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Impact of Temperature on the Physiological Status of a Potential Bioremediation Inoculant, *Arthrobacter chlorophenolicus* A6

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***Arthrobacter chlorophenolicus* A6 (A6) can degrade large amounts of 4-chlorophenol in soil at 5 and 28°C. In this study, we investigated the effects of temperature on the physiological status of this bacterium in pure culture and in soil. A derivative of A6 tagged with the *gfp* gene (encoding green fluorescent protein [GFP]) was used to specifically quantify A6 cells in soil. In addition, cyano-ditolyl-tetrazoliumchloride was used to stain GFP-fluorescent cells with an active electron transfer system ("viable cells") whereas propidium iodide (PI) was used to stain cells with damaged membranes ("dead cells"). Another derivative of the strain (tagged with the firefly luciferase gene [*luc*]) was used to monitor the metabolic activity of the cell population, since the bioluminescence phenotype is dependent on cellular energy reserves. When the cells were incubated in soil at 28°C, the majority were stained with PI, indicating that they had lost their cell integrity. In addition, there was a corresponding decline in metabolic activity and in the ability to be grown in cultures on agar plates after incubation in soil at 28°C, indicating that the cells were dying under those conditions. When the cells were incubated in soil at 5°C, by contrast, the majority of the cells remained intact and a large fraction of the population remained metabolically active. A similar trend towards better cell survival at lower temperatures was found in pure-culture experiments. These results make *A. chlorophenolicus* A6 a good candidate for the treatment of chlorophenol-contaminated soil in cold climates.**

Bacteria inoculated into soil (for bioremediation applications, for example) are often negatively impacted by suboptimal environmental factors (such as pH, nutrient availability, and temperature). The cells can become stressed, resulting in reductions in their ability to generate energy (18) and to divide (13, 34). When subjected to stressful conditions, however, some bacteria can enter a reversible state of low-level metabolic activity and high-level stress tolerance (18). The transition into this physiological state (often referred to as a state of dormancy) and resuscitation from it have previously been correlated to environmental factors such as temperature and nutrient availability (18).

Arthrobacter chlorophenolicus A6 (A6) is a bacterium capable of degrading large amounts of 4-chlorophenol as its sole source of carbon and energy (36). This strain efficiently degrades 4-chlorophenol in natural soil at its optimal growth temperature of 28°C (12, 17) as well as at 5°C (3) and during temperature fluctuations between 5 and 28°C (3). These traits make *A. chlorophenolicus* A6 a promising candidate for cleanup of chlorophenol-contaminated soil in temperate climates, where low and fluctuating temperatures can otherwise limit microbial degradation.

Previous studies (3) indicated that the A6 strain survived better in 4-chlorophenol-contaminated soil at 5°C than at 28°C. A similar increase in survival of bacteria at low temperatures has been found in other studies (6, 9, 11, 33), and it has been suggested that cold-induced proteins "cross-protect" the cells against nutrient deprivation or other kinds of stresses found in the soil environment (14, 39). However, little is known about the impact of low temperatures on the physiological status of specific bacterial populations in soil.

Traditionally, bacteria introduced into environmental samples have been monitored by growth on selective agar media and lack of growth has been assumed to correlate to cell death. However, recent developments in molecular tools and culture-independent methods have made it clear that stressed cells can remain present and metabolically active, although undetectable on agar plates (2, 7, 9, 11, 12, 19, 22), a state often referred to as viable but nonculturable (24). Due to the risk that viable but nonculturable cells might remain undetected by plate cultivation, alternative approaches are needed to monitor such populations.

One promising tool is the use of marker genes for discriminating specific microorganisms of interest from indigenous soil microorganisms (6, 14, 16, 20). For example, cells tagged with the firefly luciferase gene (*luc*) can be detected on the basis of light production catalyzed by the luciferase enzyme upon addition of the substrate luciferin (14). Due to the dependence of light output (or bioluminescence) on cellular energy reserves, luciferase activity has been used to estimate the bulk metabolic status of cells introduced into soil (14, 20). Another biomarker, the *gfp* gene, encodes the green fluorescent protein (GFP), which emits green fluorescence after illumination in blue light independently of the cell's energy status. This allows for tracking of inoculants, for instance, by fluorescence microscopy (2, 4) and flow cytometry (23, 31).

One major bottleneck in understanding how the physiological status of specific bacterial populations is affected by different conditions in soil has been the lack of efficient methods

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to monitor bacterial physiological status at the single-cell level. Lowder et al. (23) proposed that the physiological status of single cells of bacterial inoculants marked with the *gfp* gene could be monitored in environmental samples by simultaneously measuring GFP fluorescence together with fluorescence emitted from viability stains. This idea has been further investigated by Arana et al. and Banning et al. (2, 4), who monitored the survival of GFP-tagged *Escherichia coli* (stained with different viability stains) in river- or groundwater microcosms by epifluorescence microscopy. However, these tools have not yet been demonstrated for monitoring of the physiology of specific microbial populations in soil systems.

In this study, we investigated the impact of temperature on the physiological status of A6 cells in pure cultures and in soil by flow cytometry to discriminate and quantify stained viable and dead fractions of a GFP-tagged cell population. In addition, another derivative of the strain, previously marked with the *luc* gene (12), was used to monitor the metabolic activity of the population under these conditions. Our hypothesis was that the use of these techniques would enable us to better understand how the physiological status of A6 is impacted during incubation in cold soil. The results of this study have wider implications, since the methodology demonstrated should also be applicable for monitoring the physiological status and for optimizing the survival of other environmental inoculants.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strain A6 was previously marked with the firefly luciferase gene (A6L strain) or the GFP gene (A6G strain) (12). In addition, both strains carry a kanamycin resistance gene (*nptII*).

Prior to each experiment, strain A6 cells were inoculated from −80°C glycerol stocks onto 1.5% agar plates consisting of GM minimal medium (2.10 g of K_2HPO_4, 0.40 g of KH_2PO_4, 0.50 g of NH_4NO_3, 0.20 g of $MgSO_4 \cdot 7H_2O$, 0.023 g of $CaCl_2 \cdot 2H_2O$, 0.002 g of $FeCl_3 \cdot 6H_2O$ per liter of distilled water) containing 4-chlorophenol (150 μg ml^{-1}) and kanamycin (35 μg ml^{-1}) according to a previously described method (1). All plates were incubated at 28°C.

Pure-culture experiments. For pure-culture experiments, strain A6 colonies were inoculated into 200 ml of Luria-Bertani (LB) medium containing kanamycin (50 μg ml^{-1}). The cultures were incubated at 28°C (150 rpm) overnight and were thereafter used to inoculate 2 liters of LB medium containing kanamycin (50 μg ml^{-1}). After overnight incubation at 28°C, the cells had reached stationary phase and were collected by centrifugation (5,000 × *g* for 15 min). The cells were then resuspended in 2 liters of phosphate-buffered saline (PBS) buffer and starved overnight at either 5 or 28°C. Thereafter, the cells were collected by centrifugation and resuspended in 2 liters of LB medium containing kanamycin (50 μg ml^{-1}). Three replicate cultures (100 ml each) were incubated at 5 or 28°C (150 rpm), and samples were taken for luminometry, plate counting, and flow cytometric measurements performed as described below.

Resuscitation experiment. After incubation in LB medium for 9 days at 5 or 28°C (as described above), the cell suspensions were supplemented with yeast extract (0.1% [vol/vol]). Samples were taken for flow cytometry, luminometry, and plate cultivation measurements prior to the addition of yeast extract as well as after 10, 30, and 150 min of incubation at 5 and 28°C in the presence of yeast extract.

Soil microcosm experiments. For soil microcosm experiments, the strain A6 inoculum was grown in GM medium containing 4-chlorophenol (150 μg ml^{-1}) and yeast extract (0.1%). The cultures were then transferred into fresh GM medium containing 4-chlorophenol (250 μg ml^{-1}) and incubated at 28°C. The cells were harvested at late exponential phase by centrifugation (5,000 × *g*, 15 min), washed, and resuspended in 30 ml of PBS buffer (pH 7.4). The suspensions were divided into two portions and incubated (without shaking) at 5 or 28°C for 12 h. After incubation, 4 ml of the cell suspensions was added to the soil microcosms as described below. The cell concentration added to each soil microcosm was as follows: for strain A6G at 28°C, 1.8×10^{10} cells ml^{-1}; for strain A6L at 28°C, 2.0×10^{10} cells ml^{-1}; for strain A6G at 5°C, 2.3×10^{10} cells ml^{-1}; and for strain A6L at 5°C, 1.15×10^{10} cells ml^{-1}.

The soil used for soil microcosm experiments was a previously described (29) Ullerâker sandy loam, which was collected in April 2002. The soil was frozen at −20°C immediately after collection and thawed overnight at 4°C before use. Soil microcosms consisted of 105 g of soil (100 g dry weight) in 200-ml Pyrex flasks with tightened screw-cap tops. Sodium phosphate buffer (11.5 ml; 0.5 M; pH 7.4) was added to increase the soil pH to 7.4. Sterile double-distilled water was added to achieve a final moisture content of 35%. Triplicate microcosms for each strain and temperature were prepared, and the microcosms were incubated overnight at the respective incubation temperature (28 or 5°C) prior to inoculation with strain A6L or A6G cells. Control microcosms were prepared by adding PBS buffer instead of strain A6 cells. After being mixed by stirring, samples were taken as described below to monitor the proportion of cyano-ditolyl-tetrazoliumchloride (CTC)- or propidium iodide (PI)-stained green fluorescent A6 cells, luciferase activity, and CFU.

Extraction of bacterial cells from soil. Cells from triplicate 0.2-g (wet weight) soil samples were extracted using Nycodenz (Axis-Shield PoC AS, Oslo, Norway) density gradients by modification of a published protocol (12). Sterile double-distilled water (800 μl) was added to the soil sample. After being mixed by vortexing for 1 min, the suspension was centrifuged for 2 min at 380 × *g*. The liquid phase was then gently placed on top of 700 μl of Nycodenz (0.8 g/ml; final density of 1.26 g/ml) in a new microcentrifuge tube. After centrifugation at 16,000 × *g* for 20 min, the upper 375 μl was discarded and approximately 675 μl of the remaining liquid, including the brownish bacterial layer between the water and the Nycodenz phase, was transferred to a 2-ml microcentrifuge tube containing 1,350 μl of sterile double-distilled water. The tube was mixed gently and then centrifuged at 16,000 × *g* for 20 min. Subsequently, 1.7 ml of the supernatant was discarded and the pellet was resuspended in the remaining supernatant. The suspensions of the three parallel extractions were pooled and then transferred to a new tube. Samples were taken for luminometry, plate count measurements, and flow cytometry measurements as described below.

Luminometry. Samples (0.5 ml) resulting from the Nycodenz extraction from soil inoculated with strain A6L cells were centrifuged for 10 min at 10,000 × *g*. The pellets were resuspended in 90 μl of citrate buffer (0.1 M; pH 5) and transferred to plastic cuvettes (Sarstedt, Landskrona, Sweden) (3.5 ml; 55-mm length by 12-mm inner diameter). The samples were incubated at room temperature for 10 min (to allow for the luciferase enzyme to become active in samples taken from soil incubated at 5°C) before addition of 10 μl of luciferin (Promega, Madison, Wis.) (10 mM) from *Photinus pyralis*. After brief mixing by vortexing, light emission was measured in a luminometer (EG&G LB 9506 MiniLumat; Berthold, Bundoora, Australia). All light-emission values are expressed as relative light units (RLU) per gram (wet weight) of soil.

Quantification of CFU in soil. Portions (100 μl) of the cell suspension obtained from Nycodenz extraction from soil inoculated with strain A6L cells were serially diluted in 900 μl of PBS buffer and inoculated onto 1.5% Luria agar plates supplemented with kanamycin (100 μg ml^{-1}) and cycloheximide (200 μg ml^{-1}). The plates were incubated at 5 or 28°C, according to the incubation temperature of the microcosms from which the samples were taken.

Flow cytometry and staining techniques. Flow cytometric measurements of GFP-fluorescent strain A6G cells stained with either of two viability stains were conducted to monitor the viable and dead fractions of the population after introduction into soil. The stains used were CTC (Polysciences Inc., Warrington, Pa.), which forms a red fluorescent formazan derivative upon reduction by dehydrogenases and components of the electron transfer chain in cells with an active electron transfer system ("viable cells") (10, 20), and PI (Sigma-Aldrich Sweden AB, Stockholm, Sweden), a red fluorescent stain which can only enter and stain the nucleic acids of cells with damaged or leaky cell membranes (13). To confirm that PI stains dead A6 cells, GFP fluorescence intensity (per cell) and the number of PI-stained cells were quantified after killing the cells with a mixture of lysozyme (5 mg ml^{-1}) and sodium dodecyl sulfate (10%). Concentrations of 80 μM for PI and 4.5 mM for CTC were shown to be best for staining both culture samples and cell suspensions extracted from soil samples (data not shown), and these stain concentrations were used in all experiments.

Pure-culture samples (3 ml) were centrifuged at 2,400 × *g* for 10 min, and the pellets were resuspended in 3 ml of NaCl (0.9%). The cells were washed two additional times (at 7,700 × *g*) before resuspension in 3 ml of NaCl (0.9%). Cells extracted from soil samples by Nycodenz extraction were centrifuged for 10 min at 16,000 × *g* and then suspended in 1.5 ml of NaCl (0.9%). The washed cell suspensions were divided into three portions (1 ml for pure-culture samples and 0.5 ml for soil samples), and the cells were collected by centrifugation at 13,700 × *g* for 8 min. The three cell pellets per sample were treated as follows: pellet 1 was suspended in 1 ml of NaCl (0.9%), pellet 2 was suspended in 1 ml of NaCl

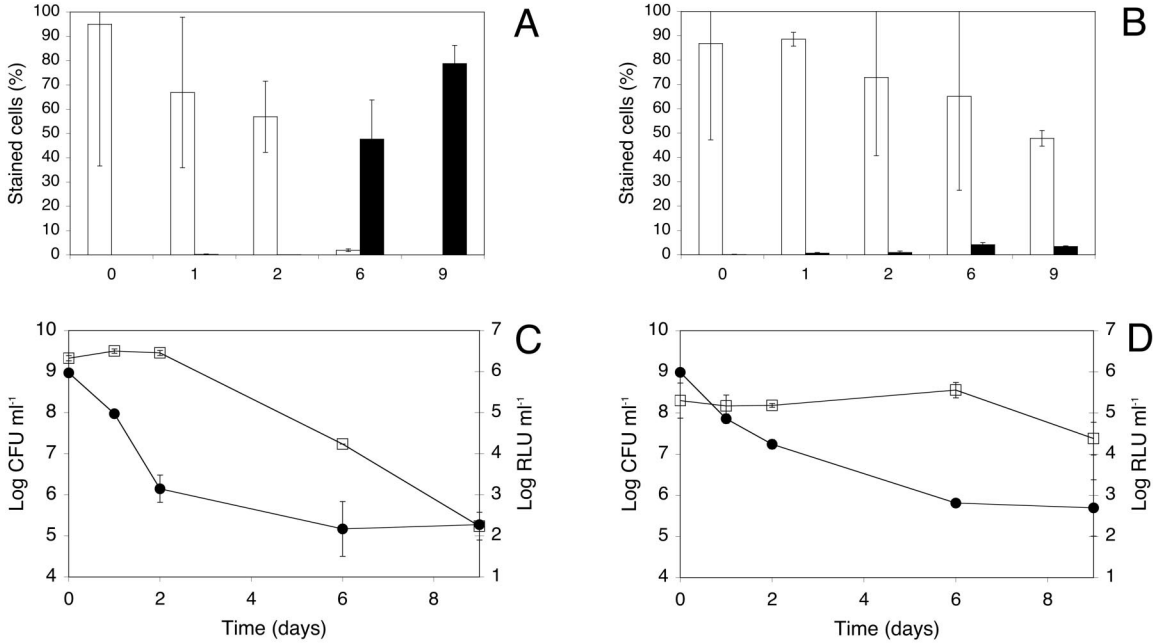

FIG. 1. Physiological status of *A. chlorophenolicus* A6 in LB medium incubated at 28°C (A and C) or 5°C (B and D). The proportions of viable cells (CTC stained; white bars) and dead cells (PI stained; black bars) of the GFP-fluorescent A6G population are shown in panels A and B. The metabolic activity (RLU [●]) and the number of colonies formed on selective agar plates (CFU [□]) of the A6L population are shown in panels C and D. The data presented in panels A and B represent the averages of duplicate samples, whereas the data in panels C and D represent the averages of triplicate samples. Error bars show the standard deviations of the means.

(0.9%) containing 4 μl of 20 mM PI stock solution (final concentration, 80 μM), and pellet 3 was suspended in 100 μl of NaCl (0.9%) and 900 μl of 5 mM CTC stock solution (final concentration, 4.5 mM). The samples were incubated at room temperature in the dark for 2 h. Thereafter, 100 μl of the suspensions was transferred to new tubes (Becton Dickinson, Oxford, United Kingdom) (5-ml polystyrene round-bottomed tubes; 12 by 75 mm) containing 900 μl of PBS buffer and 1 μl of 2.2-μm-diameter fluorescent microspheres (Duke Scientific, Palo Alto, Calif.) as an internal standard for quantitation of cells by flow cytometry, as previously described (31). The concentration of the microspheres added was in the range of 10^6 ml^{-1}, and the exact concentration of microspheres added to the samples was determined by epifluorescence microscopy before measurement by flow cytometry.

The incubated cell suspensions were injected into a FACSCalibur flow cytometer (Becton Dickinson) equipped with a 15-mW, air-cooled argon ion laser excitation light source (488 nm). For each measurement, data for 10,000 events were collected. Forward scatter was collected using a photodiode with an amplification factor of 10 and was processed in log gain. Side scatter was detected in log gain with a photomultiplier tube set at 392 V. GFP fluorescence in the range of 525 to 550 nm was detected using a HQ525/50BP filter (Chroma Technology Corp., Brattleboro, Vt.), with FL1 fluorescence detected at a photomultiplier tube voltage of 700 V with logarithmic gain. Orange-red (FL2) and red (FL3) fluorescence from PI or CTC staining in log gain was detected using a photomultiplier tube set at 521 and 700 V, respectively. The number of GFP-fluorescent cells was enumerated, and the fractions of GFP-fluorescent cells that stained with CTC or PI were quantified to monitor the viable and dead fractions, respectively, of the population. The unstained cell suspensions were used as a control to verify the total number of GFP-fluorescent cells and the GFP fluorescence per cell.

RESULTS

Confirmation of viability stains. Initial studies were performed to confirm the legitimate use of CTC and PI as indicators of viable and dead strain A6G cells, respectively. When GFP-tagged A6G cells were killed using a mixture of lysozyme and sodium dodecyl sulfate, a dramatic decrease in the intracellular GFP concentration was observed simultaneously with an increase in the number of PI-stained cells up to 100% of the total number of cells (data not shown). This indicates that PI staining is adequate for staining dead strain A6 cells. Also, approximately 95 and 100% of the viable cell population could be stained with CTC after 1 day of incubation in LB medium at 5 and 28°C, respectively (data not shown). Therefore, CTC staining provided an adequate estimation of cell viability for this bacterium.

Physiological status in pure cultures at different temperatures. Prior to soil microcosm experiments, pure-culture experiments were performed at 5 and 28°C to optimize the methodology and to study the physiological status of the cells under those conditions. The total number of GFP-fluorescent strain A6G cells remained constant at both temperatures during the time of the experiment (data not shown). The proportions of viable A6G cells were initially high at both 28 and 5°C (Fig. 1A and B, respectively) but rapidly declined in cultures incubated at 28°C simultaneously with an increase in the fraction of cells with damaged membranes ("dead cells") (Fig. 1A). At 5°C, the fraction of viable cells remained relatively high (over 45%) during the length of the experiment and only a minor fraction of the population died (Fig. 1B). A proportion of the GFP-fluorescent A6G cells did not stain with either CTC or PI at both temperatures, although the number of unstained cells at the end of the experiment was higher at 5°C (49%) than at 28°C (21%).

Experiments with the luciferase-tagged strain A6L derivative showed that the metabolic activity of the A6L population in LB medium declined at both temperatures (Fig. 1C and D),

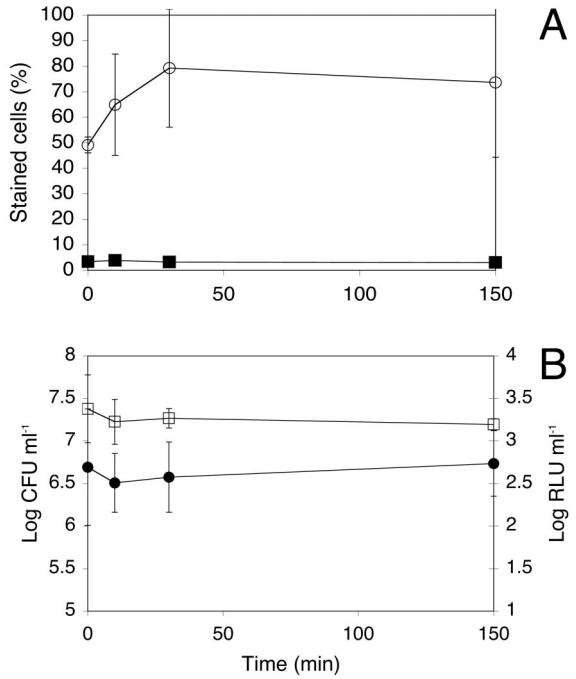

FIG. 2. Resuscitation of possibly dormant *A. chlorophenolicus* A6 cells (formed during incubation in LB medium at 5°C) by addition of yeast extract. (A) The proportions of viable (CTC-stained [○]) and dead (PI-stained [■]) GFP-fluorescent A6G cells are shown. (B) The metabolic activity (RLU [●]) and the number of colonies formed on selective agar plates (CFU [□]) of the A6L population are shown. The data represent the averages of triplicate samples, with error bars showing the standard deviations of the means.

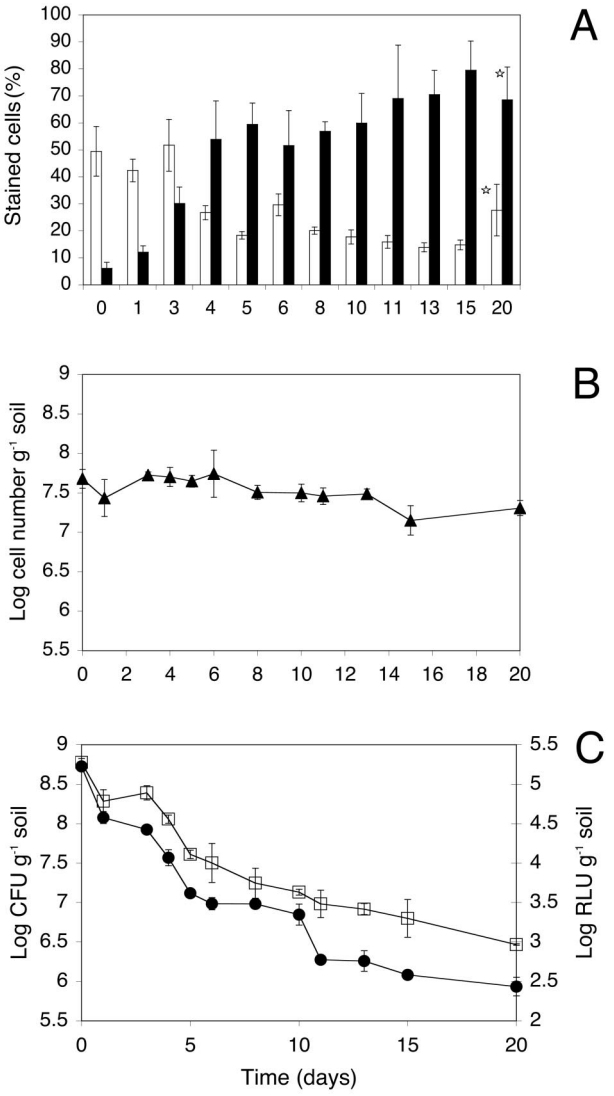

FIG. 3. Physiological status of *A. chlorophenolicus* A6 in soil microcosms incubated at 28°C. (A and B) The proportions of viable cells (CTC stained; white bars) and dead cells (PI stained; black bars) of the GFP-fluorescent strain A6G population (A), as well as the total number of GFP-fluorescent cells (B), are shown. (C) The metabolic activity (RLU [●]) and the number of colonies formed on selective agar plates (CFU [□]) of the strain A6L population are shown. The soil was inoculated with 7.2×10^8 strain A6G cells g^{-1} of soil (A and B) or 8.0×10^8 strain A6L cells g^{-1} of soil (C). The data represent the means of triplicate microcosms (except for the columns marked with stars in panel A, for which the mean was obtained from duplicate samples), and error bars show the standard deviations of the means.

although to a smaller extent at 5°C (Fig. 1D). The ability to form colonies on agar plates declined in cultures incubated at 28°C, reaching values approximately 4 log units lower than the initial value at the end of the experiment (Fig. 1C). At 5°C, the number of CFU remained relatively constant throughout the experiment (Fig. 1D).

Resuscitation studies. Yeast extract was added to the strain A6G cultures incubated at 5°C (presented in Fig. 1B) after 9 days of incubation to study whether the unstained fraction of the population could be resuscitated to an active state. The fraction of cells that were stained with CTC rapidly increased (after 10 min) from 50% to almost 80% of the population (Fig. 2A), although the total number of GFP-fluorescent cells remained relatively constant (log 9.0 and 8.9 cells ml^{-1} at 0 and 10 min, respectively). In addition, the fraction of PI-stained strain A6G cells remained constant (Fig. 2A). When yeast extract was added to A6L cultures grown for 9 days in LB medium at 5°C (presented in Fig. 1D), the bioluminescence yield remained constant (Fig. 2B), as did the number of CFU (Fig. 2B). For the cultures grown at 28°C (Fig. 1A and C), all parameters (the total number of cells, the fractions of viable and dead cells, the bioluminescence of the population, and the CFU) remained constant after addition of yeast extract (data not shown).

Physiological status in soil. By gating GFP-fluorescing cells that stained with either CTC or PI in the flow cytometry output, it was possible to quantify the fraction of viable and dead A6G cells in soil microcosms incubated at different temperatures. At 28°C, the proportion of viable strain A6G cells (as measured by CTC staining) decreased after 3 days of incubation and then became relatively stable at levels of 14 to 27% of the total cell population (Fig. 3A). The decrease in the number of viable A6G cells was accompanied by an increase in the proportion of dead A6G cells, which reached population values as high as 80% before termination of the experiment (Fig. 3A). The number of GFP-fluorescent strain A6G cells in soil incubated at 28°C declined gradually (Fig. 3B), reaching values

at the end of the experiment approximately 23% lower than initial values. In addition, the average relative GFP fluorescence intensity of the cells decreased from a relative value of 28 to a relative value of 19 from the beginning to the end of the experiment. In parallel studies with the *luc*-tagged strain, the bulk metabolic activity of the strain A6L population (measured as bioluminescence) and the ability of the cells to form colonies on agar plates declined immediately after introduction into soil and continued to decline by approximately 2 log units during the experiment (Fig. 3C).

At 5°C, the proportion of viable cells (measured as CTC-stained cells) initially decreased, stabilizing after 4 days of incubation at population values between 21 and 30% (Fig. 4A). By contrast, the proportion of dead cells gradually increased over time to a relatively constant population level of 16 to 36% after 11 days of incubation (Fig. 4A). The total number of GFP-fluorescing strain A6G cells (Fig. 4B), the GFP fluorescence intensity per cell (approximately 29 relative units), and the metabolic activity (measured as bioluminescence) of the *luc*-tagged derivative (Fig. 4C) decreased only slightly during the length of the experiment. Plate counts were highly variable at 5°C, differing by approximately 2.5 log units over the length of the experiment (Fig. 4C).

At both temperatures there was a large proportion of A6G cells in the soil that were not stained by either CTC or PI (Table 1). However, at the end of the experiment the proportion of unstained cells was more pronounced at 5°C (56%) than at 28°C (20%).

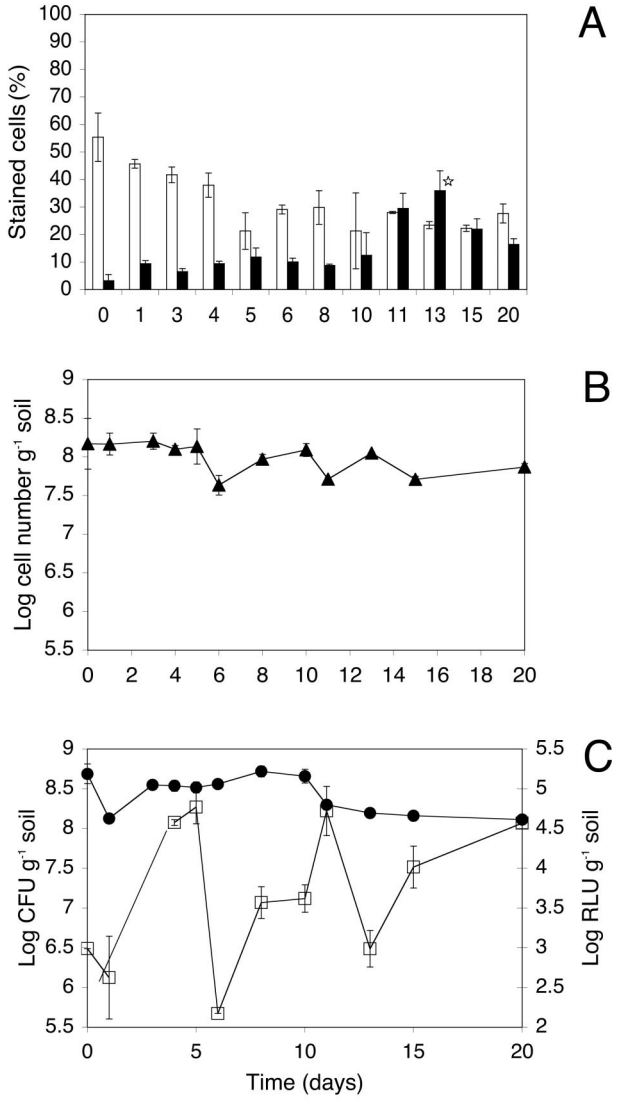

FIG. 4. Physiological status of *A. chlorophenolicus* A6 in soil microcosms incubated at 5°C. (A and B) The proportions of viable cells (CTC stained; white bars) and dead cells (PI stained; black bars) of the GFP-fluorescent A6G population (A), as well as the total number of GFP-fluorescent cells (B), are shown. (C) The metabolic activity (RLU [●]) and the number of colonies formed on selective agar plates (CFU [□]) of the A6L population are shown. The soil was initially inoculated with 9.2×10^8 strain A6G cells g^{-1} of soil (A and B) or 4.6×10^8 strain A6L cells g^{-1} of soil (C). The data represent the means of triplicate microcosms (except for the column marked with a star in panel A, for which the mean was obtained from duplicate samples), and error bars show the standard deviations of the means.

DISCUSSION

The aim of this study was to investigate the impact of temperature on the physiological status of the 4-chlorophenol-degrading bacterium *A. chlorophenolicus* A6. We used a variety of approaches to study how the strain A6 cells were specifically impacted by temperature during incubation in soil.

In soil incubated at 28°C, a majority of the strain A6 cells showed signs of cell deterioration or cell death, as shown by a decline in metabolic activity, cell integrity, and culturability. When introduced into soil incubated at 5°C, by contrast, the majority of the cells remained intact and metabolically active. These results indicate that A6 survives better in soil incubated at 5°C than at 28°C, as similarly suggested in a previous study in which plate counting was used to enumerate strain A6 cells inoculated into 4-chlorophenol-contaminated soil incubated at the same two temperatures (3).

In this study, we were able to distinguish between active and viable, dead, and possibly dormant physiological states of the *A. chlorophenolicus* population during incubation in soil. The strain A6G cells could be specifically detected in nonsterile soil on the basis of their green fluorescent phenotype (resulting from the GFP marker). Interestingly, approximately half of the A6G population in cold soil consisted of intact cells without measurable respiratory activity (i.e., cells that did not stain with either CTC or PI). Such cells were also detected after inoculation into soil incubated at 28°C and in LB cultures, although to a lesser extent than in cold soil. To further characterize the nature of the unstained cells, yeast extract was added as a substrate to activate cultures incubated at 5°C that consisted of approximately 45% unstained cells. The proportion of respiration-active, CTC-stained cells in the population immediately rose from 50 to 80%, whereas the fraction of unstained cells in the population declined to approximately 17%. There was no cell growth during this short incubation period, as confirmed by a constant number of total strain A6G cells. This implies that the intact but inactive cells were reactivated as a result of the addition of nutrients. This resuscitation period was apparently too short for the cells to replenish their energy reserves, as the *luc*-tagged A6L strain showed no increase in biolumi-

TABLE 1. Effect of incubation temperature on the average proportion (%) of unstained *A. chlorophenolicus* A6G cells in soil

Time (days)	Fraction of unstained cells (%) at:	
	28°C	5°C
0	44	41
1	46	45
3	18	52
4	19	53
5	48	67
6	19	61
8	23	61
10	22	66
11	15	43
13	16	53[a]
15	6	56
20	20[a]	56

[a] The mean of duplicate samples. All other data represent the means of triplicate samples. The standard deviation was less than 10% of the mean.

nescence during the same time period. When yeast extract was added to cultures incubated at 28°C that consisted of approximately 28% unstained cells, by contrast, no activation was observed. Therefore, it appears that a large fraction of the *A. chlorophenolicus* A6 population entered a dormant state during incubation at 5°C but not at 28°C. Our hypothesis is that the unstained A6 cells in soil incubated at 5°C also represented a fraction of the population that was dormant. *Arthrobacter* species are generally considered to be good survivors in harsh environments (5), and the formation of a dormant fraction of the population that might be reactivated should conditions become favorable may partly explain this survival capacity. An alternative explanation for the unstained cells in soil is that they are dying cells that have lost their respiratory activity but are not yet sufficiently damaged to be stained by PI.

The high-level variation in plate counts observed for strain A6L cells extracted from soil microcosms incubated at 5°C could have been due to difficulties encountered by intact but inactive A6 cells attempting to divide and form visible colonies on agar plates at that temperature. In a previous study Backman and Jansson found that A6L cells plated from soil supplemented with 4-chlorophenol were able to grow with little variation on agar medium incubated at 5°C (3). Therefore, nutrient availability in soil might impact the ability of this bacterium to grow under cold-stress conditions.

We found that the methodology used in this study contributed useful information on different aspects of bacterial physiology, but it is important to take some precautions before applying these tools for other bacteria. For instance, it is necessary to verify that the bacterial cells take up and/or retain the viability stains and to determine whether there are possible toxic effects for the cells of the viability stains. As an example, nucleic acids (to which PI binds) can be condensed or degraded due to stress conditions, resulting in insufficient cellular concentrations of PI for detection of the cells (8, 14, 24). This was not likely the case in this study, however, since unstained cells could be detected even under nutrient-rich conditions (LB medium), when nucleic acid concentrations should have been adequate for detection. Another factor that theoretically could explain the observed unstained cells in the present study is an inability of CTC to penetrate the cell membrane. However, since approximately 95 to 100% of a strain A6G population could be stained by CTC at both 5 and 28°C, this was most likely not the case for this bacterium. This observation also rules out the possibility that CTC is toxic to the cells, something that has been observed for some other bacteria (15, 28, 32).

This work for the first time demonstrates the applicability of flow cytometry in combination with viability stains and *gfp* tagging for studying the physiology of specific microbial populations in soil at a single-cell level. An alternative to using the GFP phenotype to distinguish the inoculated bacteria is to use strain-specific antibodies or probes, although the usefulness of these strategies can be limited due to risks of insufficient specificity of the probes and antibodies (16, 27, 30). Multistaining and cell sorting can be used to further increase the quality of the output data, since respiratory activity, membrane depolarization, cell integrity, etc., can be monitored on the same cell (21, 25, 26, 35, 37, 38). Therefore, this methodology is very promising for future studies of specific microbial populations in the fields of microbial ecology and environmental biotechnology and might lead to a greater understanding of bacterial physiology, dormancy, and cell death in the environment.

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