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Biocontrol strain *Pseudomonas fluorescens* CHA0 and its genetically modified derivative with enhanced biocontrol capability exert comparable effects on the structure of a *Sinorhizobium meliloti* population in gnotobiotic systems

Abstract The impact of biocontrol strain *Pseudomonas fluorescens* CHA0 and of its genetically modified, antibiotic-overproducing derivative CHA0/pME3424 on a reconstructed population of the plant-beneficial *Sinorhizobium meliloti* bacteria was assessed in gnotobiotic systems. In sterile soil, the final density of the reconstructed *S. meliloti* population decreased by more than one order of magnitude in the presence of either of the *Pseudomonas* strains when compared to a control without addition of *P. fluorescens*. Moreover, there was a change in the proportion of each individual *S. meliloti* strain within the population. Plant tests also revealed changes in the nodulating *S. meliloti* population in the presence of strains CHA0 or CHA0/pME3424. In both treatments one *S. meliloti* strain, f43, was significantly reduced in its root nodule occupancy. Analysis of alfalfa yields showed a slight but statistically significant increase in shoot dry weight when strain CHA0 was added to the reconstructed *S. meliloti* population whereas no such effect was observed with CHA0/pME3424.

Key words Ecological impact · Genetically modified organisms · Microcosm studies · Symbiotic nitrogen fixation · Plant-beneficial bacteria · *Sinorhizobium meliloti*

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Introduction

Certain strains of fluorescent pseudomonads are able to suppress a variety of plant diseases caused by soil-borne pathogens, and, hence, are of considerable agricultural value (Kloepper 1993). For instance, *Pseudomonas fluorescens* strain CHA0 effectively protects wheat from *Gaeumannomyces graminis* var. *tritici* in greenhouse and field experiments and is active against a variety of other root diseases (Défago et al. 1990). The strain produces several secondary metabolites with antimicrobial activity such as hydrogen cyanide, 2,4-diacetylphloroglucinol (Phl) and pyoluteorin (Plt). Analyses of mutant strains affected in the synthesis of antibiotics established the relevance of antibiotic production for biocontrol ability of *P. fluorescens* CHA0 (Keel et al. 1992). A derivative of *P. fluorescens* CHA0 carrying chromosomal CHA0-DNA cloned in cosmid pME3090 was shown to overproduce the antibiotics Phl and Plt and also to display enhanced biocontrol capability in certain host-pathogen systems (Maurhofer et al. 1992, 1995). Subsequent studies established that the enhanced biocontrol ability conferred by plasmid pME3424, a subclone of pME3090, was due to the amplification of the *rpoD* gene, which encodes the housekeeping sigma factor (σ^{70}) of *P. fluorescens* (Schnider et al. 1995).

Based on the increasing knowledge about the molecular mechanisms that underlie antibiotic production and thus biocontrol, the use of biocontrol agents improved by genetic engineering in agriculture seems feasible. The deliberate release of genetically modified organisms (GMOs), however, requires a thorough analysis of their potential ecological impact (Smit et al. 1992). Laboratory-based microcosm experiments have to be conducted prior to the field release of GMOs. As part of these analyses the issue of whether GMOs could affect non-target organisms needs to be addressed. One group of soil microorganisms which is of importance for soil fertility are bacteria of the genera *Bradyrhizobium* or *Sinorhizobium*, which fix atmospheric nitrogen in symbiosis with important leguminous crop plants such as soybean or alfalfa (Long 1989). Hence, it is

mandatory to evaluate whether GMOs might exert a negative influence on these plant-beneficial soil bacteria.

In this work, the impact of *P. fluorescens* strain CHA0 and of its genetically modified, antibiotic-overproducing derivative CHA0/pME3424 on plant-beneficial *S. meliloti* bacteria was assessed using gnotobiotic systems. Three *S. meliloti* field isolates which differed in their sensitivity to Plt were chosen to represent a reconstructed *S. meliloti* population. Impact analyses were performed at the subpopulation level to monitor changes in a vegetatively growing as well as an alfalfa-nodulating *S. meliloti* population. In addition, the influence of biocontrol strains on alfalfa plant yield was assessed.

Materials and methods

Bacterial strains, media and growth conditions

Wild-type strains f23, f43 and f62 are members of a natural *S. meliloti* population sampled from root nodules of alfalfa plants grown on a mining recultivation field near Cologne (Kosier et al. 1993). Field isolate f23 belonged to a major infective group, whereas strains f43 and f62 belonged to minor infective groups of this population. Strains f231, f431 and f621 were derivatives of wild-type strains f23, f43 and f62 with a spontaneous resistance to streptomycin (Sm^r), spectinomycin (Sp^r) or neomycin (Nm^r), respectively. Antibiotic-resistant derivatives were obtained by plating 500 µl late log-phase cultures on LB plates supplemented with the appropriate antibiotics. Spontaneous antibiotic-resistant mutants were purified by three single-colony passages on selective media. All experiments in this study were conducted with variants of biocontrol strain *P. fluorescens* CHA0 and its antibiotic-overproducing derivative CHA0/pME3424 (Tc^r), which are characterized by a spontaneous resistance to rifampicin (Natsch et al. 1994; Schnider et al. 1995). *S. meliloti* and *P. fluorescens* strains were grown at 30°C on LB medium (Miller 1972). For solid media 15 g agar l^{-1} medium was added. The final concentrations of antibiotics per litre medium were 500 mg streptomycin, 200 mg spectinomycin and 100 mg neomycin for *S. meliloti* and 100 mg rifampicin and 100 mg tetracycline for *P. fluorescens.* Minimal inhibitory concentration (MIC) tests to assess the sensitivity of *S. meliloti* strains to Phl and Plt were carried out as described by Keel et al. (1992) except that LB medium was used instead of nutrient yeast broth.

Polymerase chain reaction fingerprint analysis

Sinorhizobium meliloti strains were typed with the help of the ERIC polymerase chain reaction (PCR) fingerprinting method (De Bruijn 1992) using only primer ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) in the PCR reaction. The PCR primer was synthesized in a Gene Assembler Special (Pharmacia). The DNA template was obtained from a lysate of a single colony by heating the cells in a lysis buffer [0.25% sodium dodecyl sulphate (SDS)/0.05 *M* NaOH] at 95°C for 15 min. One microlitre of a 1:10 dilution of the resulting lysate was used per PCR reaction. Eight microlitres of each PCR probe was separated in horizontal 2% agarose gels in TRIS-acetate buffer for 150 min at 70 V. The gels were stained with ethidium bromide and photographed on a UV transilluminator (312 nm) using a charge coupled device (CCD) camera (Cybertech, Germany).

Microcosm studies

Sterile soil microcosms

Microcosm studies to assess the impact of *P. fluorescens* CHA0 and CHA0/pME3424 on vegetatively growing *S. meliloti* strains were performed according to Selbitschka et al. (1995) using the small microcosm system described by Henschke and Schmidt (1989). In these studies antibiotic-resistant derivatives of field strains were used. In order to ensure that the spontaneous mutations did not affect the strains' growth characteristics, the resistant strains were tested in competition experiments against their parental strains in LB medium and sterile soil microcosms. No differences between wild-type isolates and their spontaneous antibiotic-resistant derivatives could be observed, indicating that the mutations did not affect the competitive growth abilities of resistant strains (data not shown). Antibiotic-resistant derivatives of field strains were grown overnight in LB medium and bacterial cell densities were determined by measuring the optical densities (OD_{580}) using a Novaspec II photometer (Pharmacia). Bacterial cells were washed twice with sterile bidistilled water and microcosms were inoculated at a cell density of approximately 10^5 CFU g^{-1} soil. The number of cultivable cells of the individual strains was determined by plating on LB plates supplemented with the proper antibiotics. Microcosm experiments were run in triplicate.

Sterile vermiculite microcosms

For evaluation of the impact of *P. fluorescens* CHA0 and CHA0/pME3424 on alfalfa nodulation by a reconstructed *S. meliloti* population, a newly developed larger microcosm system was used. The microcosm consisted of an open glass column with a length of 20 cm and a diameter of 3 cm. A fritted glass filter served as a bottom to fix the vermiculite in the microcosm. In order to achieve a constant water supply, a cotton thread which was passed through a hole in the middle of the glass filter connected the vermiculite with a water reservoir. Vermiculite was washed 3 times in distilled water and dried for 24 h at 160°C. Four grams vermiculite (dry weight) was used per microcosm. One millilitre plant nodulation medium (Rolfe et al. 1980) was added per microcosm and the assembled microcosms were autoclaved for 20 min at 121°C. After inoculation, two *Medicago sativa* seeds which had been surface sterilized according to Kosier et al. (1993) were added per microcosm. Microcosms were incubated in a growth chamber [70% relative humidity with 16 h light (20°C) followed by an 8-h dark (18°C) period]. After 8 weeks of plant cultivation all root nodules were harvested, crushed and the bacteria reisolated as described previously (Kosier et al. 1993). The reisolated bacterial strains were identified using the ERIC PCR fingerprinting method (see above). For determination of shoot dry weights, shoots of plants derived from 12 microcosms (24 plants; control) or 18 microcosms (36 plants; *P. fluorescens* CHA0 or CHA0/pME3424, respectively) were dried for 6 days at 37°C and subsequently weighed.

Statistical analysis of data

Statistical analyses of experimental data were performed using the *F*-test and Student's *t*-test. Significance was determined at the $P \leq 0.05$ level.

Results and discussion

Pseudomonas fluorescens strains CHA0 and CHA0/pME3424 influence the structure of a reconstructed *S. meliloti* population in a sterile soil microcosm

In order to test whether biocontrol strain *P. fluorescens* CHA0 or its genetically modified derivative CHA0/pME3424 affected the structure of a reconstructed *S. meliloti* population, growth of *S. meliloti* strains in sterile soil was monitored. Previous studies had revealed that various Gram-negative and Gram-positive soil bacteria displayed sensitivity to the *P. fluorescens* antibiotic Phl in an in vitro

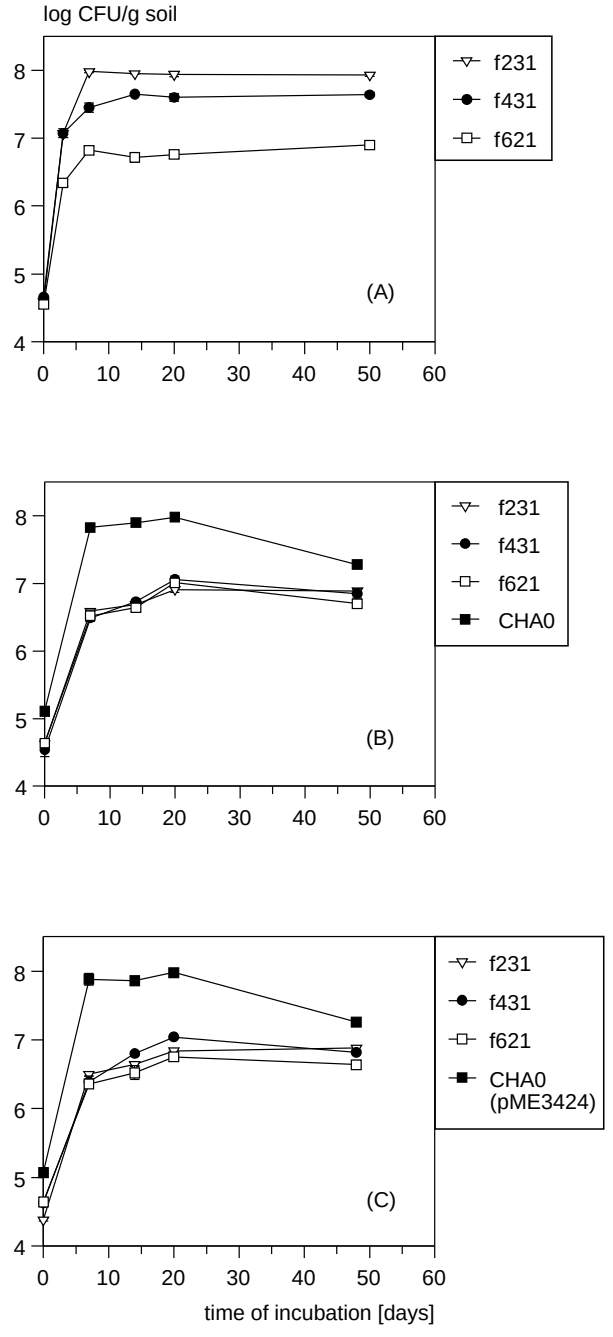

Fig. 1A–C Growth competition among *S. meliloti* strains f231, f431 and f621 as determined by selective plate counts without (**A**), and with coinoculation of *P. fluorescens* CHA0 (**B**) or its antibiotic-overproducing derivative CHA0/pME3424 (**C**) in sterile soil microcosms. Strains were always coinoculated in equal quantities. Data are the mean of three independent experiments ± standard deviations (*bars*)

test (Keel et al. 1992). Correspondingly, *S. meliloti* strains f23, f43 and f62 responded differently to the antibiotics Phl and Plt. Whereas all three strains showed a comparable insensitivity to high concentrations of Phl (>1000 μg^{-1} ml), the sensitivity to Plt differed among strains. The MIC values of strains f23, f62 and f43 were 50, 10 and 5 μg^{-1} ml, respectively.

Sinorhizobium meliloti strains f231 (Sm^r), f431 (Sp^r) and f621 (Nm^r) were coinoculated with *Pseudomonas* biocontrol agents in a 1:1:1:1 ratio in sterile soil microcosms. An experiment where *S. meliloti* strains were inoculated in a 1:1:1 ratio served as the control. In the control experiment strain f231 multiplied to the highest cell density of approximately. 9×10^7 CFU g^{-1} soil within 50 days of incubation and hence displayed the best growth competitiveness of the strains tested. Strain f431 reached a slightly lower cell density of approximately 5×10^7 CFU g^{-1} whereas strain f621 reached a significantly lower ($P \leq 0.05$) cell density of approximately 8×10^6 CFU g^{-1} soil (Fig. 1). The *S. meliloti* population reached its highest total count of 1.48×10^8 CFU g^{-1} soil 10 days after microcosm inoculation and remained essentially constant during the rest of the experiment.

In microcosms where *P. fluorescens* wild-type strain CHA0 or its genetically engineered derivative CHA0/pME3424 were included, each *S. meliloti* strain reached a comparable density of approximately 3×10^6 CFU g^{-1} soil (Fig. 1). Thus, in the presence of *P. fluorescens* differences in the growth competitive abilities of strains diminished. Moreover, the final size of the *S. meliloti* population was more than one order of magnitude below that of the control experiment (1×10^7 vs 1.48×10^8 CFU g^{-1} soil). This result might be explained by the faster growth of *P. fluorescens* compared to the *S. meliloti* strains and the concomitant depletion of nutrients.

The enhanced biocontrol ability of strain CHA0/pME3424 is a plasmid-borne trait. In order to exclude the possibility that the identical effects exerted by both *P. fluorescens* strains were due to plasmid instability, reisolates were tested for their resistance to tetracycline. Approximately 70% of the CHA0 cells still harboured plasmid pME3424, and hence the comparable effects exerted by both strains on the reconstructed *S. meliloti* population were very probably not attributable to instability of plasmid pME3424.

Pseudomonas fluorescens strains CHA0 and CHA0/pME3424 influence *S. meliloti* strains' root nodule occupancy in a gnotobiotic system

Since spontaneous antibiotic resistance mutations might affect the strains' symbiotic properties (e.g. Bromfield et al. 1985), wild-type strains f23, f43 and f62 were used to assess the impact of *P. fluorescens* strains CHA0 and CHA0/pME3424 on the alfalfa-nodulating *S. meliloti* population. For this purpose each *Pseudomonas* strain was inoculated in a 1:1:1:1 ratio with wild-type strains f23, f43 and f62 in sterile microcosms containing vermiculite and two *M. sativa* plants. Initially, sterile soil was used but since nodulation of *M. sativa* plants was virtually abolished, vermiculite was used as the carrier material. Microcosms containing the three field isolates inoculated in a 1:1:1 ratio served as a control.

In the control experiment, strain f23 occupied approximately 55% of nodules, whereas strains f62 and f43 occu-

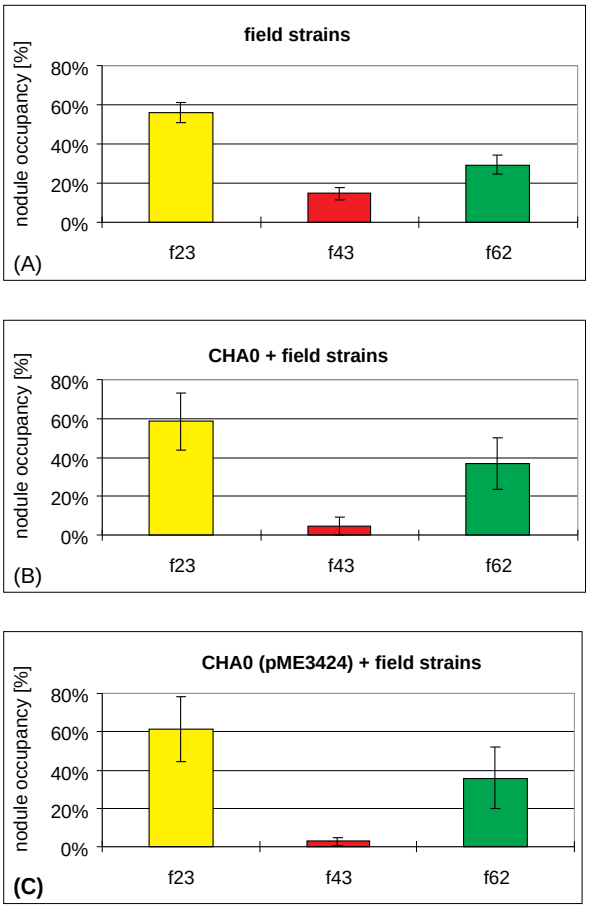

Fig. 2A–C Root nodule occupancy of *S. meliloti* strains f23, f43 and f62 as determined by ERIC PCR fingerprinting of strains reisolated from alfalfa plants grown in a gnotobiotic system without (**A**) and with coinoculation of *P. fluorescens* CHA0 (**B**) or its antibiotic-overproducing derivative CHA0/pME3424 (**C**), respectively. Strains were always coinoculated in equal quantities. Data are the mean of 4 independent experiments with 6 microcosms and 12 plants (94 nodules were tested; **A**), 3 independent experiments with 6 microcosms and 12 plants (71 nodules were tested; **B**) or 3 independent experiments with 6 microcosms and 12 plants (74 nodules were tested; **C**) ± standard deviations (*bars*)

pied only approximately 30% and 15% of root nodules, respectively (Fig. 2). The proportion of the individual strains to total root nodules induced seems to reflect their status in the natural *S. meliloti* population sampled from alfalfa nodules (Kosier et al. 1993). It is worth noting that strain f23, which belonged to a major infective group of this field population, also displayed a superior nodulation competitiveness in these microcosm experiments.

In microcosms where wild-type strain CHA0 or its antibiotic-overproducing derivative CHA0/pME3424 were included, root nodule occupancy of strains f23 and f62 slightly increased whereas strain f43 occupied significantly fewer root nodules. In both treatments strain f43 occupied only approximately 4% (CHA0) and 2.5% (CHA0/pME3424) of nodules, respectively. It remains to be determined whether strain's f43 decrease in nodule occupancy is correlated with its sensitivity to the antibiotic Plt. The potential of strain CHA0 to synthesize antimicrobial metabolites in situ i.e., in the rhizosphere, has been demonstrated for Plt and Phl (Keel et al. 1992; Maurhofer et al. 1995).

Both *P. fluorescens* strains multiplied to a density of approximately 7×10^7 CFU g^{-1} vermiculite. Again more than 70% of CHA0 cells harboured pME3424 as indicated by the presence of the tetracycline resistance marker. Similar results on the stability of plasmid pME3090 in strain CHA0 grown in plant rhizospheres have been reported previously [Maurhofer et al. 1992, 1995; plasmids pME3090 and pME3424 are both derivatives of the same basic replicon pVK100 (Schnider et al. 1995)].

Analyses to assess the influence of biocontrol strains on alfalfa shoot dry weight revealed that plant yield was not affected by coinoculation of the reconstructed rhizobial population with genetically modified strain *P. fluorescens* CHA0/pME3424 compared to the control without *P. fluorescens* (200 ± 43 mg vs 206 ± 24 mg). In contrast, addition of wild-type strain CHA0 significantly ($P\leq0.05$) improved shoot dry weight of alfalfa plants (237 ± 37 mg vs 206 ± 24 mg). Stimulation of plant growth by coinoculation of alfalfa with *S. meliloti* and *P. fluorescens* has been reported previously (Liste 1993). Conversely, it has been shown that strain CHA0/pME3424 in some cases may exert a negative influence on the growth of certain plant species (Maurhofer et al. 1992, 1995). Thus, the absence of a plant growth stimulating effect by CHA0/pME3424 might be attributable to such reasons.

In summary, our results show that no GMO-specific effect of *P. fluorescens* CHA0/pME3424 displaying enhanced biocontrol capability on the structure of a growing or alfalfa-nodulating *S. meliloti* population occurred. Moreover, no negative effect of CHA0/pME3424 on alfalfa yield was observed.

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References

Bromfield ESP, Lewis DM, Barran LR (1985) Cryptic plasmid and rifampin resistance in *Rhizobium meliloti* influencing nodulation competitiveness. J Bacteriol 164:410–413

De Bruijn F (1992) Use of repetitive (Repetitive Extragenic Palindromic and Enterobacterial Repetitive Intergeneric Consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl Environ Microbiol 58:2180–2187

Défago G, Berling C-H, Burger U, Haas D, Kahr G, Keel C, Voisard C, Wirthner P, Wüthrich B (1990) Suppression of black root rot of tobacco and other diseases by strains of *Pseudomonas fluorescens*: Potential applications and mechanisms. In: Hornby D, Cook RJ, Henis Y, Ko WH, Rovira AD, Schippers B, Scott PR (eds) Biological control of soil-borne plant pathogens. CAB International, Oxon, UK, pp 93–108

Henschke RB, Schmidt FRJ (1989) Survival, distribution, and gene transfer of bacteria in a compact soil microcosm system. Biol Fertil Soils 8:19–24

Keel C, Schnider U, Maurhofer M, Voisard C, Laville J, Burger U, Wirthner P, Haas D, Défago G (1992) Suppression of root diseases by *Pseudomonas fluorescens* CHA0: importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. Mol Plant-Microbe Interact 5:4–13

Kloepper JW (1993) Plant-growth promoting rhizobacteria as biological control agents. In: Metting FB Jr (ed) Soil microbial ecology. Dekker, New York, pp 255–274

Kosier B, Pühler A, Simon R (1993) Monitoring the diversity of *Rhizobium meliloti* field and microcosm isolates with a novel rapid genotyping method using insertion elements. Mol Ecol 2:35–46

Liste HH (1993) Stimulation of symbiosis and growth of lucerne by combined inoculation with *Rhizobium meliloti* and *Pseudomonas fluorescens.* Zentralbl Mikrobiol 148:163–176

Long SR (1989) *Rhizobium* genetics. Annu Rev Genet 23:483–506

Maurhofer M, Keel C, Schnider U, Voisard C, Haas D, Défago G (1992) Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHA0 on its disease suppressive capacity. Phytopathology 82:190–195

Maurhofer M, Keel C, Haas D, Défago G (1995) Influence of plant species on disease suppression by *Pseudomonas fluorescens* strain CHA0 with enhanced antibiotic production. Plant Pathol 44:40–50

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, New York, 466 pp

Natsch A, Keel C, Pfirter HA, Haas D, Défago G (1994) Contribution of the global regulator gene *gacA* to persistence and dissemination of *Pseudomonas fluorescens* biocontrol strain CHA0 introduced into soil microcosms. Appl Environ Microbiol 60:2553–2560

Rolfe BG, Gresshoff PM, Shine J (1980) Rapid screening for symbiotic mutants of *Rhizobium* and white clover. Plant Sci Lett 19:277–284

Schnider U, Keel C, Blumer C, Troxler J, Défago G, Haas D (1995) Amplification of the housekeeping sigma factor in *Pseudomonas fluorescens* CHA0 enhances antibiotic production and improves biocontrol abilities. J Bacteriol 177:5387–5392

Selbitschka W, Dresing U, Hagen M, Niemann S, Pühler A (1995) A biological containment system for *Rhizobium meliloti* based on the use of recombination-deficient (*recA*-) strains. FEMS Microbiol Ecol 16:223–232

Smit E, van Elsas JD, van Veen JA (1992) Risks associated with the application of genetically modified microorganisms in terrestrial ecosystems. FEMS Microbiol Rev 88:263–278