (Maurhofer et al., 1995; Sarniguet et al., 1995; Schnider et al., 1995). In *Pseudomonas fluorescens* CHA0, this was achieved following the introduction into CHA0 of an extra copy of the strain's own *rpoD* gene (which encodes the housekeeping sigma factor σ^{70}), using an oligo-copy plasmid as vector (Maurhofer et al., 1995; Schnider et al., 1995). Compared with the wild-type pseudomonad, one of the strains thus constructed (i.e. CHA0(pME3424)) overproduced the antimicrobial polyketides Phl (330 versus 100 ng $(10^8$ CFU$)^{-1}$, on malt agar) and Plt (530 versus 78 ng $(10^8$ CFU$)^{-1}$, on King's B agar (King et al., 1954)) and displayed enhanced suppression of *P. ultimum*-mediated damping-off of cucumber in soil microcosms (Schnider et al., 1995).

Since the commercial use of biocontrol agents into the soil ecosystem implies the release of large number of cells, biosafety aspects of these inoculations need to be considered, regardless of whether wild-type strains or genetically-improved derivatives are to be used (Cook, 1996; Tiedje et al., 1989; Défago et al., 1997). In the case of biocontrol pseudomonads producing antimicrobial polyketides, the fact that Phl and Plt can inhibit various microorganisms (Keel et al., 1992; Shanahan et al., 1992; Reddi et al., 1969) needs to receive particular attention. So far, the ecological impact of such biocontrol pseudomonads has been assessed in the rhizosphere of inoculated plants (Natsch et al., 1997, 1998; Moënne-Loccoz et al., 1998a) and subsequent rotation crops (Moënne-Loccoz et al., 1998b; Naseby et al., 1998). For instance, Natsch et al. (1997) showed that both the spontaneous rifampicin-resistant mutant *P. fluorescens* CHA0-Rif and its genetically-modified derivative CHA0-Rif(pME3424) caused a transient

modification to the resident community of culturable fluorescent pseudomonads in the rhizosphere of cucumber.

However, whether biocontrol pseudomonads producing antimicrobial polyketides can also have an ecological impact at non-target sites, i.e. in habitats where inoculant dissemination can take place, has not been assessed so far. Field experiments have shown that *P. fluorescens* CHA0-Rif, released as soil inoculant into a well-structured soil, could be transported vertically to deeper soil layers and potentially to groundwater level when a thunderstorm was simulated immediately after inoculation (Natsch et al., 1996). One day after inoculation, the pseudomonad was recovered at levels up to 7 log CFU per g of soil at a depth of 1.5 m. Preferential flow of rainwater through cracks and biopores (Gish and Shirmohammadi, 1991) was probably responsible for the rapid vertical transport of CHA0-Rif through soil. In another study, low cell numbers of strain CHA0-Rif (2 to 4 log cells per ml of lysimeter effluent water at a depth of 2.5 m) were recovered after an important rainfall that took place several months after soil inoculation (Troxler et al., 1998).

In soils where preferential flow is significant, nutrients (e.g. nitrate) derived from manure application have been documented to contribute to groundwater contamination (Bronswijk et al., 1995; Foran et al., 1993). Irrigation with wastewater effluent can cause an input of dissolved organic carbon (DOC) compounds into aquifers (Amiel et al., 1990; Pye and Kelly, 1984). Bacteria naturally present in the groundwater may utilize DOC and nitrate as nutrients for growth (Bulger et al., 1989). 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 (Trevors et al., 1989).

The objective of the current work was to investigate the ecological impact of *P. fluorescens* CHA0-Rif and its genetically-modified derivative CHA0-Rif(pME3424) on resident culturable bacteria in groundwater microcosms. The pseudomonads were 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 (Natsch et al., 1996; Troxler et al., 1998). 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 the inoculants, in an effort to maximize the potential ecological impact of the introduced pseudomonads (i.e. worse-case scenario).

Materials and Methods

Bacterial inoculants and growth conditions

P. fluorescens CHA0-Rif (Natsch et al., 1994) is a spontaneous rifampicin-resistant mutant of the biocontrol agent CHA0 (Stutz et al., 1986) that grows like the wild-type in laboratory media (Natsch et al., 1994). Plasmid pME3424 was constructed (Schnider et al., 1995) by introducing a copy of the *rpoD* gene of CHA0 into the IncP vector pVK100 (Knauf and Nester, 1982). Presence of pME3424 in the pseudomonad resulted in enhanced production of Phl and Plt, as well as improved disease-suppression ability (Schnider et al., 1995). CHA0-Rif(pVK100) was obtained by mobilizing pVK100 into CHA0-Rif, as described (Schnider et al., 1995), and was used in the experiment to distinguish between potential effects 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 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 (i.e. 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^{10} cells ml^{-1} based on OD measurements at 600 nm (Sharifi-Tehrani et al., 1998).

Preparation and inoculation of groundwater 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; Troxler et al., 1997b), 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 was described in detail by Troxler et al. (1997b). It 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. Chemical properties of lysimeter effluent water collected on 8 December 1996 were as follows: 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+}.

Groundwater microcosms consisted of 100 ml lysimeter effluent water in previously-autoclaved 125-ml serum bottles. 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) 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). Indeed, the pseudomonad synthesizes mostly one type of polyketide (Phl or Plt) in the majority of laboratory media tested so far (Keel et al., 1996; Schnider et al., 1995, Sharifi-Tehrani et al., 1998; Duffy and Défago, 1999). Similar amounts of Phl and of Plt are produced by CHA0-Rif and CHA0-Rif(pVK100) when grown in NGB, whereas CHA0-Rif(pME3424) overproduces the two metabolites in this medium (Fig. 1). 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 (Trevors et al., 1989; van Overbeek et al., 1990). 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 cell suspension of CHA0-Rif, CHA0-Rif(pME3424) or CHA0-Rif(pVK100) was added per microcosm, and thus each strain was added at the rate of 10^4 or 10^7 CFU per ml of groundwater microcosm. Uninoculated microcosms (controls) received 100 µl of sterile distilled water. The microcosms were closed with sterile cotton stoppers and placed at 8°C in the dark in an incubator.

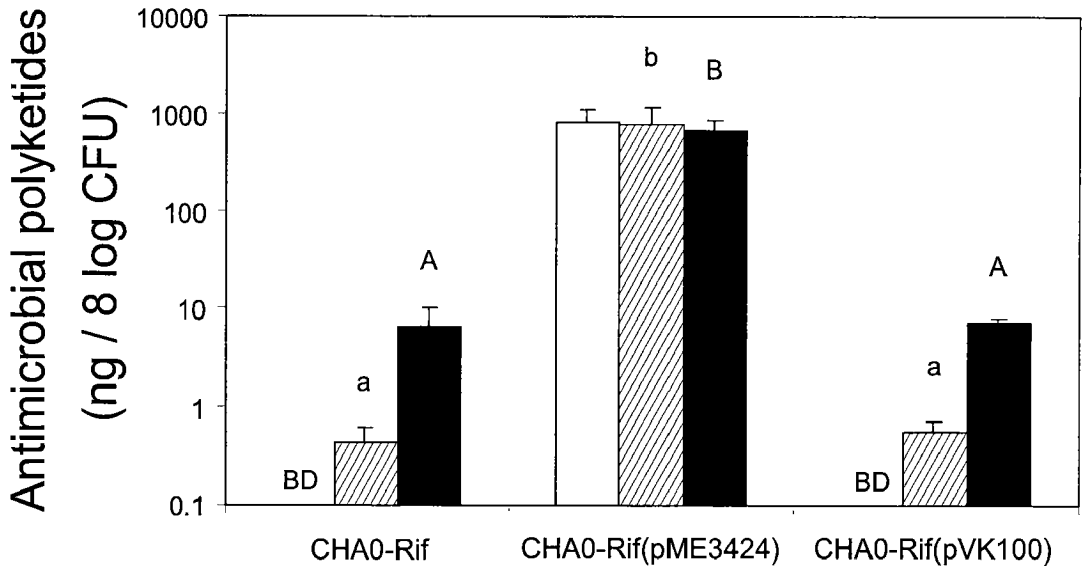

Fig. 1. Amounts of the antimicrobial polyketides monoacetylphloroglucinol (Mphl;□), 2,4-diacetylphloroglucinol (Phl;▨), and pyoluteorin (Plt;■) produced by *P. fluorescens* CHA0-Rif, CHA0-Rif(pME3424), and CHA0-Rif(pVK100) in nutrient glycerol broth (NGB). Data for Phl and Plt were processed separately, by analysis of variance and Tukey's HSD test ($P < 0.05$), and differences between treatments are shown with letters a and b (Phl) and A and B (Plt). BD: below detection limit.

Sampling, chemical analysis and bacterial cell counts

At each sampling time, three microcosms were studied per treatment (destructive sampling). First, the cotton stoppers were removed from the microcosms and two 500-µl aliquots were taken for determination of pH (at 50 and 112 d) and the concentration of dissolved oxygen (at 20, 50 and 112 d). The pH was measured with a Knick Digital pH-meter (Auer Bittmann Soulié AG, Zürich, Switzerland), after readings had stabilized (i.e. about one min). Dissolved oxygen was measured with an oxygen electrode (Rank Brothers, Bottisham, UK), using distilled water as a reference. The results obtained with the oxygen electrode were confirmed by determinations done with the colorimetric test kit *visocolor*® Oxygen SA 10 (Macherey-Nagel, Dueren, Germany). For further processing, the bottles 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, S1 agar (Gould et al., 1985) and tryptic soy agar (Oxoid, Basingstoke, UK) at one-tenth strength (i.e. 10% TSA;

containing 12 g agar l^{-1}) to count culturable cells of the inoculants, the total numbers of culturable fluorescent pseudomonads (CFP) and of culturable aerobic bacteria (CAB), respectively. Colonies were counted after incubation of plates for 6 d at 20°C. No colony was found on KBA + Rif100 when samples from uninoculated microcosms were plated (detection limit of about 1 log CFU ml^{-1}). Plasmid maintenance in culturable cells of CHA0-Rif(pME3424) and CHA0-Rif(pVK100) was checked by comparing CFUs on KBA + Rif100 and KBA + Rif100 + Tet125 at 20 and 50 d. At 112 and 175 d, this approach was completed by replica plating randomly-selected colonies from KBA + Rif100 onto KBA + Rif100 and KBA + Rif100 + Tet125. The presence of pME3424 or pVK100 in colonies resistant to Tet125 was confirmed by electrophoresis on agarose gels after extraction of plasmids by alkaline lysis (Sambrook et al., 1989). Tetracycline-sensitive colonies were also studied to check if pME3424 and pVK100 were absent.

The presence of Phl, its precursor monoacetylphloroglucinol (Mphl), and Plt in microcosms was assessed on days 20 and 50, by extracting 50 ml from each microcosm studied, using ethyl acetate, and analyzing the extract by reverse-phase HPLC, as described elsewhere (Keel et al., 1992; Maurhofer et al., 1992). The detection limit was 45 ng (ml microcosm)$^{-1}$ for Mphl (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). In addition, the total CAB resistant to Phl at 1 µg ml^{-1} (i.e. Phl1) or Plt at 1 µg ml^{-1} (i.e. Plt1) were quantified by colony counts on 10% TSA plates 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 (Natsch et al., 1997).

The possibility that the inoculants persisted in groundwater microcosms as non-culturable cells was assessed by indirect immunofluorescence (IF) microscopy combined with Kogure's viability test (Kogure et al., 1979), as described by Troxler et al. (1998) and Hase et al. (1999). 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 (Troxler et al., 1997a) 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 population growth, as shown previously (Troxler et al., 1997b; Hase et al., 1999). 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 (Hobbie et al., 1977) 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 (Johnson et al., 1982), and were studied using a Zeiss Axioskop epifluorescence microscope (filters 450-490 nm). Kogure's direct viable counts (DVCs) 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 DVCs (×400 magnification), as enlarged cells were easier to detect.

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). Each treatment was studied in triplicate at each sampling time (destructive sampling) and the location of the microcosms in the incubator followed a randomized design. 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 for Windows (version 5; SYSTAT Inc., Evanston, IL).

For pH, the total CFP, the total CAB, and the percentages of the total CAB sensitive to Phl or to Plt, all 14 treatments were compared together at each sampling time studied. Inoculant persistence was studied as follows: First, analyses were carried out to compare the influence of cell enumeration method (i.e. total IF counts, DVCs and CFUs) within each treatment at each sampling time. Second, for each type of cell count the influence of inoculum level and nutrient addition was studied for each inoculant at each sampling time. Third, persistence of the three strains was compared for each type of cell count, at each combination of inoculum level × nutrient addition at each sampling time.

Results

Effect of nutrient amendment and inoculum level on the survival of P. fluorescens CHA0-Rif, CHA0-Rif(pME3424) and CHA0-Rif(pVK100) in groundwater microcosms

When introduced into microcosms without added nutrients, *P. fluorescens* CHA0-Rif declined in time, as population levels reached detection limit or below (at low inoculum level; Fig. 2A), or stabilized at cell numbers of 3-4 log cells ml^{-1} (at high inoculum level; Fig. 2G). 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. 2D,J). 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. 2A) and 1.4 log units (Fig. 2G).

When used at low inoculum level, CHA0-Rif(pME3424) was below detection limit at 20 d already, even when nutrients had been added (Fig. 2B,E). When introduced at high level, the strain was recovered up to 20 d, by total IF counts only (when no nutrients had been added) or all three cell count methods (in nutrient-amended microcosms) (Fig. 2H,K). At low inoculum level, CHA0-Rif(pVK100) was detected by colony counts up to 20 d (when no nutrient had been added; Fig. 2C) or 50 d (in nutrient-amended microcosms; Fig. 2F), but CFUs were lower than the corresponding ones found for CHA0-Rif (Fig. 2A,D). At high inoculum level however, CHA0-

Rif(pVK100) was recovered throughout the 175-d experiment (Fig. 2I,L) and cell numbers of the strain were as high as those of CHA0-Rif (Fig. 2G,J), regardless of the cell count method used.

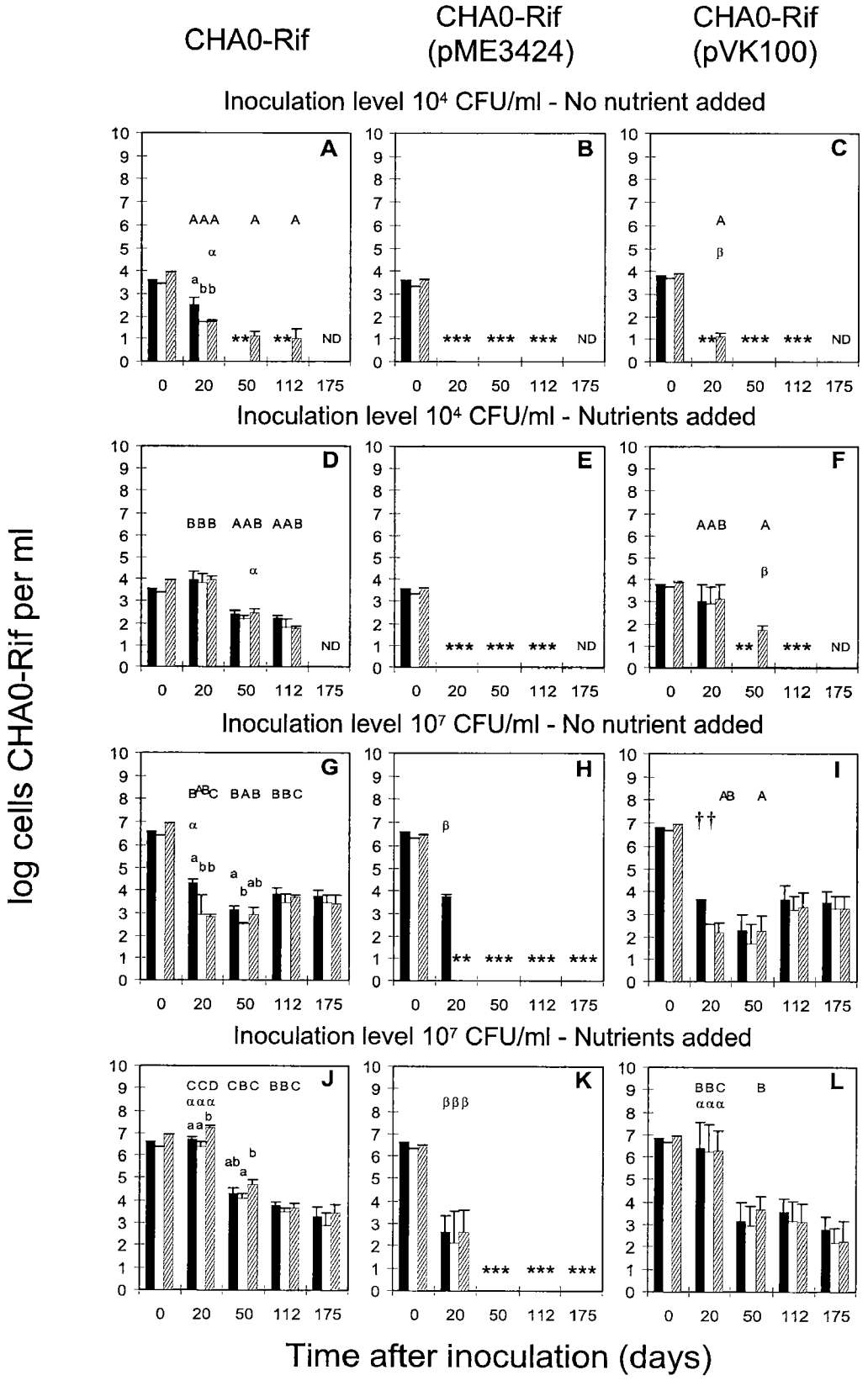

FIG. 2.

Fig. 2. Effect of nutrient amendment and inoculum level on the survival of *P. fluorescens* CHA0-Rif (in A,D,G,J), CHA0-Rif(pME3424) (in B,E,H,K), and CHA0-Rif(pVK100) (in C,F,I,L) in groundwater microcosms prepared with lysimeter effluent water. The pseudomonads were introduced at 4 (in A-F) or 7 log CFU ml^{-1} (in G-L) in unamended (in A-C and G-I) or nutrient-amended microcosms (in D-F and J-L). The inoculants were monitored by total IF counts (■), DVCs (□) and CFUs (▨). Stars denote counts below the detection limits of 2.0 (total IF counts), 1.6 (DVCs) and 1.0 log cells ml^{-1} (CFUs). Error bars signify standard deviations. Total IF counts and DVCs at 20 d in I (indicated with †) were obtained from two replications only (one data missing) and were not included when doing statistical analyses (the corresponding error bars are not shown). Data were processed by analysis of variance followed (when differences were found) with Tukey's HSD test ($P < 0.05$), as follows: First, the three cell count methods were compared for each strain at each sampling time (i.e. within each box), and differences are shown using a and b. Second, the influence of inoculum level and nutrient addition on the survival of each inoculant was investigated for each type of cell count at each sampling time (i.e. vertically), and significant differences are indicated with A, B, and C. Third, the three inoculants were compared within each type of cell count at each combination of inoculum level × nutrient addition at each sampling time (i.e. horizontally) and significant differences are denoted with α and β. ND: not done.

In conclusion, CHA0-Rif declined during the 175-d experiment, but cell numbers depended on inoculum level and the addition of nutrients. The pseudomonad was compromised for survival in groundwater microcosms when it contained pME3424, and the negative effect of the plasmid was mainly due to the presence of *rpoD*.

Maintenance of plasmids pME3424 and pVK100 in culturable cells of the inoculants

Maintenance of pME3424 was investigated only at one sampling (i.e. 20 d), in nutrient-amended microcosms where CHA0-Rif(pME3424) had been introduced at high inoculum level, because culturable cells of the inoculant (on KBA + Rif100) were not found on other occasions. Colony counts of introduced cells on KBA + Rif100 and KBA + Rif100 + Tet125 were statistically identical, indicating that pME3424 was stable in the pseudomonad (data not shown).

Likewise, CFUs on KBA + Rif100 and KBA + Rif100 + Tet125 were statistically identical in the two treatments where CHA0-Rif(pVK100) was used at low inoculum level. At high inoculum level, maintenance of pVK100 was 81% (in microcosms without added nutrients) and 89% (in nutrient-amended microcosms) at 175 d (Fig. 3), as shown by replica-plating randomly-selected colonies from KBA + Rif100 onto KBA

+ Rif100 + Tet125 (or onto KBA + Tet125; data not shown). The presence (or absence) of pVK100 was confirmed by plasmid profile analysis. Interestingly, a significant proportion of the cells of CHA0-Rif(pVK100) present in the microcosms and capable of growing on KBA + Rif100 had lost their ability to grow directly on KBA + Rif100 + Tet125, although they could form a colony on this medium once grown first on KBA + Rif100 (Fig. 3).

Effect of nutrient amendment and inoculation with P. fluorescens CHA0-Rif, CHA0-Rif(pME3424) or CHA0-Rif(pVK100) on dissolved oxygen content and pH in groundwater microcosms

The content in dissolved oxygen, which amounted to 0.35 mM in the lysimeter effluent water used to prepare the microcosms, was approximately 0.26 mM at 20 d, regardless of whether or not microcosms had been inoculated and/or amended with nutrients. Similar values were recorded at 50 d (i.e. 0.28 mM) and at 112 d (i.e. 0.27 mM), in all 14 treatments.

The pH of lysimeter effluent water was 7.6 and that measured in the microcosms was about 7.8 at 50 d, regardless of the treatment studied. At 112 d however, pH in unamended microcosms was statistically higher (i.e. about 8.1) where CHA0-Rif, CHA0-Rif(pVK100) or CHA0-Rif(pME3424) had been introduced at high inoculum level compared with uninoculated microcosms (i.e. 7.9) or microcosms inoculated at low level (i.e. about 8.0), regardless of the strain used for inoculation (Fig. 4A). pH values of 8.1-8.2 were recorded at 112 d for all seven treatments in nutrient-amended microcosms (Fig. 4B), and they were statistically identical to those found in unamended microcosms at high inoculum level.

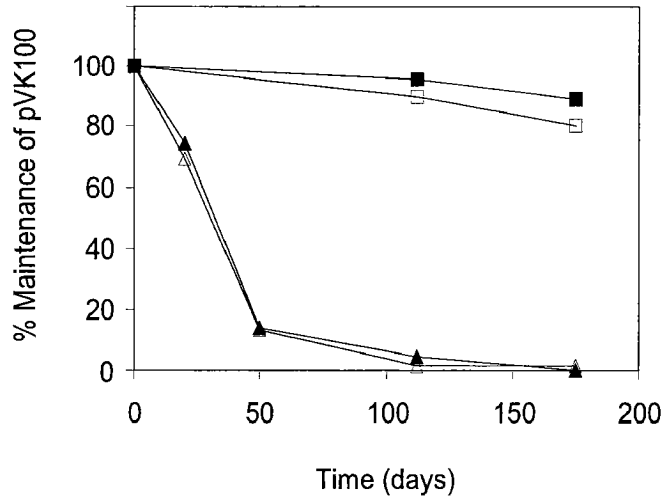

Fig. 3. Maintenance of the plasmid vector pVK100 in *P. fluorescens* CHA0-Rif(pVK100) introduced at 7 log CFU ml^{-1} into groundwater microcosms prepared with unamended (open symbols) or nutrient-amended lysimeter effluent water (filled symbols). Plasmid maintenance was obtained by direct comparison of colony counts on KBA + Rif100 and KBA + Rif100 + Tet125 (△,▲), as well as by calculating the percentage of colonies on KBA + Rif100 capable of subsequent growth on KBA + Rif100 + Tet125 (□,■). At 175 d, plasmid maintenance reached 8% (in unamended microcosms) and 7% (in nutrient-amended microcosms) when derived by comparing colony counts on KBA + Tet125 (instead of KBA + Rif100 + Tet125) and KBA + Rif100.

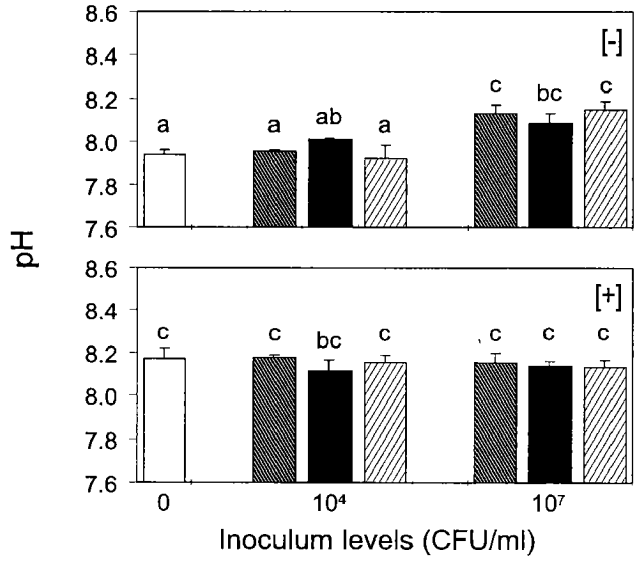

Fig. 4. Effect of nutrient amendment and inoculation with *P. fluorescens* CHA0-Rif (▨), CHA0-Rif(pME3424) (■) or CHA0-Rif(pVK100) (▨) at 112 d on the pH of groundwater microcosms prepared with lysimeter effluent water. Some of the microcosms were not inoculated (□). Microcosms were kept unamended ([-]) or were amended with nutrients ([+]). All 14 treatments were compared using analysis of variance followed with Tukey's HSD test ($P < 0.05$), and significant differences are indicated with a, b and c.

Effect of nutrient amendment and inoculation with P. fluorescens CHA0-Rif, CHA0-Rif(pME3424) or CHA0-Rif(pVK100) on the total culturable populations of fluorescent pseudomonads and aerobic bacteria in groundwater microcosms

The impact on resident CFP was studied since the inoculants were likely to interact and compete (for a similar ecological niche) primarily with fellow pseudomonads naturally present in the microcosms. The addition of nutrients had a positive effect on the total number of CFP in groundwater microcosms (Fig. 5A,C,E,G). This effect was observed for all seven treatments at 20 and 50 d (Fig. 5A,C), but only for one of seven at 112 d (i.e. CHA0-Rif at low inoculum level; Fig. 5E) and two of five at 175 d (i.e. CHA0-Rif at low inoculum level and the uninoculated control; Fig. 5G).

In contrast, inoculation of groundwater microcosms with *P. fluorescens* CHA0-Rif, CHA0-Rif(pME3424) or CHA0-Rif(pVK100), at low or high inoculum level, had little or no effect on the number of total CFP when compared with the uninoculated control (Fig. 5A,C,E,G). At 20 d, 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. 5A[+]), but the increase resulted essentially from the contribution of CHA0-Rif colonies on S1 plates considering CFUs of the strain on KBA + Rif100 (Fig. 2J). At 50 d, when no nutrients were added, the total number of CFP in all three inoculated treatments at high inoculum level exceeded that in the control by about 0.8 log units (Fig. 5C[-]), but this time the increase did not result from the inclusion of the inoculants in colony counts of S1 plates considering population levels of the introduced strains (Fig. 2G). At 175 d, when no nutrients were added, the number of total CFP in the CHA0-Rif(pVK100) treatment at high inoculum level was higher than in the control by about 0.7 log units (Fig. 5G[-]), and again the increase was not caused by the direct contribution of CHA0-Rif(pVK100) colonies on S1 plates considering CFUs of the strain on KBA + Rif100 (Fig. 2I).

Since CHA0-Rif(pME3424) was not found on plates at 50 d (Fig. 2), potential dissemination of pME3424 to resident CFP was studied at 112 d, by spread-plating samples on S1 containing low amounts (25 $\mu g\ ml^{-1}$) of tetracycline. No colony was 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.

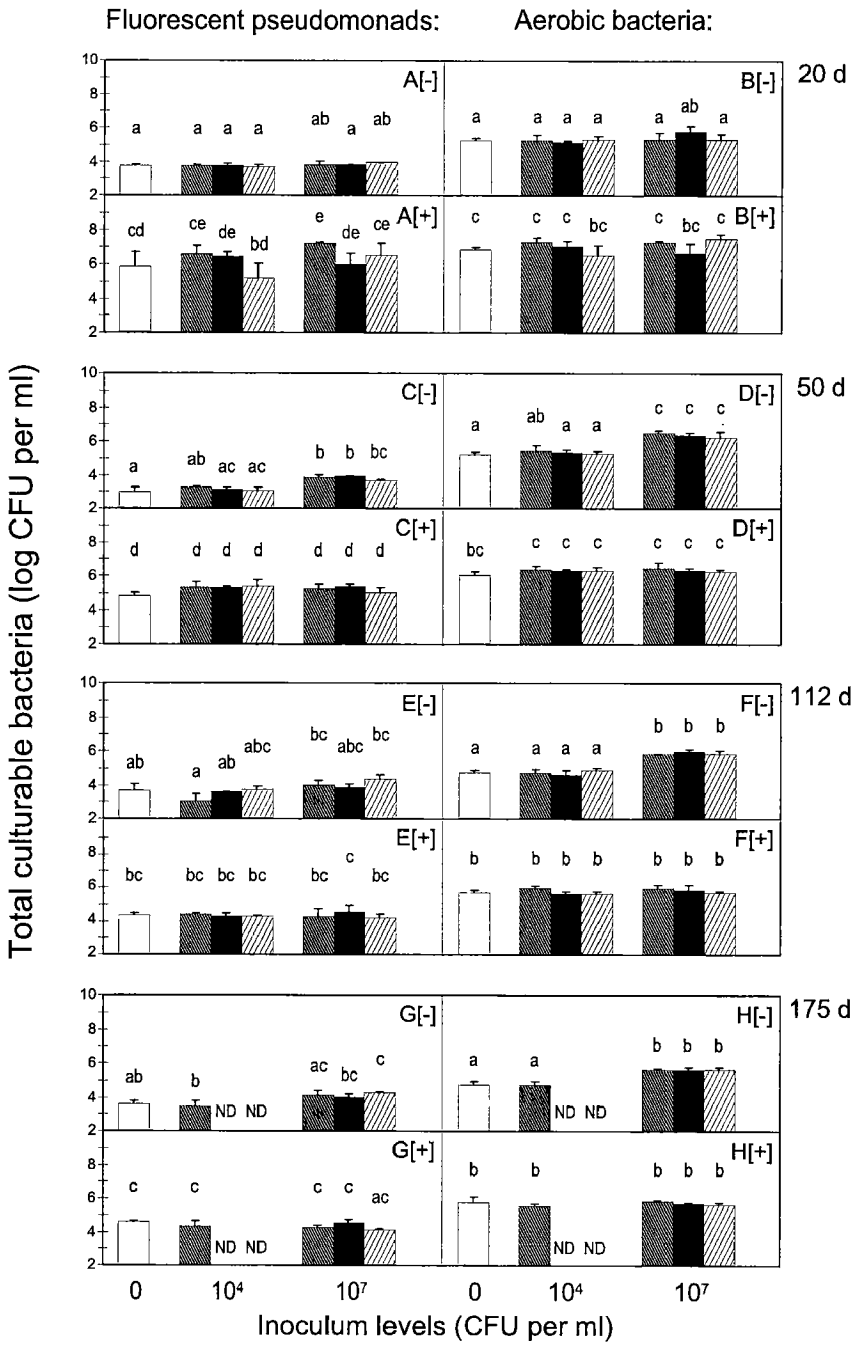

Fig. 5. Effect of nutrient amendment and inoculation with *P. fluorescens* CHA0-Rif (▧), CHA0-Rif(pME3424) (■) or CHA0-Rif(pVK100) (▨) on the total numbers of culturable fluorescent pseudomonads (CFP; in **A**,**C**,**E**,**G**) and culturable aerobic bacteria (CAB; in **B**,**D**,**F**,**H**) in groundwater microcosms. Some of the microcosms were not inoculated (□). The total numbers of CFP and CAB in the lysimeter effluent water used to prepare the microcosms were 2.7 and 4.2 log CFU ml^{-1}, respectively. Microcosms were kept unamended ([-]) or were amended with nutrients ([+]). The effect of treatments on the numbers of CFP and CAB were analyzed separately, as follows: At each sampling time, the 14 treatments were compared using analysis of variance followed with Tukey's HSD test ($P < 0.05$), and significant differences are indicated with a-e.

The impact on resident CAB was investigated to determine the potential of the inoculants to interact with distantly-related bacterial taxa naturally present in the microcosms. As for the total CFP, the addition of nutrients had a positive effect on the total number of CAB in groundwater microcosms (Fig. 5B,D,F,H). At 20 d, this effect was significant for all seven treatments but one (i.e. CHA0-Rif(pME3424) at high inoculum level; Fig. 5B), and was of less magnitude than the effect on the total CFP (Fig. 5A). 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. 5D,F,H).

Inoculation of groundwater microcosms with *P. fluorescens* CHA0-Rif, CHA0-Rif(pME3424) or CHA0-Rif(pVK100), at low or high inoculum level, had no effect on the number of total CAB at 20 d (Fig. 5B). 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. 5D). Interestingly, the increase did not result from the contribution of colonies of the inoculants or of resident pseudomonads on 10% TSA plates considering CFUs of the introduced strains (Fig. 2G,H,I) and the total CFP (Fig. 5C[-],E[-],G[-]).

Production of antimicrobial polyketides by P. fluorescens CHA0-Rif, CHA0-Rif(pME3424) and CHA0-Rif(pVK100) in groundwater microcosms and effect on the total culturable aerobic bacteria sensitive to Phl or Plt

Whether or not the inoculants could have an ecological impact on resident culturable bacteria mediated by antimicrobial polyketides was investigated by HPLC analysis of the polyketides in the microcosms (at 20 and 50 d) and determination of the ability of the total CAB to grow in the presence of Phl or Plt (at 20 d). All inoculants are resistant to levels of Phl or Plt well in excess of 5 $\mu g\ ml^{-1}$, but at 20 d 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 almost the totality of the CAB on plates) or CHA0-Rif(pVK100) (which represented less than 20% of the total CAB). Chemical analysis of the microcosms indicated that Mphl, Phl and Plt were below detection limit at 20 and at 50 d, even in nutrient-amended microcosms.

In microcosms without added nutrients, most of the total CAB could grow in the presence of Phl1 at 20 d, and bacteria sensitive to Phl1 (less than 20% of the total CAB) were found in two of the six inoculated treatments (Fig. 6A[-]). In nutrient-amended microcosms, 25% of the total CAB were sensitive to Phl1 in the uninoculated control (Fig. 6 A[+]). This percentage was statistically identical to those in the six inoculated treatments, except in nutrient-amended microcosms in which CHA0-Rif(pVK100) had been introduced at high inoculum level, where 92% of the total CAB were sensitive to Phl1 (Fig. 6 A[+]).

In contrast to data obtained for Phl1, an important proportion of the total CAB recovered at 20 d were sensitive to Plt1 in unamended microcosms (Fig. 6 B[-]). The percentage of the total CAB sensitive to Plt1 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. 6 B[-]). A similar situation was found in nutrient-amended microcosms: a significant percentage of the total CAB were sensitive to Plt1 (21% to 60%; Fig. 6 B[+]) and treatments had no influence on this percentage

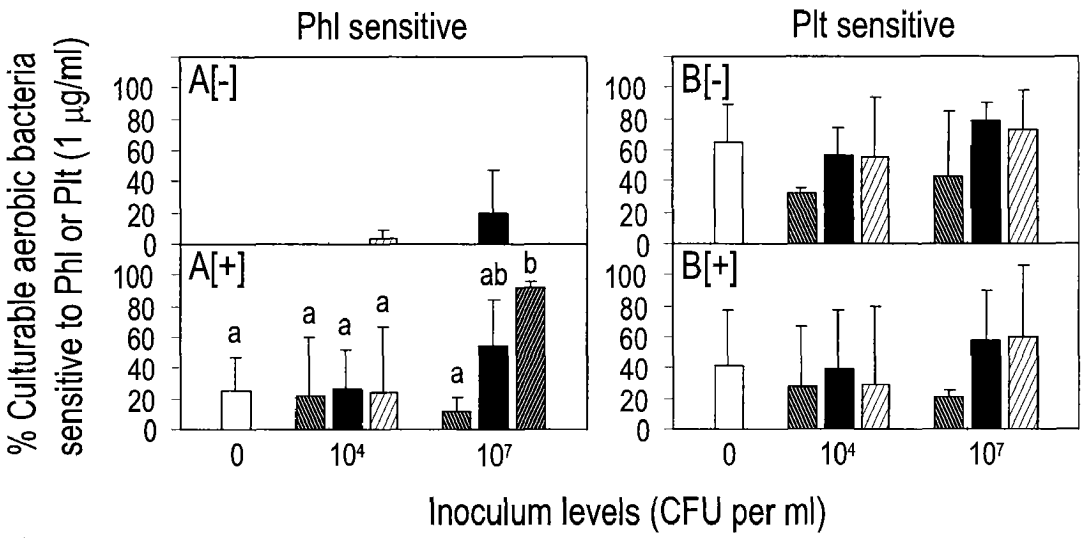

Fig. 6. Effect of nutrient amendment and inoculation with *P. fluorescens* CHA0-Rif (▧), CHA0-Rif(pME3424) (■) or CHA0-Rif(pVK100) (▨) on the percentages of the total culturable aerobic bacteria (CAB) sensitive to Phl (in **A**) or Plt (in **B**) at 1 µg ml^{-1} at 20 d in microcosms prepared with lysimeter 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, and the seven treatments in **A[+]** were compared using analysis of variance followed with Tukey's HSD test ($P < 0.05$); significant differences are indicated with a and b). The 14 treatments were compared together in **B**. The inoculants accounted for a small proportion of the total CAB studied, except for CHA0-Rif used in nutrient-amended microcosms (i.e. in **A[+]** and **B[+]**) at high inoculum level and that represented almost the totality of the CAB (which accounts for the difference between the CHA0-Rif and CHA0-Rif(pVK100) treatments at high inoculum level in **A[+]**). 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, whereas those sensitive to 5 µg Plt ml^{-1} corresponded to 81% (nutrient added) and 94% (no nutrient added) of the total CAB.

Discussion

Under field conditions, the biocontrol strain *P. fluorescens* CHA0-Rif can be transported to deeper soil layers and potentially to shallow groundwater (Natsch et al., 1996; Troxler et al., 1998), but still little is known on the capacity of the pseudomonad to survive at this non-target site (Troxler et al., 1998). In the current work, experimental conditions were chosen to represent a worse-case scenario regarding potential dissemination of a biocontrol pseudomonad and leaching of nutrients through the soil profile following application of e.g. manure to the soil surface. The results indicate that inoculum level and/or nutrient amendment had a significant influence on the survival of CHA0-Rif in groundwater microcosms. Similarly, the addition of nutrients to microcosms prepared with agricultural drainage water delayed the decline of the rhizosphere isolate *P. fluorescens* R2f(RP4) inoculated at 6 log cells ml^{-1} (Trevors et al., 1989). Strain R2f(RP4) could still be detected in microcosms one year after inoculation (van Overbeek et al., 1990). Here, the decline of CHA0-Rif did not result in the formation of viable but non-culturable cells, as in another study (Troxler et al., 1998) in which microcosms were prepared using lysimeter effluent water exhibiting different water characteristics (e.g. nitrate concentration more than four times lower, number of resident CFP lower by 0.5 log unit). Similarly, viable but non-culturable cells of *Sinorhizobium meliloti* and *Agrobacterium tumefaciens* occurred in Baltimore tap water but not in Charlotte tap water (Manahan and Steck, 1997).

Since CHA0-Rif could persist at significant population levels in groundwater 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. 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 (de Leij et al., 1994). In soil microcosms, CHA0-Rif affected microbial populations naturally present in the rhizosphere of cucumber, as (i) part of the resident CFP were displaced (Natsch et al., 1997) and (ii) the potential catabolic activity of the microbial community was modified (Natsch et al., 1998). 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 groundwater microcosms, 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 d, where the inoculant survived at low cell numbers. Similarly, the effect (small but statistically significant) of inoculation with CHA0-Rif on water pH was apparent at 112 d but not at 50 d (Fig. 4). 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 (Short et al., 1991; Doyle and Stotzky 1993) and may result from the complexity of interactions in natural communities (Ives 1995), although certain ecosystems (e.g. the rhizosphere) seem able to buffer the effects of such ecological perturbations (Natsch et al., 1997, 1998). 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 d or at 50 d, even when nutrients promoting production of Phl and Plt had been added. Indeed, significant population levels of resident CAB sensitive to low concentrations (1 $\mu g\ ml^{-1}$) of Phl or Plt were found in a majority of treatments (Fig. 6). 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*. The positive effect of the inoculant on the number of resident CAB resulted probably from the availability of nutrients released during the decline of CHA0-Rif. In conclusion, the impact of the inoculant was not transient, became apparent mostly after the decline of the pseudomonad, and was unlikely to involve the antimicrobial polyketides Phl and Plt.

One objective of this work was to compare the ecological impact of CHA0-Rif with that of the genetically-modified derivative CHA0-Rif(pME3424). Strain CHA0-Rif(pME3424) is a model genetically-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 (Schnider et al., 1995; Natsch et al.,

1997). In the current work however, CHA0-Rif(pME3424) was clearly compromised for survival in groundwater microcosms. This 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 d when incubated in sterile water containing 0.3% NGB (data not shown). Another phenomenon observed only with CHA0-Rif(pME3424) was the 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. 2H). 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. rhizosphere) rather than stress conditions, as could suggest the fact that at high inoculum level culturable cells of the pseudomonad at 20 d were found only when microcosms had been amended with nutrients (Fig. 2K).

Another unexpected observation when studying inoculant survival was that cells containing pVK100 and used at high inoculum level progressively lost their ability to grow in the presence of both Rif100 (chromosomal marker) and Tet125 (plasmid vector marker) (Fig. 3) and, to a smaller extent, to grow in the presence of Tet125 alone (at 175 d; see legend of Fig. 3). This loss was linked to the environmental conditions experienced by the strain in groundwater microcosms, as cells grew well on KBA + Rif100 + Tet125 once cultured on KBA + Rif100. This observation is analog to the apparent loss of rifampicin resistance (i.e. rifampicin-resistance masking) and of resistance to both mercury and tetracycline observed for pseudomonads recovered from inner root tissues of certain crop plants (McInroy et al., 1996) and well water microcosms (Caldwell et al., 1989), respectively. In these two studies, gnotobiotic systems were used and the inoculants were recovered on non-selective plates. In the current work however, antibiotic resistance-masking was shown for bacteria present in microcosms containing resident microbiota and that were recovered on selective plates i.e. containing the antibiotic rifampicin. Interestingly, antibiotic-resistance masking in CHA0-Rif(pVK100) did not take place when counts were done on plates containing kanamycine (kanamycine resistance is another phenotype conferred by pVK100), as

CFUs on KBA + Rif100 + kanamycine (25 μg ml^{-1}) at 175 d were statistically identical to those on KBA + Rif100 (data not shown). This observation has important implications, as plasmid-borne antibiotic-resistant phenotypes have been used (in combination with chromosomal resistance) to monitor survival of genetically-modified pseudomonads in various experiments (Cronin et al., 1997), including some with plasmid vectors derived from pVK100 (Natsch et al., 1997; Beyeler et al., 1999).

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 analyse the ecological impact of inoculation. Indeed, the presence of pME3424 represented a suicide function rather than a functional modification potentially changing interactions with the resident microbiota. Results indicated that 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) that the effects of CHA0-Rif on the number of resident CAB and on pH, in the treatment where the strain was introduced at high inoculum level in unamended microcosms, were due to the presence of high cell numbers of the inoculant at the early stage of the experiment.

In conclusion, introduction of high cell numbers (i.e. worse-case scenario) of the biocontrol strain *P. fluorescens* CHA0-Rif or its genetically-modified derivative CHA0-Rif(pME3424) caused a delayed impact on pH and the resident CAB in groundwater microcosms. The impact on the resident CAB was not due to the production of the antimicrobial polyketides Phl or Plt, and a comparable effect was observed when laboratory medium was added to the microcosms to mimick leaching of nutrients to the groundwater. In fields where soil and weather conditions do not promote extensive water percolation by preferential flow, the ecological impact of biocontrol *Pseudomonas* inoculants on resident culturable bacteria at groundwater level is likely to be marginal (if any), all the more as groundwater conditions tend not to be conducive to production of antimicrobial secondary metabolites such as Phl and Plt *in situ*.

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DISCUSSION

1. Autecology of *P. fluorescens* CHA0-Rif in Bulk Soil and Groundwater Microcosms

Survival of P. fluorescens CHA0-Rif

The survival of the biocontrol *Pseudomonas fluorescens* CHA0-Rif was investigated in soil microcosms prepared with nonsterile bulk soil and in groundwater microcosms consisting of effluent water collected from 2.5-m deep outdoor lysimeters. A decline in cell numbers of the inoculant was observed after its introduction into both soil and groundwater microcosms (Chapters 1-3). CHA0-Rif dropped to lower cell numbers in groundwater than in soil within 50 d in the majority of the treatments studied. Interestingly, population dynamics of the inoculant were somewhat similar in soil and in groundwater microcosms for 50 d, when the laboratory medium nutrient glycerol broth (i.e. NGB) had been added to groundwater microcosms at a final concentration of 0.3% (Chapters 1 and 3). This may lead to the speculation that a lower content of nutrients in unamended groundwater microcosms than in soil, could have led to nutrient deprivation in CHA0-Rif cells and consequently to the accelerated decline of the strain in groundwater microcosms. However, removing the resident microbiota by filter sterilisation from lysimeter effluent water collected in summer or in winter prevented CHA0-Rif cells from declining after their introduction into microcosms prepared with that water (Chapter 2). Furthermore, no decline of CHA0-Rif was found during a 7-d incubation period of the cells in liquid culture lacking a single (C, S, N, or P) nutrient or in a NaCl solution (Chapter 1). Thus, nutrient deprivation of CHA0-Rif cells may not affect the survival of the strain in aquatic environments.

Introduced into soil microcosms, CHA0-Rif cells survived at inoculation level for 14 d (Chapter 1). At subsequent samplings, the inoculant was found at significantly lower cell numbers. This survival pattern of CHA0-Rif in soil was similar, regardless of whether log-phase cells or single-nutrient deprived cells had been introduced into soil. In contrast, deprivation of CHA0-Rif cells of multiple nutrients in liquid culture accelerated the decline of CHA0-Rif cells, once introduced into soil. The mechanisms leading to decrease in cell numbers of CHA0-Rif in soil are not clear.

The morphology of CHA0-Rif cells was studied previously by Troxler et al. (1997b). They found CHA0-Rif mostly as small spherical cells with a diameter of 1.5 μm in the surface horizon of large outdoor lysimeters 56 d after release of the strain. Those cells were significantly smaller than CHA0-Rif cells harvested from overnight cultures, which were rods with a length of about 3.6 μm. A decrease in the length of CHA0-Rif cells occurred also during a 7-d period of deprivation of C, S, N or multiple nutrients in liquid culture (Chapter 1). It cannot be excluded that the soil in the surface horizon of the lysimeters displayed nutrient contents low enough to cause a reduction of the size of CHA0-Rif cells persisting in that soil, and that these cells might have been deprived of nutrients. However, it remains to be ascertained whether nutrient deprivation in CHA0-Rif cells could be linked to the decline of the inoculant in soil, which was observed in microcosms in the current study as well as in the surface horizon of the lysimeters.

Reduction of the length of cells of another strain of *P. fluorescens* (i.e. R2f Rp^r) after introduction into soil in microcosms or subjection to carbon starvation in liquid culture was shown to be accompanied by the development of protection of those cells against several types of abiotic stress (i.e. exposition to ethanol, high temperature, osmotic tension and oxidative stress; Van Overbeek et al., 1995). The ability to survive in soil of cells of the same pseudomonad was improved when the cells had been deprived for multiple nutrients prior to introduction into soil (Van Elsas et al., 1994). Perhaps, a nutrient-deprivation-induced development of resistance to abiotic stress conditions prevailing in soil was responsible for the improved survival of strain R2f Rp^r in soil. Whether in the current study, nutrient deprived CHA0-Rif cells could have been similarly protected against abiotic stress may be of interest, as biocontrol bacteria can be exposed to abiotic stress when the inoculum is prepared, formulated, stored, or applied. Abiotic stress encountered by biocontrol inocula may include suboptimal pH and temperature as well as drought, and may affect both performance (e.g. secondary metabolite production) and strain survival (Slininger and Shea-Wilbur, 1995; Slininger et al., 1996; Moënne-Loccoz et al., 1999; Rodham et al., 1999; Hase et al., submitted). Even the target site (i.e. the rhizosphere) of a biocontrol strain could represent abiotic stress for the colonising bacteria, as a wide range of conditions can prevail on the roots of different crops and in different zones along the root (Marschner et al., 1997; Dekkers et al., 1998).

An improvement of storage and/or post-application survival of soil inoculants has been shown to be due to the fact that the inoculum was supplemented with nutrients during its production or formulation (Graham-Weiss et al., 1987; Caesar and Burr, 1990; Fouilleux et al., 1996), although this may have negative effects on biocontrol (Fuchs et al., 1999; Moënne-Loccoz et al., 1999). Nutrient availability is probably an important parameter to consider during manufacturing of the inoculum, or when choosing a formulation for storage and application of strain CHA0. However, availability for CHA0-Rif cells of the single nutrients C, S, N and P during inoculum preparation was not an important factor for the stability of the inoculum in soil microcosms.

In groundwater microcosms prepared with lysimeter effluent water, the decline of introduced CHA0-Rif cells was shown to be closely linked to the synecology of the strain (i.e. to interactions with the resident microbiota in the microcosms) (Chapter 2). Here, the decrease of CHA0-Rif cells may involve predation by protozoa (Alexander, 1981), or other negative interactions like competition and/or antagonism with resident microorganisms. If in the current study predation of bacteria had occurred, the high *Pseudomonas* inoculum might have been reduced to a size similar to that of the natural population of pseudomonads present in the groundwater microcosms. In uninoculated groundwater microcosms, the numbers of the resident culturable populations of fluorescent pseudomonads and of aerobic bacteria remained at constant levels, with fluctuations not exceeding one log unit. In inoculated microcosms, the numbers of inoculant cells decreased below those levels. Perhaps, inoculant cells were preferentially predated. Indeed, there are reports on selective grazing by nanoflagellates in aquatic environments on e.g. bacteria with a particular length (Kinner et al., 1998) or starved cells of an introduced *P. fluorescens* strain (Christoffersen et al., 1995). Pronounced predation by protozoa is suggested to require a threshold size of the bacterial community (i.e. 6 log cells ml^{-1}; Alexander, 1981). Moreover, flocculation is suggested to prevented inoculated bacteria from predation by micro animals in aquatic microcosms (Inamuri et al., 1996). In the current study, The presence and activity of predators in the groundwater microcosms were not investigated. Further studies will be needed to determine if predation played a major role in the inoculant decline in groundwater microcosms.

The survival of CHA0-Rif in groundwater microcosms varied greatly depending on i) the inoculum level (Chapter 3), ii) the time of the year water had been collected and iii) the soil type in the lysimeter (Chapter 2). Some bacterial populations present in the lysimeter effluent water as well as its chemical composition were analysed for correlation with the survival of CHA0-Rif in groundwater microcosms. Correlations were found between chemical water parameters (nitrate, magnesium, calcium) and cell numbers of CHA0-Rif (total IF counts, DVCs, CFUs), but they were never significant at more than one sampling. However, inoculant survival at the first two samplings (i.e. total IF counts and DVCs at 20 d and at 50 d, CFUs at 20 d) correlated well (and positively) with the number of resident culturable aerobic bacteria determined prior to inoculating the microcosms. Perhaps, predictive models based on groundwater characteristics could be developed for describing survival of biocontrol pseudomonads in groundwater, as *in situ* monitoring of inoculants is difficult and, depending on the applied method, can fail to record certain phenotypes of persisting cells (Thiem et al., 1994; Troxler et al., 1998).

Another question of relevance for risk assessment is whether conditions can occur under which a released biocontrol strain, when translocated to groundwater, is able to grow and to produce secondary metabolites. The suggestion that initial survival of CHA0-Rif in groundwater microcosms amended with a small amount of NGB (i.e. final concentration of 0.3%) was favoured by cell divisions of the bacterium is compatible with the fact that CHA0-Rif can grow in this medium (Chapter 3). Strain CHA0-Rif produces the antimicrobial polyketides 2,4-diacetylphloroglucinol (Phl) and pyoluteorin (Plt) in the rhizosphere (Maurhofer et al., 1995) and in NGB (Chapter 3). In groundwater microcosms, those compounds were not detected by HPLC analysis of microcosm water even when microcosms had been inoculated with a high number of CHA0-Rif cells (7 log CFU ml^{-1}) and amended with 0.3% NGB (i.e. simulation of a worse-case scenario). The detection limit was 28 ng (ml microcosm)$^{-1}$ for Phl (i.e. 133 nM) and 70 ng (ml microcosm)$^{-1}$ for Plt (i.e. 261 nM). These values are in the same order of magnitude as the amounts of Phl and Plt produced by the strain in the rhizosphere (i.e. per g of rhizosphere; Maurhofer et al., 1995). Thus, it cannot be completely excluded that CHA0-Rif had produced small amounts of Phl and Plt in groundwater microcosms.

In unamended groundwater microcosms, linear regression between CFUs and time revealed an increase in culturable cells of CHA0-Rif and a derivative, which contains the wide host range cloning vector pVK100 (Knauf and Nester, 1982) (i.e. CHA0-Rif(pVK100)) from 20 d on (P = 0.013 and P = 0.015 for CHA0-Rif and CHA0-Rif(pVK100), respectively) (Chapter 3). This increase took place after an initial decline of cells of those inoculants introduced into groundwater microcosms at high inoculum level. Introduced into microcosms prepared with agricultural drainage water, the *P. fluorescens* rhizosphere isolate R2f containing the broad host range plasmid RP4 (Saunders and Grinsted, 1972) (i.e. R2f(RP4)) declined to undetectable numbers of culturable cells within one month (Trevors et al., 1989). This strain was recovered on plates about one year later (Van Overbeek et al., 1990). The authors speculated that resuscitation of nonculturable cells of the strain had taken place. However, resuscitation has never clearly been shown for *Pseudomonas* (Mukamolova et al., 1998). Monitoring of CHA0-Rif or CHA0-Rif(pVK100) was done with respect to the potential occurrence of nonculturable inoculant cells. At the first sampling, a significant amount of cells, which had lost the ability to form colony on agar and to respond in Kogure's viability test were found in groundwater microcosms to which no medium had been added. These cells, however, had disappeared by the following sampling, and there is no evidence that they had any significance in the survival of CHA0-Rif. Cryptic growth (Postgate, 1967) was suggested to be responsible for subsequent strain growth after initial decline of *Enterobacter agglomerans* and *Burkholderia cepacia* (both containing the plasmid R388::Tn1721 with resistances to tetracycline and trimethoprim) in sterile well water (Caldwell et al., 1989). This implies that growth of *E. agglomerans* and *B. cepacia* may have occurred due to nutrients released during cell death of those strains. A similar phenomenon may have been involved in the delayed growth of CHA0-Rif and CHA0-Rif(pVK100) in non-sterile, unamended groundwater microcosms. However, this phenomon was only observed in groundwater microcosms i) prepared with effluent water collected from a well drained lysimeter in winter (Chapter 2) and ii) inoculated with a high inoculum level (Chapter 3).

In conclusion, decline of CHA0-Rif cells introduced into soil may involve nutrient deprivation in the persisting CHA0-Rif cells. In groundwater microcosms, inoculant survival was closely linked to the synecology of CHA0-Rif, as cell numbers did not decline in the absence of the resident microbiota. The survival of CHA0-Rif transported

through soil to groundwater level may be predicted by taking into account particular water characteristics, such as the population level of resident culturable aerobic bacteria. Groundwater conditions tend not to be conducive to the production of Phl and Plt at an extent, to which these compounds are produced by the strain at its target site.

Occurrence of nonculturable cells of P. fluorescens CHA0-Rif

In several previous studies strain CHA0-Rif was found to persist as VBNC cells (i.e. cells, which were unable to form visible colonies on plate although responding in Kogure's viability test) in the field (Troxler et al. 1997a and 1997b). In contrast, essentially no VBNC cells of the pseudomonad were found in microcosms regardless of whether they consisted of soil or groundwater in the current study (Chapters 1-3). It has been suggested that the occurrence of VBNC cells in soil may not be predictable from laboratory studies (Natsch et al., 1998b). In spite of that, VBNC cells of other *Pseudomonas* strains (Binnerup et al., 1993) and of CHA0-Rif were found in soil microcosms under certain conditions (Fabio Mascher, personal communication). Moreover, in a previous study VBNC cells of CHA0-Rif (Troxler et al., 1998) and of other bacterial species (Cho and Kim, 1999) have been detected in groundwater microcosms. Those results indicate that it may be difficult to predict the occurrence of VBNC cells from one experiment to the next even when performed in a similar experimental system. The mechanisms, which could have led to the occurrence of VBNC cells of CHA0-Rif in groundwater microcosms are not yet understood (Troxler et al., 1998). From results recently obtained with CHA0-Rif, it seems reasonable to assume that stress factors other than nutrient starvation are necessary for the appearance of VBNC cells of CHA0-Rif (Mascher et al., submitted). The ecological significance of VBNC cells of *Pseudomonas* is unknown and there is no clear evidence for resuscitation and ecological effects of VBNC cells in *Pseudomonas* (Natsch et al., 1998b).

Cells of CHA0-Rif, which had lost the ability to respond in Kogure's viability test and to form colony on agar appeared towards the end of the incubation period of CHA0-Rif cells in bulk soil (i.e. when culturable CHA0-Rif cells had declined; Chapter 1). Culturable CHA0-Rif cells declined within a few hours after introduction into soil

when the inoculum had been deprived of multiple nutrients. Here, in parallel with the rapid initial decline of culturable inoculant cells, significant amounts of CHA0-Rif cells occurred which were unable to respond in the viability test and to form colony on agar. The number of those cells of CHA0-Rif remained relatively constant until the end of the experiment compared with the number of culturable CHA0-Rif cells. Perhaps, CHA0-Rif cells had lost the ability to respond in Kogure's viability test, but were not dead, as dead bacterial cells introduced into soil do not remain as long in soil (Cleyet-Marel and Crozat, 1982; Turpin et al., 1993). Such inactive/dormant cells have been found with the same pseudomonad in the surface horizon of field lysimeters (Troxler *et al.*, 1997a). The significance of this physiological state is unknown. In groundwater microcosms, CHA0-Rif cells, which had lost the ability to respond in Kogure's viability test and to form colony on agar occurred at a few early samplings in unamended microcosms (Chapter 2). Here, those cells declined similarly, as did culturable cells of the strain, perhaps indicating that they were not inactive/dormant, but dead. Introduced into groundwater microcosms, also a *Burkholderia cepacia* inoculum declined, regardless of the cell count method used to monitor the inoculant (Winkler et al., 1995).

In conclusion, essentially no VBNC cells of CHA0-Rif were found in the current study, suggesting that stress factors inducing the VBNC state in this strain had not prevailed under the investigated conditions. CHA0-Rif cells, which had lost the ability to respond in Kogure's viability test and to form colony on plate were found in soil and groundwater microcosms. Whereas in soil those cells might have been in an inactive/dormant state, in groundwater the occurrence of those cells probably corresponded more to a moribund condition than a survival strategy.

2. Autecology of *P. fluorescens* CHA0-Rif(pME3424) in Groundwater Microcosms

The plasmid pME3424 consists of the plasmid vector pVK100 (harbouring e.g. resistance to tetracycline and kanamycine) and a copy of CHA0's *rpoD* gene (encoding sigma factor σ^{70}) (Schnider et al., 1995). The presence of the extra *rpoD* gene in CHA0(pME3424) leads to i) improved biocontrol efficacy in soil microcosms and ii) enhanced production of the antimicrobial polyketides Phl and Plt *in vitro* of the derivative compared with its parental strain. The persistence of CHA0-Rif(pME3424)

was studied in groundwater microcosms prepared with effluent water collected from a well-drained lysimeter in winter (Chapter 3). CHA0-Rif(pME3424) was introduced into microcosms either at a high cell number (as may be transported to groundwater level due to a heavy rainfall occurring shortly after the release of the strain into soil; Natsch et al., 1996) or at a low cell number (perhaps reaching groundwater after some residence time of the cells in soil; Troxler et al., 1998). To one set of microcosms, the laboratory medium nutrient glycerol broth (i.e. NGB), which promotes inoculant growth and production of the antimicrobial polyketides Phl and Plt by CHA0-Rif and CHA0-Rif(pME3424) was added at a final concentration of 0.3%.

Persistence of P. fluorescens CHA0-Rif(pME3424) in groundwater microcosms

Inoculated into groundwater microcosms, CHA0-Rif(pME3424) declined to undetectable cell numbers within 50 d, regardless of the inoculum level × nutrient addition (i.e. 0.3% NGB) combination studied. As for CHA0-Rif in the current study, no VBNC cells of CHA0-Rif(pME3424) were found in groundwater microcosms. In contrast, the presence of the plasmid pFAC510 in *P. fluorescens* 10586(pFAC510) was involved in the occurrence of VBNC cells of this strain in sterile river water microcosms, but a *luxABC* gene on pFAC510 was not responsible for the occurrence of VBNC cells (Oliver et al., 1995). The authors speculated that multiple copies of pFAC510 present in the bacterium might have been responsible for the survival pattern of that strain. Probably also multiple copies of pME3424 are present in CHA0-Rif(pME3424) (Schnider et al., 1995). It is unknown so far how the presence of plasmid DNA may lead to the VBNC state in bacterial cells. After inoculation of unamended (i.e. without the addition of NGB) groundwater microcosms with a high inoculum level, about 4 log cells CHA0-Rif(pME3424) per ml were detected at 20 d. These cells neither responded in Kogure's viability test, nor formed colony on agar. As no CHA0-Rif(pME3424) cells were found in microcosms at subsequent samplings, the non-responsive and nonculturable cells found at 20 d might have correspond to dead inoculant cells remaining in the microcosms before the disappeared.

The rapid disappearance of CHA0-Rif(pME3424) was due to the presence of the extra *rpoD* gene in pME3424, as indicated by the fact that CHA0-Rif(pVK100)

persisted similarly in groundwater microcosms as did CHA0-Rif. The *rpoD* gene encodes for the housekeeping sigma factor σ^{70} of *P. fluorescens* and is essential in CHA0 as indicated by the failure of all attempts to inactivate the single chromosomal *rpoD* gene of CHA0 (Schnider et al., 1995). Introduction of a plasmid containing the cloned *rpoS* gene (encoding for the stationary-phase sigma factor σ^{S}) of *P. fluorescens* Pf-5 into an *rpoS*$^{-}$ mutant of that strain improved biocontrol efficacy compared with the wildtype, but led to a smaller population colonising the root (Sarniguet et al., 1994). Similarly, lower numbers of culturable cells of CHA0-Rif(pME3424) than of CHA0-Rif were found in nutrient amended groundwater microcosms (Chapter 3), in bulk soil (Natsch et al., 1998b), in the rhizosphere (Natsch et al., 1998a) and after growth in full strength NGB (CFUs were four times lower than those of CHA0-Rif).

Housekeeping sigma factors are constantly expressed (Jishage and Ishihama, 1995; Tanaka et al., 1993) and target the expression of housekeeping genes (Ishihama, 1988; Savioz et al., 1990). In contrast, genes encoding stationary-phase sigma factors (σ^{S}) are mainly expressed during the transition between log phase and stationary phase and under certain stress conditions (Hengge-Aronis, 1993) including nutrient deprivation (McCann et al., 1991; O'Neal et al., 1994). It was speculated that in *P. fluorescens* i) the ratio between the two types of sigma factors may be particularly important for gene expression in the stationary phase (Schnider et al., 1995) and ii) altered induction of *rpoS* at the transition between exponential and stationary phase may be critical to the development of resistance to environmental stress (Whistler et al., 1998). It cannot be excluded that mutation in, or introduction into the cell of, genes encoding for those sigma factors may cause an imbalance of their relative amounts, perhaps resulting in an altered pattern of gene expression. Following this hypothesis, the restricted survival of CHA0-Rif(pME3424) compared with that of its parental strain might have been due to a changed pattern of gene expression caused by the presence of the extra *rpoD* gene on pME3424. In *E. coli*, some genes, which are induced in response to starvation conditions and, which seem to be associated with resistance to several stresses are triggered by stationary-phase specific sigma factors (Matin, 1991). However, it is not clear whether under the conditions prevailing in groundwater microcosms, in the current study, gene expression in CHA0-Rif(pME3424) could have been regulated in a similar way.

The antimicrobial polyketides Phl and Plt, whose overproduction by CHA0(pME3424) *in vitro* was suggested to be the result of a gene dosage effect for *rpoD* (Schnider et al., 1995) were not detected by HPLC analysis of microcosm water. Even in microcosms amended with NGB, those compounds were not found. As no Phl and Plt was detected also in microcosms inoculated with CHA0-Rif or CHA0-Rif(pVK100), it remains unclear whether the extra *rpoD* gene on pME3424 bears the potential to influence the production of Phl and Plt by CHA0-Rif(pME3424) in groundwater. Interestingly, another secondary metabolite (i.e. salicylic acid) was produced by CHA0-Rif and CHA0-Rif(pVK100), but not by CHA0-Rif(pME3424) in full strength NGB (data not shown). A similar effect on the synthesis of the antibiotic pyrrolnitrin was observed after inactivation of the *rpoS* gene in *P. fluorescens* Pf-5 (Sarniguet et al., 1994). For a better understanding of the influence of the extra *rpoD* gene in CHA0-Rif(pME3424) on the ability of the bacterium to survive in groundwater, it would be important to know the genes expressed by CHA0-Rif and CHA0-Rif(pME3424) under such conditions.

In order to put a time limit on the possible hazard of large-scale releases of (e.g. genetically engineered) inoculants attempts are made to restrict their survival in the environment (Smit et al., 1992). Active biological containment systems are based on killing genes inducible upon the presence or the absence of specific substrates to be degraded by the microorganisms (Recorbet et al., 1993; Szafranski et al., 1997; Ramos et al., 1997). However, many of those systems perform insufficiently due to an incomplete killing of inoculant populations (Ronchel et al., 1995; Molina et al., 1998), the occurrence of resistant cells (Jensen et al., 1993; Recorbet et al., 1993; Contreras et al., 1991) or the loss of the killing genes (Atlas et al., 1989). Moreover, those systems are triggered by specific compounds, which may restrict a successful application for biocontrol bacteria. Therefore, alternative types of containment systems triggered by environmental influences like the temperature (Ahrenholtz et al., 1994; Jechlinger et al., 1998) or general growth conditions (Tedin et al., 1995) may be promising. It remains to be ascertained to which extent the extra *rpoD* gene in CHA0-Rif(pME3424) could represent a suicide function once the bacterium is translocated to non-target sites.

In conclusion, survival of CHA0-Rif(pME3424) was strongly affected in groundwater microcosms due to the presence of the extra *rpoD* gene on pME3424. To understand the mechanisms leading to the potential suicide function of pME3424 in

CHA0-Rif(pME3424) at non-target sites, a better knowledge on the gene expression regulated by RpoD in the derivative and its parental strain is needed.

Plasmid maintenance in P. fluorescens CHA0-Rif(pME3424) and CHA0-Rif(pVK100) in groundwater microcosms

The plasmid pME3424 was lost in 13% of the CHA0-Rif(pME3424) cells surviving in the rhizosphere of cucumber 52 d after inoculation of the strain (Natsch et al., 1998a). In contrast, pME3424 was not lost in the culturable CHA0-Rif(pME3424) cells detected in groundwater microcosms, as CFUs on plates with rifampicin and on those with both rifampicin and tetracycline were statistically identical. Whether the nonculturable cells of CHA0-Rif(pME3424) found at 20 d in unamended groundwater microcosms still contained the plasmid was not studied, but plasmid loss in nonculturable cells was not evidenced for other bacteria in aquatic environments (Morgan et al., 1991; Byrd et al., 1992). Since plasmid transfer may take place in groundwater (Nakatsu et al., 1995), it was investigated whether pME3424 could have been transferred to resident culturable fluorescent pseudomonads. After CHA0-Rif(pME3424) had disappeared in groundwater microcosms, no culturable fluorescent pseudomonads resistant to low amounts of tetracycline were found, suggesting that pME3424 was not disseminated to those bacteria. It cannot be ruled out that the plasmid may have been present in cells of genera other than *Pseudomonas*, or in bacteria, which may have lost culturability after uptake of pME3424, or even as free DNA in the microcosms. However, genetically engineered plasmid DNA is rapidly degraded in the presence of DNAse-producing microflora in aquatic environments (Alvarez et al., 1996).

Maintenance of the plasmid vector pVK100 in CHA0-Rif(pVK100) cells inoculated at high level into groundwater microcosms decreased towards the end of the incubation period. The extent of loss of pVK100 in CHA0-Rif(pVK100) cells in groundwater microcosms was comparable with the extent at which pME3424 was lost in CHA0-Rif(pME3424) cells in the rhizosphere (Natsch et al., 1998a). Plasmid maintenance in CHA0-Rif(pVK100) cells decreased similarly in nutrient-amended or unamended groundwater microcosms. It is not clear whether the loss of pVK100 was

due to the longer time CHA0-Rif(pVK100) cells persisted in groundwater microcosms compared with CHA0-Rif(pME3424) cells, or the absence of the *rpoD* gene on pVK100. Since the *rpoD* gene turned out to have a negative effect on the survival of CHA0-Rif(pME3424) in groundwater microcosms, it is unlikely that stability in the host would be less for pVK100 than for pME3424. Loss of pVK100 from *Rhodospirillum rubrum* occurred rapidly when culturing the transconjugant anaerobically (Saegesser et al., 1992). Since there was no evidence that conditions in groundwater microcosms had become anaerobic towards the end of the study, other factors might have been involved in the loss of pVK100 in CHA0-Rif(pVK100).

The rate of maintenance of pVK100 was determined by transferring rifampicin-resistant (chromosomal marker) colonies of CHA0-Rif(pVK100) onto plates containing also tetracycline (plasmid vector marker). Selecting for bacterial resistances to both rifampicin and tetracycline on plates led to a strong underestimation of cell numbers of CHA0-Rif(pVK100) in groundwater microcosms. This observation has important implications, as plasmid-borne antibiotic-resistant phenotypes have been used (in combination with chromosomal resistance) to monitor the survival of genetically modified pseudomonads in various experiments (Cronin et al., 1997), including some with plasmid vectors derived from pVK100 (Natsch et al., 1997; Beyeler et al., 1999). However, CHA0-Rif(pVK100) could be quantified correctly in groundwater microcosms when utilising the kanamycin-resistant phenotype conferred by pVK100. Resistance to kanamycin in bacteria can be mediated by modification of a ribosomal subunit or by acetylation of the drug (Misumi and Tanaka, 1980; Nakano et al., 1989). In contrast, resistance to tetracycline involves an inner membrane protein and a proton motive force-dependent efflux of the drug that keeps it away from its intracellular target, the ribosome (McMurry et al., 1986). Expression of the tetracycline-resistance requires energy and an intact cell membrane. The proton motive force could be inhibited in *E. coli* by nutrient starvation and specific inhibitors, which resulted in 80 to 90% tetracycline-sensitive *E. coli* cells. Hypothetically, cells of CHA0-Rif(pVK100) persisting in groundwater microcosms may have been progressively deenergized, due perhaps to low nutrient availability in the microcosms.

However, analogous observations were made with resistances to rifampicin or to mercury that failed to be expressed upon the recovery of pseudomonads from inner root tissues of certain crop plants and well water microcosms (McInroy et al., 1996;

Caldwell et al., 1989). There, gnotobiotic systems were used and higher numbers of the inoculants could be found when plates without the antimicrobial compound were used. Thus, resistance in pseudomonads failed to be expressed i) against differently acting antibiotics or even to heavy metals and ii) after persistence of the cells in very different environments. This points to the existence of several mechanisms, which can lead to a loss of the resistant phenotype in *Pseudomonas* cells. It would be interesting to know whether bacterial adaptation to certain environmental conditions (e.g. low nutrient availability) may include modifications leading to the loss of antibiotic-resistant phenotypes.

In conclusion, the rapid disappearance of CHA0-Rif(pME3424) in groundwater microcosms seems to restrict the dissemination potential of pME3424. In groundwater, the plasmid vector pVK100 may not ensure the stability of pME3424 in CHA0-Rif(pME3424) cells if those cells were able to survive for longer periods than observed in the current study. As found for the tetracycline-resistance on pVK100, loss of resistance-marker expression of the introduced genes may affect a correct monitoring of genetically modified cells persisting in groundwater.

3. Synecology of *P. fluorescens* CHA0-Rif and CHA0-Rif(pME3424) in Groundwater Microcosms

Significance of the addition of nutrients to groundwater microcosms

Nutrient glycerol broth (i.e. NGB) was added at a final concentration of 0.3% to one set of groundwater microcosms to mimic nutrient leaching into groundwater (Chapter 3). Since there was no impact in groundwater microcosms inoculated at low inoculum level, in this chapter, the impact of inoculation with a high number of CHA0-Rif or CHA0-Rif(pME3424) cells is compared with the impact of nutrient addition. The effects caused in microcosms by the addition of nutrients were essentially similar in magnitude to those resulting from inoculation. Both treatments led to an increase in the size of culturable indigenous bacterial populations and in a rise of the pH in groundwater microcosms. Perhaps, the same mechanisms were involved in the occurrence of the impact, as death of high numbers of inoculant cells could have

supplied the inoculated microcosms with additional nutrients. Indeed, cell death mediated by the presence of a killing gene in the *P. putida* strains CMC4 and CMC12 involved the release from the cells of some cytoplasmatic contents to the extracellular medium (Ronchel et al., 1998).

The number of resident culturable fluorescent pseudomonads in groundwater microcosms was significantly increased after inoculation at one sampling (i.e. at 50 d) and after nutrient amendment at three samplings (i.e. 20 d, 50 d and 175 d) compared with untreated microcosms. The impact on the resident culturable fluorescent pseudomonads like that on the pH was not lasting, regardless of the treatment, which caused the effect. In contrast, already at an early stage of the experiment, resident culturable aerobic bacteria displayed cell numbers, which were about one log unit higher than those in the untreated control treatment, and this effect lasted until the end of the experiment. The effect on numbers of resident culturable aerobic bacteria became obvious in inoculated microcosms at 50 d and in nutrient amended microcosms at 20 d. The addition of a laboratory medium (i.e. Lennox L broth) at a concentration similar to that used in the current study, caused only a short-term increase in the number of resident culturable aerobic bacteria in microcosms prepared with river water (Leung et al., 1995). In contrast, inoculation of these microcosms with 4.7 log CFU ml^{-1} of recombinant *Pseudomonas* spp. did not affect the population levels of resident culturable aerobic bacteria. Also in the current study, the population levels of resident culturable fluorescent pseudomonads and of resident culturable aerobic bacteria in groundwater microcosms were more affected by the nutrient amendment than by the inoculation. Interestingly, even the combination of those two treatments (i.e. both inoculation and nutrient amendment) could not increase the magnitude of the effects on resident culturable bacteria. This indicates that either a maximal magnitude of that type of impact was caused by the nutrient amendment or that the nutrient supply to the microcosms was much larger from NGB addition than from inoculation. Indeed, the dry weight of introduced inoculant cells does not reach the amount of NGB added to the microcosms (i.e. about 3 mg $(microcosm)^{-1}$). *In situ* injection of nutrients (i.e. 160 g acetic acid and 560 mg of each, nitrate and phosphate) into a 0.6-m thick, clay-rich sediment led to several, but transient (within 85 d) increases of the heterotrophic bacteria population in groundwater (Capuano et al., 1995). It might be interesting to know how substances, which unintentually could reach groundwater *in situ* (e.g. manure

or pesticides) might affect resident bacterial populations and/or simultaneously introduced biocontrol pseudomonads. In any case, it was surprising that the addition of NGB (which promotes growth and polyketide production of the inoculants *in vitro*) to groundwater microcosms had a longer lasting influence on the population levels of indigenous than of introduced bacteria, regardless of the number at which inoculants had been introduced.

In conclusion, inoculation or nutrient amendment caused effects, which were similar regarding their magnitude and the parameters, which were affected, perhaps indicating similar mechanisms such as nutrient supply being involved in the occurrence of those effects in groundwater microcosms. However, the impact caused by the introduction of biocontrol inoculants into groundwater microcosms may not occur *in situ* except when several factors are concatenated (e.g. the occurrence and the death of high numbers of inoculant cells in groundwater).

Comparison of the impact of P. fluorescens CHA0-Rif with that of its genetically modified derivative CHA0-Rif(pME3424) in groundwater microcosms

The introduction of either CHA0-Rif or CHA0-Rif(pME3424) caused a transient displacement of a part of the resident culturable fluorescent pseudomonads and modified the potential catabolic activity of the microbial community in the rhizosphere (Natsch et al., 1997 and 1998a). Those effects were similar for CHA0-Rif and its derivative. Also in groundwater microcosms, no difference was found between the effects of CHA0-Rif and those of CHA0-Rif(pME3424), regardless of the inoculum level × nutrient addition combination studied. This was surprising when considering the different survival of the two strains in groundwater microcosms. It seems, therefore, that the culturable cells of CHA0-Rif found from days 20 to 175 in unamended groundwater microcosms had a marginal or no influence on the numbers of resident culturable aerobic bacteria and on pH. This implies that this ecological impact was mainly caused by high cell numbers of the inoculants present in the microcosms at an early stage of the experiment (inoculation at 7 log CFU/ml). Thus, the presence of pME3424 in CHA0-Rif(pME3424) in groundwater microcosms did not represent a functional modification changing interactions with the resident microbiota or ecosystem

parameters. Biological containment systems based on suicide genes are designed to reduce the causing of e.g. an impact on natural populations to a limited time or to site-specific conditions. In groundwater microcosms, inoculation with a high number (but not with a low number) of CHA0-Rif(pME3424) cells exhibiting characteristics of a suicide strain did not prevent from undesired long-term effects on resident culturable aerobic bacteria.

For risk assessment, the prediction of ecological changes potentially occurring in groundwater after field release of biocontrol inoculants, in particular of GMOs, would be of interest. In the current study, introduction into groundwater microcosms of a high number of genetically modified or parental strain cells caused an identical impact. To my knowledge an impact on population levels of indigenous bacteria caused by a genetically modified inoculant, but not by its parental strain has never been reported for groundwater environments. In the current study, the impact caused by CHA0-Rif depended on the season, as no increase in population levels of resident bacteria following inoculation with CHA0-Rif was found in groundwater microcosms prepared with lysimeter effluent water collected at other times during the year (Chapter 2). No apparent effect on the population density of indigenous bacteria was caused by the introduction of the *P. putida* strain PpY101 into aquatic microcosms (Iwasaki et al., 1993). The genetically modified derivative PpY101(pSR134) displayed a synecology, which was not different from that of its parental strain. In the current study, it seems that the seasonal fluctuation of the composition of lysimeter effluent water, rather than the genotype of the inoculant was influential on the occurrence of effects on population levels of resident microorganisms in groundwater microcosms. In the following, an attempt is made to find a correlation between the water composition and the population of resident culturable aerobic bacteria that was affected by nutrient amendment or occasionally by inoculation with CHA0-Rif in groundwater microcosms. The relationship between the numbers (i.e. log numbers) of the total culturable aerobic bacteria detected at the end of the experiments (i.e. at 175 d, when CHA0-Rif comprised not more than a small percentage of the resident culturable aerobic bacteria in inoculated groundwater microcosms) and properties of the lysimeter effluent water was analysed using Pierson correlation coefficient (Chapters 2 and 3). For that correlation analysis numbers of culturable aerobic bacteria, which had been detected in inoculated (with CHA0-Rif at high inoculum level) and/or nutrient amended

groundwater microcosms as well as those detected in untreated control microcosms were used (i.e. resulting in a total of 11 treatments). Significant correlations were found with the contents in nitrate (r = 0.74; P = 0.009), chloride (r = 0.73; P = 0.011), potassium (r = 0.64; P = 0.035) and calcium (r = 0.72; P = 0.012) as well as with the log number of culturable aerobic bacteria of the lysimeter effluent water (r = - 0.79; P = 0.004). Perhaps knowledge on the chemical and biological composition of groundwater can help to predict the occurrence of an impact on population levels of resident bacteria in a case, in which large numbers of wildtype or genetically modified inoculant cells are introduced.

In conclusion, simulation in microcosms of a worse-case scenario of high numbers of GMO or parental strain cells being introduced into groundwater led to a lasting impact on the population level of resident bacteria. The effect was due to the high inoculum level and the conditions encountered by the inoculants in the microcosms rather than the genotype of the inoculant. Further studies should address the predictability of such effects caused by the introduction into groundwater of high numbers of inoculant cells exhibiting potential suicide functions.

Comparison between the impact of the P. fluorescens inoculants in groundwater microcosms and in the rhizosphere

The effects on resident bacterial populations in the rhizosphere and in groundwater microcosms seemed not to be due to the antimicrobial polyketides of CHA0-Rif or CHA0-Rif(pME3424) (Chapter 3; Natsch et al., 1998a). This was indicated by the fact that inoculation with CHA0-Rif or CHA0-Rif(pME3424) had not changed the proportion of resident bacteria sensitive to amounts of Phl or Plt that could be detected from these environments. Therefore, the production of those antimicrobial polyketides must have been too little (if any) to affect the numbers of the investigated resident bacteria. In both groundwater microcosms and rhizosphere, inoculation with CHA0-Rif or CHA0-Rif(pME3424) caused a short-term alteration in the numbers of resident culturable fluorescent pseudomonads (Chapter 3; Natsch et al., 1997). In the rhizosphere, a part of that population was displaced at an early sampling (i.e. at 10 d). In groundwater microcosms, the number of resident culturable fluorescent pseudomonads

was higher at one sampling (i.e. 50 d) compared with uninoculated microcosms. The displacement of a part of the resident culturable fluorescent pseudomonads caused by CHA0-Rif or CHA0-Rif(pME3424) in the rhizosphere was suggested to be due to competition for a similar ecological niche between the introduced and the resident pseudomonads. Compared with aquatic environments, the rhizosphere is likely to provide more microsites, which are not only difficult to access for predators, but also may display large fluctuations in the concentration of soluted substances. This may have implications on the interactions between microorganisms. Whereas in aquatic environments such as groundwater microcosms, the concentration of soluted substances might be widely homogenous, in small compartments existing in soil habitats, such substances may earlier become limiting to bacterial survival. This could be true equally for nutrients and for toxic compounds produced by microorganisms. Indeed, a *P. fluorescens* inoculum was shown to cause a short-term reduction in the overall microbial diversity when introduced into soil with a small, but not with a large pore neck size (White et al., 1994). In the rhizoshere, introduction of CHA0-Rif or CHA0-Rif(pME3424) did not affect numbers of resident bacteria, which are sensitive to the antimicrobial polyketides of the inoculants *in vitro* (Natsch et al., 1998a). In contrast, the population of a highly resistant *P. fluorescens* strain was found to be significantly reduced in the presence of the inoculants (Natsch et al., 1998a). This indicates that competition for a similar ecological niche between the introduced and the resident species was an important factor responsible for the displacement of the resident pseudomonad. It has been suggested that in the rhizosphere, fast growing organisms such as fluorescent pseudomonads are most sensitive to perturbations (De Leij et al., 1995). Perhaps, this property became to a disadvantage for the survival of the CHA0-Rif inoculum when introduced into groundwater microcosms as soon as the inoculated cells had to compete with the resident bacteria. Following death of high numbers of inoculant cells, a large amount of nutrients or other growth-promoting substances becoming available may have favoured the short-term increase in the number of the resident culturable fluorescent pseudomonads in groundwater microcosms.

In groundwater microcosms, but not in the rhizosphere, the introduction of the biocontrol strains resulted in a twenty-fold increase in the number of resident culturable aerobic bacteria. This impact became obvious after the inoculants had declined to low cell numbers in the microcosms and was lasting until the end of the experiment.

Stimulation of resident heterotrophic bacteria has been reported to take place upon the introduction of *P. fluorescens* inocula also in the rhizosphere, but here the effects were of short duration (Kozdrój, 1997 and 1999). Perhaps, the affected population of resident culturable aerobic bacteria in groundwater microcosms corresponded more to a slowly growing population, as microorganisms in subsurface environments must be considered to be adapted to the prevailing oligotrophic conditions, which limit bacterial growth in these environments. In subsurface environments, a large proportion of the resident bacteria is in a nonculturable state (Dodds et al., 1996). In the current study, it was not investigated to what extent resuscitation of resident nonculturable bacteria might have been contributed to the increase of the number of resident culturable aerobic bacteria in groundwater microcosms.

In several studies performed in the rhizosphere, ecological changes caused by the introduction of bacteria are compared with natural fluctuations of the investigated parameters (Liste et al., 1997; Mahaffee and Kloepper, 1997; Kozdrój et al., 1999). The effects on resident bacteria exerted by CHA0-Rif or CHA0-Rif(pME3424) in the rhizosphere of cucumber were less pronounced than natural changes of these populations occurring during plant-root growth (Natsch et al., 1997 and 1998a). In groundwater microcosms, the increase in the numbers of both the resident culturable fluorescent pseudomonads and the resident culturable aerobic bacteria following inoculation exceeded seasonal fluctuations of the respective population levels in the lysimeter effluent water used to prepare the microcosms (Chapter 2). The rhizosphere seems to be better buffered against microbial perturbations potentially caused by the introduction of the biocontrol inoculants than the non-target site, which is represented in the current study by the groundwater microcosms. A reasonable explanation for this observation may be the fact that strain CHA0 was originally isolated from a tobacco field.

In conclusion, effects on non-target bacterial populations may not be expected from antimicrobial polyketides produced in the rhizosphere or eventually at low level in groundwater by the biocontrol inoculants. In both environments an impact can result from interactions such as competition with the resident microbiota. The magnitude of potential effects of the introduction of biocontrol strains seems to be influenced by the characteristics and the size of the resident bacterial community in relation to those of the introduced population. An impact resulting from the introduction of high numbers

of inoculant cells may exhibit a greater magnitude at non-target sites where the resident microorganisms are adapted to conditions differing to a large extent from those of the target site of the inoculant.

GENERAL CONCLUSIONS

The study addressed the fate and the impact of the biocontrol inoculant *P. fluorescens* CHA0-Rif and its derivative CHA0-Rif(pME3424) at non-target sites such as nonrhizosphere soil and groundwater.

Nutrient availability was shown to be influential on the subsequent survival of CHA0-Rif in soil. Here, cells of CHA0-Rif, which neither responded in Kogure's viability test nor formed colony on plate were prevalent.

The survival of CHA0-Rif in groundwater depended largely on biological characteristics of the water. Here, inoculants persisted mainly as culturable cells. In inoculant cells bearing the plasmid vector pVK100, the tetracycline-resistant phenotype conferred by pVK100 was transiently lost.

An impact of the inoculants on resident culturable groundwater bacteria occurred only under worse-case conditions. This impact was not due to CHA0-Rif's antimicrobial polyketides, since the production of these compounds by CHA0-Rif was not favoured by groundwater conditions. Since the impact in groundwater microcosms was lasting compared with that in the rhizosphere, microbial inoculants may interact differently with their environment at the target site and at non-target sites.

This finding emphasises the importance of the synecology of inoculants at non-target sites for biosafety assessment of microbial releases into the environment.

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PUBLICATIONS

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ANNEX

Survival and cell culturability of biocontrol *Pseudomonas fluorescens* CHA0 in the rhizosphere of cucumber grown in two soils of contrasted fertility status

Abstract

The effect of cucumber roots on survival patterns of the biocontrol soil inoculant *P. fluorescens* CHA0-Rif was assessed for 22 days in two non-sterile soils, using a combination of total immunofluorescence cell counts, Kogure's direct viable counts and colony counts on plates containing rifampicin. In Eschikon soil (high fertility status for cucumber), CHA0-Rif persisted as culturable cells in bulk soil and in the rhizosphere, but colony counts were lower than viable counts and total cell counts inside root tissues. The occurrence of viable but non-culturable (VBNC) cells inside root tissues (5 log cells g^{-1} root) was unlikely to result from the H_2O_2 treatment used to disinfect the root surface, as H_2O_2 caused death of CHA0-Rif cells *in vitro*. In Siglistorf soil (low fertility status for cucumber), the inoculant was found mostly as non-culturable cells. Colony counts and viable counts of CHA0-Rif were similar, both in bulk soil and inside root tissues, whereas in the rhizosphere viable counts exceeded colony counts at the last two samplings (giving about 7 log VBNC cells g^{-1}). In conclusion, soil type had a significant influence on the occurrence of VBNC cells of CHA0-Rif, although these cells were found in root-associated habitats (i.e. rhizosphere and root tissues) and not in bulk soil.

Introduction

Population dynamics of bacterial inoculants in soil systems is often studied by colony counts on selective media (Natsch et al. 1994; McInroy et al. 1996; Cronin et al. 1997). However, recent investigations have shown that colony counts were insufficient to monitor bacterial inoculants in soil, as certain strains could persist as mixed populations of culturable and non-culturable cells (Binnerup et al. 1993; Heijnen et al. 1995; Troxler et al. 1997b). For instance, the biocontrol inoculant *Pseudomonas fluorescens* CHA0-Rif was found mostly as non-culturable cells in the surface soil horizon of large outdoor lysimeters, as indicated by the comparison of colony counts (on selective medium) and total immunofluorescence (IF) counts of the strain (Troxler et al. 1997b). Furthermore, a significant amount of the non-culturable cells of the inoculant responded positively to Kogure's viability test (Kogure et al. 1979), indicating that they were not dead (Troxler et al. 1997b). Kogure's direct viable count (DVC) 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.

Several studies have focused on the culturability of *P. fluorescens* in bulk soil (Binnerup et al. 1993; Troxler et al. 1997b; Hase et al. 1999). However, whether or not root-associated pseudomonads can occur as non-culturable cells has received little attention so far. All cells of strain CHA0-Rif present inside maize roots at crop ripening responded positively to Kogure's viability test, but only a quarter of them could be recovered on solid medium (Troxler et al. 1997c). Only circumstantial evidence is available regarding the presence of non-culturable pseudomonads in the rhizosphere (Troxler et al. 1997b). This is an important issue, because pseudomonads are typical rhizobacteria (Miller et al. 1989; de Leij et al. 1995) and several *Pseudomonas* strains represent promising biocontrol agents against soil-borne pathogens affecting plant roots (Cook et al. 1995; Dunne et al. 1997; Sharifi-Tehrani et al. 1998).

The objective of this work was to assess survival of the biocontrol agent *P. fluorescens* CHA0-Rif and its ability to persist as non-culturable cells in the rhizosphere. Two soils were used, since colony counts (van Elsas et al. 1986) and the occurrence of non-culturable cells (van Overbeek et al. 1995) of *P. fluorescens* introduced into bulk soil can vary greatly from one type of soil to the next. Furthermore, the effect exerted by plant roots on culturable soil fluorescent pseudomonads also

depends on soil type (Latour et al. 1995). Soil or rooting medium conditions will influence growth of roots and the release of root exudates, including that of exudated compounds used as nutrient or recognised as signal by soil bacteria (P•ikryl and Van•ura 1980; Richardson et al. 1988; Marschner 1991; Buerkert and Marschner 1992). Therefore, two soils displaying different physical and chemical characteristics and representing contrasted fertility status for cucumber were chosen. One of the soils (Eschikon soil) was a loam, in which cucumber grows well, and the other one (Siglistorf soil; acidic silt with high organic matter content) corresponds to a type of soil in which cucumber grows slowly (Papadopoulos 1994). *P. fluorescens* CHA0-Rif was inoculated into the soils, which were then sown with cucumber. The inoculant was monitored in bulk soil, in the rhizosphere and inside root tissues using a combination of colony counts, Kogure's direct viable counts (DVCs; Kogure et al. 1979) and total IF counts of the strain.

Materials and methods

Soils used in the experiment

Both soils were obtained from the surface horizon of cambisols. The first soil (described by Natsch et al. 1994) was collected at a fallow located in Eschikon (near Zurich) and corresponds to a loam (15% clay, 42% silt, 43% sand; pH 7.0, 3.5% organic matter). The second soil was obtained in a forest located near Siglistorf and corresponds to a silt (16% clay, 53% silt, 31% sand; pH 4.3, 10% organic matter). Plant roots were removed. The soils were passed through a 5-mm mesh screen and air-dried at 15°C until water potential (determined by the filter paper method; McInnes et al. 1994) reached approximately -0.03 MPa (i.e. water contents of 22.5% w/w and 31.6% w/w for Eschikon and Siglistorf soils, respectively).

Bacterial strains, inoculation of soil and sowing

The biocontrol agent *P. fluorescens* CHA0 was isolated from the root of tobacco grown in Morens soil (near Fribourg, Switzerland; Stutz et al. 1986). Strain CHA0-Rif is a spontaneous rifampicin-resistant mutant of CHA0 (Natsch et al. 1994).

Strain CHA0-Rif was routinely grown at 27°C with shaking (150 rev min^{-1}) in King's B broth (King et al. 1954) containing 100 µg rifampicin ml^{-1} (i.e. Rif100). The cells were used to inoculate King's B agar (i.e. KBA) containing Rif100 and 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 5×10^9 cells ml^{-1} based on OD measurements (600 nm). Bacteria were applied to the soil by spraying 20 ml of the suspension per dm^3 of soil (i.e. about 2×10^8 cells g^{-1}). In the un-inoculated control, soil was sprayed with 20 ml of sterile distilled water per dm^3 of soil. The soil was thoroughly mixed and 500 cm^3 of soil (i.e. dry weight of 410 g of Eschikon and 220 g of Siglistorf) was added per pot.

Cucumber seeds (*Cucumis sativus* L. "Sensation"; R. Geissler AG, Zürich) were surface-disinfected in 5% (w/v) sodium hypochloride for 30 min and rinsed with sterile distilled water. One seed was sown per pot, immediately after soil inoculation. The pots were placed in a growth chamber (70% relative humidity; 16 h with 160 µE m^{-2} s^{-1} at 22°C and 8 h of dark at 18°C), following a randomised design. Every second day, the water content of the soil was checked by weighing the pots and if necessary adjusted to reach about -0.03 MPa by adding sterile distilled water to the soil surface. Fresh root biomass was 1.22 g (Eschikon) and 0.071 g per plant (Siglistorf) at 22 d, confirming that the latter soil has a low fertility status for cucumber. Roots were healthy (no disease symptoms) in both soils.

Sampling of microcosms and monitoring of the inoculant

Destructive sampling was done at 2, 7, 14 and 22 d. The first 5 mm of soil in the pots was removed with a sterile spatula and discarded. Bulk soil was collected (5 g per pot). Plants were harvested and loosely-adhering soil was shaken off. Rhizosphere samples consisted of germinated seeds (at 2 d) or root systems (at subsequent

samplings) with closely-adhering soil. All samples were agitated in 1 ml (seeds) or 50 ml (roots and 5-g bulk soil samples) of sterile distilled water for 15 min at 300 rev min^{-1}. Dilution series of the extracts were prepared in sterile distilled water. At 22 d, the procedure was completed by a subsequent treatment of the roots with 10% hydrogen peroxide (15 s), which were then rinsed three times in 50 ml sterile distilled water. This treatment effectively disinfected the root surface, as indicated by the absence of colonies when the last water used to rinse the roots was afterwards spread plated on S1 agar (Gould et al. 1985). Surface-disinfected roots were ground aseptically in 10 ml of sterile distilled water, using a mortar and pestle, and cells present in root tissues were extracted by shaking the resulting samples (in 28-ml Universal bottles; 30 min at 350 rev min^{-1}; Troxler et al. 1997c).

Culturable cells of CHA0-Rif were enumerated by counting colonies on King's B agar containing Rif100, after having incubated the plates for 48 h at 27°C (two plates per dilution). No colony was found when samples from un-inoculated microcosms were plated (detection limits of 10^3 CFU g^{-1} soil, 4.6×10^3 CFU g^{-1} seed and 2.5×10^3 CFU g^{-1} root). Viable cells of CHA0-Rif were quantified using the DVC technique of Kogure et al. (1979), in which enlarged cells were counted by immunofluorescence microscopy, as described by Troxler et al. (1997b) and Hase et al. (1999). The primary antiserum used is specific for CHA0 (Troxler et al. 1997a) and no cross-reaction was found here when examining samples from un-inoculated microcosms. In brief, samples were incubated for 6 h in the presence of yeast extract (250 μg ml^{-1} sample) and nalidixic acid (20 μg ml^{-1} sample), and were fixed with formaldehyde (20 mg ml^{-1} sample). Nalidixic acid inhibits cell division in CHA0-Rif (Troxler et al. 1997b; Hase et al. 1999). The samples were passed through 0.2-μm pore size polycarbonate filters stained with Irgalan Black (Hobbie et al. 1977). The filters were treated successively with the primary antiserum (60 min) and the secondary antibody (fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins; 45 min) prior to treatment with 1,4-diazobicyclo-(2,2,2)-octan-glycerol mounting medium to prevent fading (Johnson et al. 1982). Cells of CHA0-Rif were counted using a Zeiss Axioskop epifluorescence microscope (filters 450-490 nm; at least 20 fields and/or 150 bacterial cells per sample). Elongated cells (length > 3 μm) were considered as viable cells. The same filters were used to count also the total number of cells of CHA0-Rif.

Effect of hydrogen peroxide on the inoculant

Since hydrogen peroxide was used to disinfect the surface of root samples, the effect of the compound on *P. fluorescens* CHA0-Rif was assessed under *in vitro* conditions. Cells of the inoculant were prepared, as described above (about 8×10^9 cells ml^{-1}), and exposed to hydrogen peroxide (0.1 to 10% H_2O_2) for 15 s. Total IF counts, DVCs and colony counts of the pseudomonad were determined, as described above.

Statistics

Each treatment was replicated three times in soil microcosms (i.e. three microcosms studied by destructive sampling at each sampling time) and when the strain was exposed to H_2O_2 *in vitro*. Cell counts were log-transformed. Data from soil microcosms were analysed at each sampling time, using two-factor (cell count method $\times$ soil type) analysis of variance, followed (when appropriate) with Fisher's LSD test. Similarly, data from the *in vitro* experiment were treated using a two-factor (cell count method $\times$ H_2O_2 concentration) analysis of variance and Fisher's LSD test. All statistical analyses were conducted at $P < 0.05$ using version 5.0 of SYSTAT for Windows (SYSTAT Inc., Evanston, IL).

Results and discussion

Survival and cell culturability of the inoculant in bulk soil

When Eschikon soil was used, cell numbers of *P. fluorescens* CHA0-Rif in bulk soil decreased from 8 to approximately 6.5 log cells g^{-1} soil in the 22-d experiment, regardless of the method used to count the cells (Fig. 1A). This result is in accordance with previous findings (Hase et al. 1999). In contrast, total IF counts of CHA0-Rif in bulk Siglistorf soil remained at levels of 7.6 log cells g^{-1} soil (or higher), and they exceeded the other cell counts throughout the experiment (by more than 1 log unit at 22

d; Fig. 1A). DVCs and colony counts of the pseudomonad were essentially similar at each sampling in this soil.

Despite not responding to Kogure's viability test, the non-culturable cells found in bulk Siglistorf soil were probably not dead, as most heat-killed bacterial cells tend to disappear rapidly once added to non-sterile soil (Cleyet-Marel and Crozat 1982; Turpin et al. 1993). Non-culturable cells of *P. fluorescens* R2f Rp^r occurred in Ede loamy sand, but not in Flevo silt loam (van Overbeek et al. 1995). As in the latter study, several characteristics differed between the two soils in the current investigation (i.e. texture, pH, etc.), and further work will be necessary to identify soil properties responsible for the different behaviours of CHA0-Rif. For instance, bulk soil density at 22 d was higher for Eschikon soil (i.e. 0.91) than for Siglistorf soil (i.e. 0.49), which perhaps influenced strain survival. Viable but non-culturable (VBNC) cells of the inoculant (i.e. cells that did not form a colony on the plate but responded positively to a viability test) were not found in bulk soil here, but have been reported in non-rhizosphere soil (i) for other bacteria including a pseudomonad (Binnerup et al. 1993) or (ii) when CHA0-Rif was exposed to abiotic stress in the surface horizon of lysimeters (Troxler et al. 1997b).

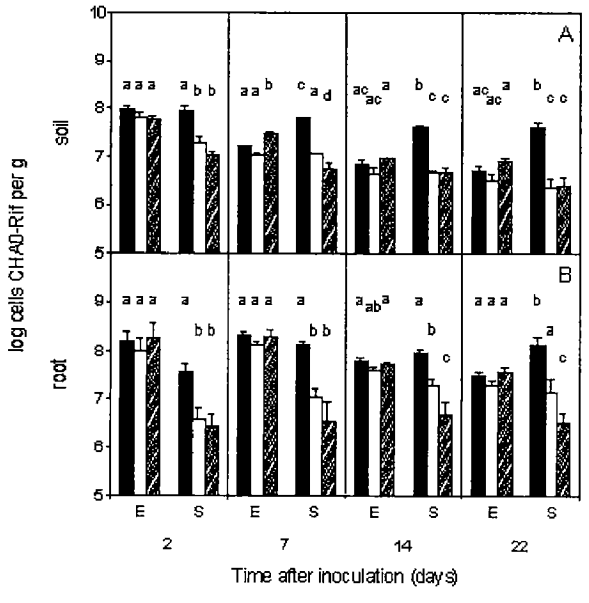

Fig. 1. Influence of soil type on the survival of *P. fluorescens* CHA0-Rif in bulk soil (A) and in the rhizosphere of cucumber (B). In both soils, the inoculant was added at 8.1 log cells g^{-1}. The strain was monitored by total IF counts (black bars), DVCs (white bars) and CFUs (hatched bars). Error bars represent standard errors. At each sampling time, data were analysed separately in A and in B, and the statistical relationship between the six treatments is indicated with letters a, b and c.

Survival and cell culturability of the inoculant in the rhizosphere

In the rhizosphere of cucumber grown in Eschikon soil, CHA0-Rif was recovered at levels comprised between 7.3 and 8.3 log cells g^{-1} root during the 22-d experiment, and no difference was found at any of the samplings between total IF counts, DVCs and CFUs of the strain (Fig. 1B). Likewise, total IF counts, DVCs and CFUs of *Flavobacterium* sp. P25 were similar in the rhizosphere of wheat in Ede loamy sand (Heijnen et al. 1995). In Siglistorf soil however, total IF counts of CHA0-Rif were higher than DVCs and CFUs throughout the experiment (as in bulk soil), and DVCs exceeded CFUs at the last two samplings. Total IF counts were lower at 2 d than at subsequent samplings. This could be due to the fact that germination was slower in that soil, with the likely consequences that exudation was less and that the amount of soil adhering to the plant at 2 d was less than that at the following samplings.

The occurrence of VBNC cells of CHA0-Rif in the rhizosphere but not in bulk soil, in Siglistorf microcosms, suggests that the presence of the root favoured maintenance of substrate-responsiveness in the subpopulation of cells undergoing a loss in culturability. An alternative hypothesis is that the root itself could be involved in the loss of colony-forming ability, resulting in the formation of VBNC cells. Indeed, unfavourable growth conditions for cucumber, linked to the physico-chemical properties of this soil (Papadopoulos 1994), resulted probably in altered patterns of root exudation compared with those in Eschikon soil. How this could have resulted in the occurrence of VBNC cells of CHA0-Rif in the rhizosphere in Siglistorf microcosms remains to be ascertained.

Survival and cell culturability of the inoculant inside the root

Strain CHA0-Rif can behave as a root endophyte (Troxler et al. 1997a), and VBNC cells of the strain were found inside roots of field-grown maize at harvest time (Troxler et al. 1997c). In the current work, total IF counts and DVCs of the strain exceeded CFUs in root tissues when Eschikon soil was used (Fig. 2). Thus, VBNC cells of CHA0-Rif occurred only inside the root in Eschikon microcosms. In contrast, total IF counts were higher than both DVCs and CFUs in root tissues of cucumber grown in

Siglistorf soil (Fig. 2). In the latter microcosms, VBNC cells were not found inside the root despite occurring in the rhizosphere.

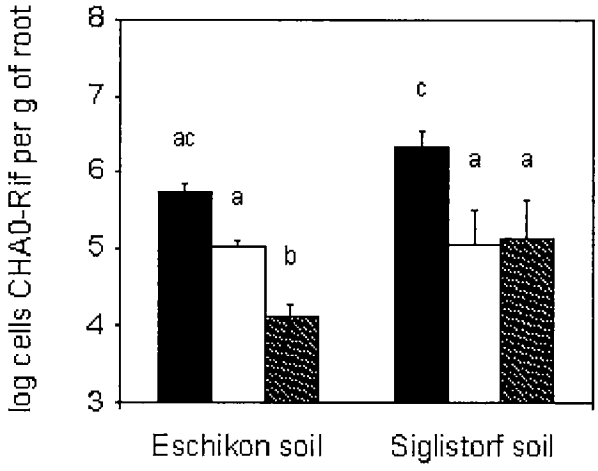

Fig. 2. Influence of soil type on cell numbers of P. fluorescens CHA0-Rif inside root tissues at 22 d after sowing. The strain was monitored by total IF counts (black bars), DVCs (white bars) and CFUs (hatched bars). Error bars represent standard errors. The statistical relationship between the six treatments is indicated with letters a, b and c.

H_2O_2, which was used to surface-disinfect root samples, had no effect on cell numbers of CHA0-Rif when the strain was exposed to 0.1% or 1% H_2O_2 *in vitro*, and cells remained culturable (Fig. 3). After a 15 s incubation of CHA0-Rif in 10% H_2O_2 however, total IF counts were still at inoculation level, whereas DVCs and CFUs were below detection limit (2.0 log cells ml^{-1} for both). The actual concentration of H_2O_2 inside the root during surface disinfection of samples was unlikely to reach this level, but nonetheless this observation raises the possibility that, in this and other studies (Troxler et al. 1997c), certain bacterial cells present in the root cortex can be exposed to H_2O_2 levels sufficiently high to result in a loss of colony-forming ability. In the current work however, the H_2O_2 treatment of samples was unlikely to explain the occurrence of VBNC cells of CHA0-Rif inside roots grown in Eschikon soil, as exposure of the strain to H_2O_2 *in vitro* did not result in VBNC cells.

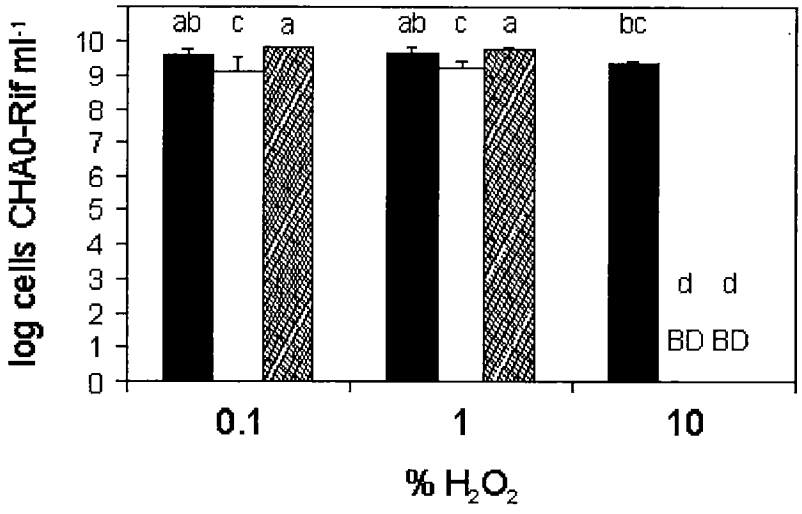

Fig. 3. Effect of H_2O_2 on cell numbers of *P. fluorescens* CHA0-Rif *in vitro*. The strain was added at 9.9 log cells ml^{-1} and monitored by total IF counts (black bars), DVCs (white bars) and CFUs (hatched bars) 15 s after exposure to H_2O_2. Colony counts on KBA (data not shown) and KBA + Rif100 were identical. Error bars represent standard errors. Statistics were done to compare the nine treatments (an arbitrary log value of zero was used when counts were below detection limit), and significant differences between them are indicated with a, b, c and d. BD, below detection limit (2.0 log cells ml^{-1}).

Several rifampicin-resistant pseudomonads recovered from root tissues of cucumber failed to grow on medium containing rifampicin, but could do so once previously cultured on rifampicin-free medium (i.e. rifampicin-resistance masking; McInroy et al. 1996). Here, colony counts of CHA0-Rif on KBA and KBA + Rif100 were identical when studying root tissue samples obtained from cucumber grown in sterile Eschikon soil (data not shown), indicating that the presence of rifampicin in plates could not account for the non-culturability of the VBNC cells mentioned above (Fig. 2). The fact that DVCs and CFUs of CHA0-Rif inside root tissues were statistically identical in the case of Siglistorf soil is further evidence that this pseudomonad does not display masking of rifampicin-resistance.

Conclusion

In conclusion, when Eschikon soil was used, *P. fluorescens* CHA0-Rif persisted as culturable cells in bulk soil and in the rhizosphere of cucumber, but VBNC cells of the strain were found inside root tissues. In contrast, non-culturable cells of CHA0-Rif were identified in bulk soil, in the rhizosphere and inside root tissues of cucumber when Siglistorf soil was used, and VBNC cells of the inoculant were found in the rhizosphere at the last two samplings. Therefore, the occurrence of non-culturable cells of CHA0-Rif was strongly influenced by the soil type, even in root-associated habitats (i.e. rhizosphere and root tissues), and VBNC cells were found only in the latter habitats.

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