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Effects of temperature on detection of plasmid or chromosomally encoded *gfp*- and *lux*-labeled *Pseudomonas fluorescens* in soil

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Pseudomonas fluorescens is a normal inhabitant of the soil rhizosphere. The use of genetically altered strains of *P. fluorescens* in bioremediation has led to the need for effective monitoring of such cells released into the environment. In this study, we present data on the persistence in soil of *P. fluorescens* harboring *gfp* (green fluorescent protein) or *lux* (bioluminescence) genes. Comparisons were made between strains marked chromosomally and strains carrying these markers on a plasmid. Overall effects of plasmid carriage on culturability were also examined. Sterile soil microcosms were inoculated with washed cells to a final concentration of *ca.* 10^6 CFU.g^{-1} and placed at 5, 23, and 35–37 °C. Samples were taken periodically and examined for culturability and viability, using the substrate responsiveness assay. Our results indicated no significant loss of culturability at 5 and 23 °C for a period of over one year. In contrast, cells of *P. fluorescens* incubated at 35–37 °C entered the viable but nonculturable state within 7 days. All cells labeled with *gfp* retained fluorescence regardless of culturability, suggesting that the green fluorescent protein can be of value in monitoring the presence of cells following their release to the environment. Because fluorescence was maintained regardless of the cells' physiological state, this protein may also be an indicator of cell viability.

Keywords: GFP / *Pseudomonas fluorescens* / viable but nonculturable (VBNC) / starvation

INTRODUCTION

Many strains of *P. fluorescens* have been shown to degrade a variety of organic compounds, and thus to be important in bioremediation. Further, strains have been genetically modified that could potentially have widespread use in the breakdown of xenobiotics, biomass conversion, and waste treatment. Research on the survival and ecological effects of genetically engineered microorganisms (GEMs) released into the field is a vital issue due to the uncertainty of their spatial distribution, the possibilities of gene transfer to indigenous organisms, and the potential disruption of the balance of the environment into which they are introduced.

Several methods have been proposed for the monitoring of GEMs. These include culturing on selective media, the use of monoclonal antibodies, genetic analysis, and the introduction of marker or reporter genes (Jansson, 1998; 1999). Such methods are time consuming, require costly equipment, or involve the addition of a substrate in order to detect the organisms (Lindemann and Suslow, 1987). While each method can be effective in detecting the presence of specific microorganisms, they may over- or underestimate the number of viable GEMs. In addition, the heterogeneity of a soil environment may interfere with the assessment of the survival of GEMs and the effects of competition with indigenous organism.

As a further complication, over 30 genera of bacteria, including several *Pseudomonas* species, have been shown to enter a viable but nonculturable (VBNC) state as a result of environmental stress, *e.g.* nutrient deprivation or detrimental temperature or osmotic levels (Oliver, 1993). Bacteria in this dormant state are unable to be cultured on routine laboratory media, but maintain viability as evidenced by various direct microscopic assays such as the direct viable count (DVC) method of Kogure et al. (1979). Such cells continue gene expression (Lleò et al., 2000; Saux et al., 2002; Yaron and Matthews, 2002) and

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following appropriate treatment, are capable of again becoming culturable (Oliver, 1993; 2000b). The VBNC state has been observed in bacteria in aquatic, terrestrial, and epiphytic environments and tracking of GEMs may be adversely affected by their entry into this dormant state (Oliver, 2000a).

The use of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, and *lux* genes from *Vibrio fischeri,* has become common in the study of eukaryotic and prokaryotic cells in a variety of environments (Amin-Hanjani et al., 1993; Bloemberg et al., 1997; Chalfie et al., 1994; Cho and Kim, 1999; de Weger et al., 1991; Ducan et al., 1994; Grant et al., 1992; Leff and Leff, 1996; Meikle et al., 1992; Normander et al., 1999; Unge et al., 1997; 1999). The GFP protein is stable, requires no additional substrate, and is expressed constitutively with little or no stress to the organism (Bloemberg et al., 1997; Bokman and Ward, 1981; Chalfie et al., 1994). We have found that the introduction of *gfp* into bacteria in aquatic environments has value both as a marker and as an indicator of cell activity and viability (Lowder and Oliver, 2001; Lowder et al., 2000). Thus, using *gfp*-labeled microorganisms could be of value in tracking cells released to soil environments, even when the cells are present in the VBNC state. Similarly, *P. fluorescens* cells marked with bioluminescence genes have been shown to be detectable in rhizosphere soils (de Weger et al., 1991; Meikle et al., 1992), although loss of luminescence appears to be fairly rapid during stress (Ducan et al., 1994; Meikle et al., 1992).

This study examined the persistence of *gfp*- and *lux*-labeled *P. fluorescens* cells in soil microcosms at various temperatures. Temperatures were those that bacteria would be expected to encounter in the environment, including during winter (5 °C), summer (35–37 °C), and moderate climatic months (23 °C). Comparisons were made between parent cells and those where the marker genes were on the chromosome or on a plasmid.

RESULTS

Growth curves of parent and marked strains

With the exception of *P. fluorescens* 10586 pSMC21, all strains exhibited almost identical growth curves, regardless of the presence of *lux* or *gfp* genes (Fig. 1). This was true whether the cells were grown with or without kanamycin (data not shown). In contrast, *P. fluorescens* 10586 pSMC21 demonstrated a significantly reduced

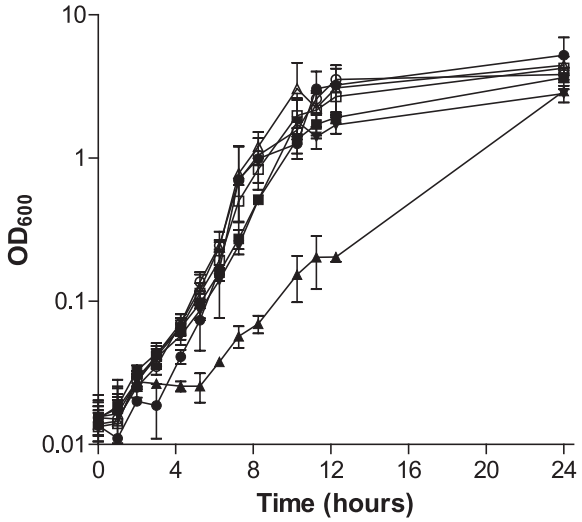

Figure 1. Growth curves of *P. fluorescens* strains grown in LB broth at 23 °C. Shown are A506 (○), A506::gfp2 (□), A506 pSMC21 (△), 10586 (●), 10586 pFAC510 (▼), 10586 pSMC21 (▲), and 10586::pFAC510 (■).

generation time over the first 12 hours, although by 24 hours the cell density of this strain was not markedly different than the other strains examined. Interestingly, the presence of this same plasmid in *P. fluorescens* A506 did not result in such a metabolic load (Fig. 1). The reason for this difference is not known, but could result from a difference in copy number (high for strain 10586 pSMC21 and low for A506 pSMC21). The growth rate difference observed, however, did not appear to have any significant effect on the rate at which these cells entered the VBNC state at elevated temperature (see below).

Survival of *gfp*- and *lux*-marked *P. fluorescens* cells in soil

Cells of *P. fluorescens* A506 maintained high levels of culturability in soil when incubated at 5 or 23 °C (Fig. 2A). Cells in which the chromosome contained two copies of *gfp* also remained fully culturable at these temperatures (Fig. 2B), suggesting that the presence of *gfp* had no effect on survival in soil. Long-term (1 year) culturability studies (Fig. 3) indicated that these strains were capable of prolonged survival, although cells at the lower (5 °C) temperature remained at a somewhat higher level. As evidenced during direct microscopic examination, GFP fluorescence was maintained even after this long-term incubation in soil. Cells of *P. fluorescens* 10586 responded

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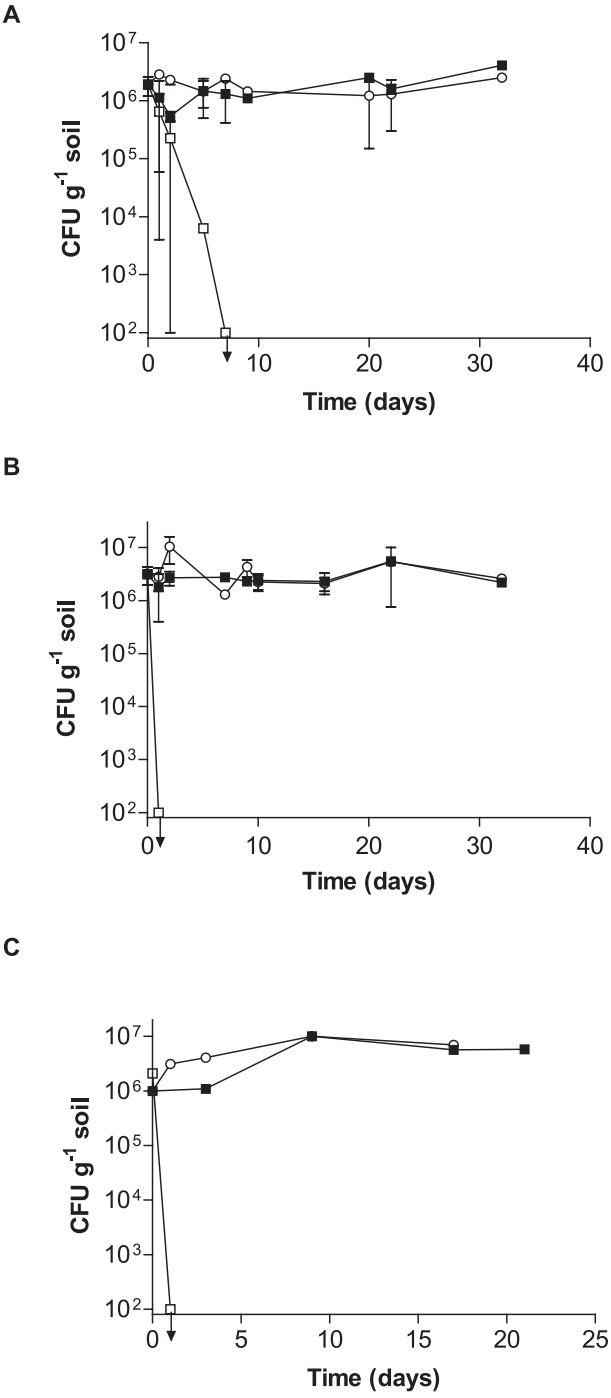

Figure 2. Culturability in soil of *P. fluorescens* A506 at 5 °C (■), 23 °C (○), and 37 °C (□). (A) Parent strain A506. (B) Strain A506::*gfp*2. (C) Strain A506 pSMC21(*gfp*). Except for (C), which is the result of a single study but showed the same pattern as A506 and A506:*gfp2*, error bars represent standard deviation of two experiments. Down arrows indicate levels of culturability below detection.

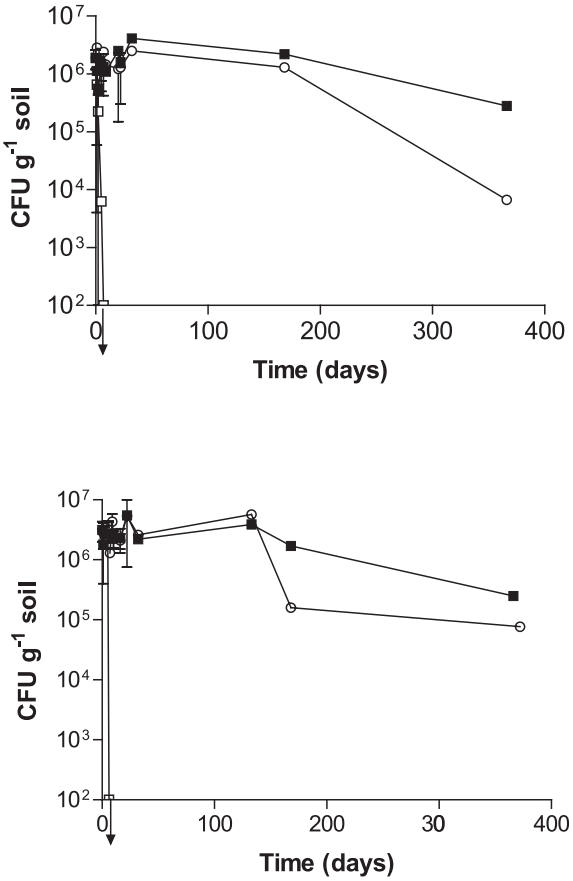

Figure 3. Long-term culturability in soil of *P. fluorescens* at 5 °C (■), 23 °C (○), and 37 °C (□). (A) Parent strain A506. (B) Strain A506::*gfp*2. Error bars represent standard deviation of two experiments. Down arrows indicate levels of culturability below detection.

similarly (Fig. 4), with all strains maintaining high levels of culturability at least through 25–50 days of this study when incubated at either 5 or 23 °C.

In contrast to the results seen when cells were incubated at 5 or 23 °C, *P. fluorescens* A506, A506::*gfp*2, and A506 pSMC21 all rapidly became nonculturable when incubated in soil at 35–37 °C (Fig. 2). To determine whether these cells were dead or had entered into the VBNC state, we employed a substrate responsiveness viability assay. In this method, cells are incubated in the presence of yeast extract, which serves as a nutrient source, and nalidixic acid which inhibits DNA synthesis and thus prevents cell division. With the addition of a fluorescent dye or the constitutive production of a fluorescent product such as the GFP, viable cells can be identified microscopically by their elongation, even in mixed populations. Using this assay, 40–43% of the cells

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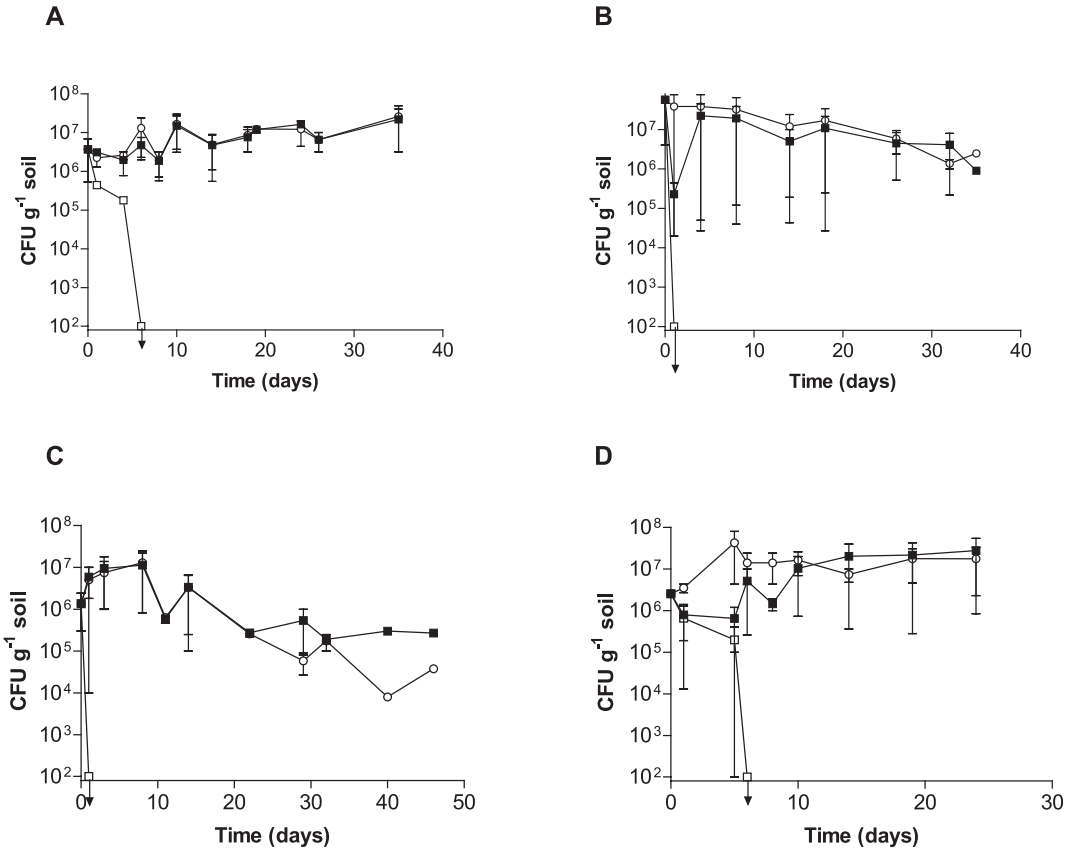

Figure 4. Culturability in soil of *P. fluorescens* 10586 at 5 °C (■), 23 °C (○), and 37 °C (□). (A) Parent strain 10586. (B) Strain 10586 pFAC510 (*lux ABE*). (C) Strain 10586 pSMC21 (*gfp*). (D) Strain 10586::pFAC510 (*lux ABE*). Error bars represent standard deviation of two experiments and down arrows indicate levels of culturability below detection.

incubated at 35–37 °C were shown to be viable, indicating entrance of these cells into the VBNC state. Such values were obtained even for cells that had been in the VBNC state for up to 60 days. A typical example is shown in Figure 5. While culturability of cells of *P. fluorescens* A506::*gfp* declined to <100 CFU.g^{-1} soil within 24 h (Fig. 5A), total cell counts and the substrate responsiveness assay remained high (>10^5 cells.g^{-1} soil). Entrance into the VBNC state was observed to be highly temperature-dependent. As seen in Figure 5B, cells of *P. fluorescens* became nonculturable in one day at 37 °C, required 50 d at 30 °C, but remained at >10^6 CFU.g^{-1} when incubated at 23 °C.

Like *P. fluorescens* A506 strains, cells of *P. fluorescens* 10586, 10586 pFAC510, 10586 pSMC21, and 10586::pFAC510 all rapidly became nonculturable when incubated in soil at 35–37 °C (Fig. 4). Results of the substrate responsiveness assay indicated that all strains had entered the VBNC state, with 36–46% of these populations being viable.

Effect of plasmids on survival in soil

P. fluorescens 10586 harboring the multi-copy (*ca.* 10) *lux*-labeled pFAC510 exhibited a slow decline in culturability at both 5 and 23 °C (Fig. 4B). A similar decline was seen for *P. fluorescens* with the *gfp*-labeled plasmid, pSMC21 (Fig. 4C). In contrast, *P. fluorescens* 10586::pFAC510, in which only a single copy of plasmid pFAC510 was present (due to spontaneous integration into the chromosome), exhibited no such decline in culturability at either temperature (Fig. 4D). This suggests that the decline in culturability seen in the plasmid-bearing strains may be a consequence of the metabolic load induced by these plasmids, as opposed to any direct consequence of either *lux* or *gfp* in these cells.

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The presence of the same *gfp*-labeled plasmid, pSMC21, did not appear to have this effect on the culturability of *P. fluorescens* A506 (Fig. 2C), with the plasmid-bearing cells exhibiting culturability comparable to the parent (Fig. 2A) at both 5 and 23 °C. Similarly, two copies of the *gfp* gene in the chromosome did not affect culturability of strain A506 over the short term at these temperatures (Fig. 2B).

As noted above, *P. fluorescens* 10586,10586 pFAC510, and 10586 pSMC21, and 10586::pFAC510 all rapidly entered the nonculturable state at 37 °C (Fig. 4). Whether plasmid pFAC510 was extrachromosomal (Fig. 4B) or integrated into the chromosome (Fig. 4D) did not appear to impact this finding. Similarly, strain A506 pSMC21 entered this dormancy state within a few days at 35 °C (Fig. 2C) similar to the parent strain (Fig. 2A). In all of the above studies, samples plated onto agar amended with kanamycin revealed counts similar to samples plated on agar without antibiotics, indicating that these plasmids were maintained during this temperature stress.

DISCUSSION

This study documents the long-term survival of *P. fluorescens* in soil, and its entry into the VBNC state at elevated temperature. *P. fluorescens* cells labeled with the *gfp* gene were found to retain culturability for at least a year at temperatures up to 23 °C. It also indicates the reliability of GFP as a marker for long-term monitoring of GEMs in the presence of environmental stresses. GFP fluorescence was maintained and thus cells could be observed microscopically in soil samples without the addition of nutrients or other exogenous substrates.

P. fluorescens cells marked with bioluminescence genes were similarly shown to be detectable in soil for extended periods. However, it has been suggested that *lux*-labeled cells are not able to synthesize sufficient substrate to continuously support the luminescent reaction due to the high energy demand (de Weger et al., 1991). Although addition of nutrients has been demonstrated to increase light output (Ducan et al., 1994; Meikle et al., 1992), this is impractical in field studies due to the potential bias it could create for the marked microorganisms. A strain of *P. fluorescens* dually marked with *luxAB* and *gfp* has been employed in soil studies, allowing simultaneous monitoring of population densities and metabolic activity (Unge et al., 1999). Cells in that study were detected in soil by GFP fluorescence for up to 30 days, but luciferase activity declined over the incubation period. This again implies reduced energy production by the cells, likely due to exhaustion of nutrients over the course of the study.

Strains in this study were shown to maintain plasmid DNA even after prolonged incubation in soil. Previous studies on this topic have reported varied results. *P. putida* was shown to maintain both a natural and recombinant TOL plasmid for up to one month when introduced into soil microcosms (Ramos et al., 1991). In contrast, a study using *P. fluorescens* bearing a bioluminescent plasmid showed dramatic (80 to 90%) plasmid loss after only six days in rhizosphere soil (de Weger et al., 1991). In our experiments, the presence of pSMC21 led to moderate (2–3 log) decreases in the culturability of *P. fluorescens* 10586 (Fig. 4C), but appeared to have no such effect on *P. fluorescens* A506 (Fig. 2C). It seems likely that the metabolic load imparted by pSMC21 on strain 10586, which not seen with strain A506 (Fig. 1), would account for the gradual loss of culturability of strain 10586 pSMC21 (Fig. 3C). Interestingly, whether pFAC510 was extrachromosomal or integrated also appeared to impact culturability of *P. fluorescens* 10586 (Fig. 4B and 4D). We previously reported that the presence of extrachromosomal (plasmid) DNA may significantly affect entry of GEMs into the VBNC state when in aqueous environments (Oliver et al., 1995). Whereas parent strains of *P. fluorescens* and *P. syringae* entered the VBNC state at 37 °C while maintaining culturability at 5 °C, the presence of plasmid pFAC510 resulted in a total reversal of this temperature effect, with cells remaining fully culturable at 37 °C but nonculturable at 5 °C (Oliver et al., 1995). Such a dramatic effect was not seen in the present soil experiments, and it may be that there are additional factors in soil that affect the entry of these strains into the VBNC state. Because other studies have shown the acquisition of plasmids from indigenous microorganisms *in situ* (Lilley and Bailey, 1997; Normander et al., 1998), our results suggest that the acquisition of extrachromosomal DNA could potentially induce changes that alter the ability to detect organisms released in the field.

The effect of temperature on the culturability of *P. fluorescens* was significant. While all *P. fluorescens* strains showed plate counts of 10^4–10^6 CFU.g^{-1} soil for extended times when held at 5 and 23 °C, these same strains all became VBNC when incubated at 35–37 °C. In the case of *P. fluorescens* A506::*gfp*, entry into this state was also documented when cells were held at 30 °C (Fig. 5B), although the rate of entry was much delayed compared to cells incubated at 37 °C. Despite the inability to culture such cells on laboratory media, their elongation after addition of yeast extract and nalidixic acid indicated their presence in the VBNC state. Prior studies have

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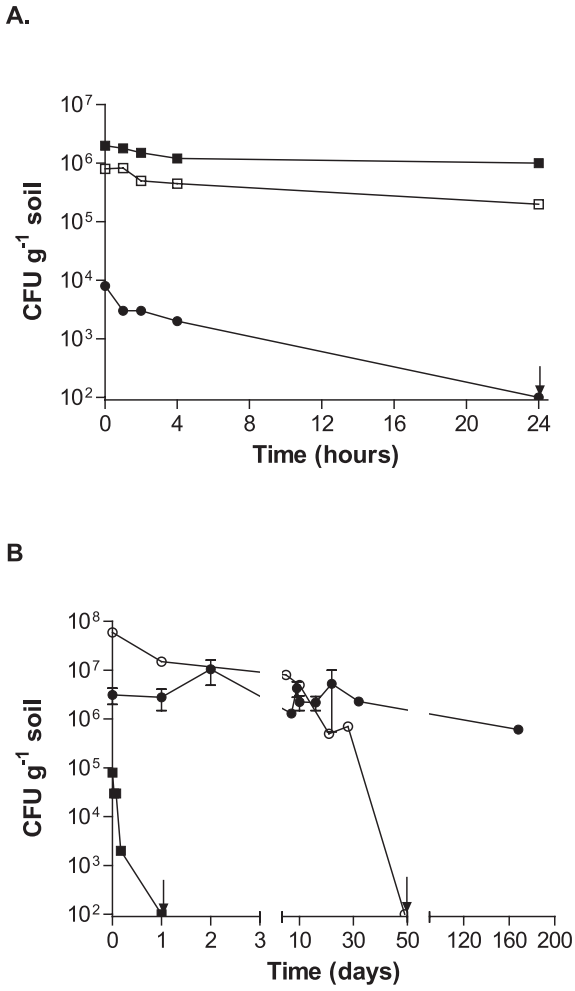

Figure 5. Entrance of *P. fluorescens* A506*::gfp* into the VBNC state. (A) Incubation at 37 °C, with measurement of (●) colony forming units (CFU.g^{-1}), (■) total microscopic cell counts, and (□) direct viable counts by the substrate responsiveness method. (B) Role of temperature in induction of the VBNC state. Shown are colony forming units of cells incubated at 23 °C (●), 30 °C (○), and 37 °C (■). Arrows indicate levels of culturability below the limit of detection. Data shown in (A) are the result of a single study, but are typical of similar studies conducted under similar conditions.

shown varying temperature effects on GEMs. Survival of a plasmid bearing strain of *P. putida* was shown to be greater at 4 and 25 °C than at 37 °C in soil microcosms (Ramos et al., 1991). A previous study from our laboratory on *P. fluorescens* and *P. syringae* strains in aquatic microcosms displayed similar patterns of survival, with cells maintaining culturability at temperatures from 4–25 °C while becoming VBNC at 35 °C (Oliver et al., 1995).

Our experiments using several GEMs demonstrate the considerable culturable variability that can result within the same species under various temperature conditions. When exposed to elevated temperatures, as would be encountered in tropical environments, for example, entry into the VBNC state by pseudomonads in both aqueous and soil environments occurs extremely rapidly, and would likely represent a significant concern in the monitoring of such cells. This would be especially true when the cells are genetically modified, as genetic exchange between GEMs in the nonculturable state and resident bacterial flora of the soil can not be excluded. Our data suggest that the use of *P. fluorescens* for soil bioremediation, whether the cells are genetically modified or not, should be done with the greatest caution when ambient temperatures might be elevated. Further, thorough characterization of strains as regards their response to the temperatures they are likely to encounter should be conducted prior to any release into the environment.

MATERIALS AND METHODS

Bacterial strains

P. fluorescens A506::*gfp*2 (Unge et al., 1997) is chromosomally marked with two copies of the mutant *gfp* gene P11 (Bokman and Ward, 1981), which results in stronger fluorescence intensity than the wild-type GFP. This strain, along with the parent *P. fluorescens* A506 (Lindemann and Suslow, 1987), were supplied to us by Janet Jansson (Swedish University of Agricultural Sciences, Uppsala, Sweden). *P. fluorescens* A506 pSMC21 [*gfp*$^+$, Ap^r, Cb^r, Kn^r] carries the *gfp* gene and kanamycin resistance on the plasmid pSMC21, a derivative of pSMC2 (Bokman and Ward, 1981), and was provided to us by George O'Toole (Dartmouth Medical School, USA) and Guido Bloemberg (Leiden University, The Netherlands). *P. fluorescens* 10586 pFAC510 [*luxABE*, Kn^r, Ap^r, Sp^r] (Amin-Hanjani et al., 1993) harbors the multi-copy (*ca.* 10) plasmid pFAC510, a pUCD4 derivative of 16.3 kb marked with resistance to kanamycin, ampicillin, and spectinomycin (Grant et al., 1992). It is marked with *luxABE* from *V. fischeri*. *P. fluorescens* 10586::pFAC510 contains a single copy of the plasmid pFAC10 present as a result of spontaneous integration into the chromosome (Amin-Hanjani et al., 1993). *P. fluorescens* 10586 and the pFAC510-bearing strains were supplied to us by Anne Glover (University of Aberdeen, Scotland). *P. fluorescens* 10586 pSMC21 [*gfp*$^+$, Ap^r, Cb^r, Kn^r] was generated in this study by

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electroporating pSMC21, isolated from *P. fluorescens* A506 pSMC21, into *P. fluorescens* 10586.

This combination of strains allowed comparisons to be made between cells with and without plasmids, with plasmid DNA that was extrachromosomal or inserted into the chromosome, and for cells harboring *gfp* or *lux* genes either chromosomally or on a plasmid.

Growth curves

To examine any possible consequences of the genetic modifications on growth of the cells, the various strains were cultured in Luria Bertani (LB) or LB with kanamycin (20 $\mu g.ml^{-1}$) at *ca.* 23 °C with shaking. Optical density values at 600 nm were monitored hourly for the first 12 hours, then again when stationary phase had been reached (*ca.* 24 h).

Cell culture and soil microcosms

Soil was collected from an agricultural site in Mt. Airy, North Carolina, between 1997 and 1999. This was a clay loam soil (30% clay, 30% sand and 40% silt) with an organic content of 0.86% and a pH of 5.2. Sterile soil microcosms were created by autoclaving 45 g of soil in screw-cap flasks. Cells were grown in LB at 23 °C with shaking to logarithmic phase and washed twice with phosphate buffered saline (PBS). Soil microcosms were inoculated drop-wise with 5 ml of the washed cells. Microcosms were placed at 5, 23, or 35–37 °C. Samples (1 g) of each microcosm were taken periodically, diluted with 9 ml of PBS, vortexed thoroughly, and centrifuged for 10 minutes at *ca.* 2500× *g*. Supernatants were collected, serially diluted in PBS, and plated onto LB agar. Duplicate samples collected from each microcosm at the same time points showed comparable levels of culturable cells, indicating that this sampling protocol provided reproducible results.

Plasmid maintenance was determined by plating cells onto LB agar containing 20 $\mu g.ml^{-1}$ kanamycin. In all cases, plates were incubated at 23 °C.

Total cell counts and viability assays

Samples for total and viable cell counts were collected as described above. Cell viability was monitored following overnight incubation with 0.025% yeast extract and 0.002% nalidixic acid, as described by Kogure et al. (1979). Cells were fixed with 50 µl formalin, stained with acridine orange, and filtered onto 0.2 µm pore-size, black polycarbonate filters. Cells were viewed by epifluorescence microscopy using an Olympus BX60 microscope. In this study, we did not routinely monitor the levels of activity of the *gfp* or *lux* markers, but instead characterized the survival of the inoculated cells harboring these reporter genes. We did examine the cells microscopically in order to confirm *gfp* expression, and colonies to confirm luminescence of the *lux* marked cells, prior to inoculation into the soil microcosms and occasionally during the studies.

Except as indicated, all studies were repeated, and the data presented are an average of samples taken from each of two replicate microcosms. Error bars represent standard error of the means of data from the replicate studies.

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