Development of AFLP-derived functional strain-specific markers to assess the persistence of 10 bacterial strains in soil microcosms

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ABSTRACT

To augment the information on commercial microbial products, we investigated the persistence patterns of high priority bacterial strains from the Canadian Domestic Substance List (DSL). Specific DNA markers for each of the 10 DSL bacterial strains were developed using the amplified fragment length polymorphism (AFLP) technique, and the fate of introduced DSL strains in soil were assessed by real-time quantitative polymerase chain reaction (qPCR). The results indicated that all DNA markers had high specificity at the functional strain level, and that detection of the target microorganisms were sensitive at a detection limitation range from $1.3 \times 10^2$ to $3.25 \times 10^5$ CFU/g dry soil. The results indicated that all introduced strains showed a trend towards a declining persistence in soil and could be categorized into three pattern types. The first was long term persistence of *Pseudomonas stutzeri* (ATCC 17587) and *Pseudomonas dinitrificant* (ATCC 13867) strain. In the second pattern, the inoculated *Bacillus subtilis* (ATCC 6051) and *Escherichia hermannii* (ATCC 700368) strain populations dropped dramatically below the detection threshold after 10 to 21 days, while the third pattern resulted in a gradual decrease falling below the detectable level within the 180-day incubation period. These patterns indicate a selection effect of microbial community related to the ecological function of introduced microbial strains in soil. As a key finding, the DSL strains can be quantitatively tracked in soil with high sensitivity and specificity at the functional strain level. This provides the basic evidence for further risk assessment of the priority DSL strains.

**Key words:** DSL microorganisms, Strain-specific marker, AFLP, persistence, soil microcosms
Recently, there have been environmental and human health concerns on the fate of commercially used microorganisms found in various environmental products such as septic tank treatment products, degreasers, compost starters, and drain cleaners. The products utilize unique characteristics of the microorganisms that could include the production of relevant enzymes (proteases, lipases, chitinase) or its function within a consortium.

The microorganisms within the products include a variety of bacteria. For example, *Pseudomonas* sp. strains are typically active denitrifiers, producers of biosurfactants (12, 37), and effective in bio-control of pathogens, petroleum bioremediation processes (12, 48); however, they can also affect human health by causing nosocomial infections (10). *Bacillus* sp. and *Enterobacter* sp. have been applied to soil to specifically enhance the phosphorus uptake of plants (4, 46); whereas, the representative *Bacillus cereus* is an opportunistic pathogen causing food poisoning manifested by diarrhea or emetic symptoms (22). Despite the potential risk of the commercial microbial products, there is little information available about the fate of microbial products in the environment. It is therefore necessary to develop a robust method to monitor the fate of commercial microorganisms in natural environments which would increase our knowledge of the persistent behavior of introduced microorganisms and aid environmental regulators on methods to assess products of biotechnology.

One of the challenges in tracking strain specific microorganisms is the development of a DNA marker with high specificity and detection sensitivity. In comparison to traditional culture-based methods that used specific physical and chemical parameters for microbial identification (33), the use of culture-independent tools like fluorescent antibodies or fluorescently labelled oligonucleotide probes has been suggested to be more advantageous for tracking microbial strains (24, 29, 43). However, the construction of a genetically modified microorganism can itself be categorized as a potential environmental hazard due to the possibility of persistence in the environment for a long time (29, 40). From a method standpoint, difficulties arise in obtaining sufficient signal intensity within discrete cells, and in transforming the target strain with the genetic marker (15, 16).

Having said this, however, the use of DNA-based markers is a more attractive method because of its culture independent nature, high specificity and sensitivity. For example, sequence-based markers directed at the 16S RNA gene (44) and 16S-23S rRNA intergenic transcribed spacer (ITS) region, have been successfully used to...
detect species of closely related taxa (17, 39). DNA markers targeted at protein-coding genes like recA (encoding the multi-functional DNA-binding protein involved in homologous recombination, 20, 41), gyrB (encoding DNA gyrase B protein, 13) and wzm (encoding inner membrane protein, 31) and rpoB (encoding the β-subunit of RNA polymerase B, 21) gene have also been adopted for the specific detection of closely related strains at the species level (35, 50). However, both of ITS regions and specific protein gene sequences still exhibit relatively conserved sequences among closely related species, although they have more sequence variation than the 16S rRNA gene. Because of this, it may be difficult or impossible to distinguish closely related strains (42). Genomic libraries (18) and DNA fragments amplified by repetitive sequence-based PCR (26) have recently been used as strain-specific DNA probes, but these methods are thought to be semi-quantitative. Only recently has the AFLP (amplified fragment length polymorphism) technique, which was firstly developed by Vos et al. (47), been used to quantitatively track the fate of introduced microorganisms in the environment (15, 29, 30). Research using AFLP have demonstrated a high specificity and sensitivity, with a limit of detection being $10^2$ CFU (colony forming units)/g soil, using quantitative real-time PCR primers against the AFLP-derived strain-specific DNA markers. With these high sensitive DNA markers, the introduced microorganisms can be monitored at the strain or functional-population level (30).

An additional challenge when assessing the persistence of microbial strains in soil is to understand why some strains persist while others do not. Research has shown that the populations of introduced microbial strains have gradually declined to undetectable levels (3, 19) and have persisted in the range of less than 72 hours (34) to up to 7 years (2, 9, 11, 14, 45). The persistence duration of the introduced strains are affected by biotic and abiotic factors, including the intrinsic physiological and genetic characteristics of the introduced microorganisms, indigenous soil organisms and the physical-chemical characteristics of soil (45). Recent investigations on the persistence of 9 Bacillus sp. and 3 Paenibacillus spp. bacterial strains in soil microcosms have demonstrated a great difference in the persistence behavior of the bacteria (30), indicating a difference in characteristics within the same genera. These results suggest that intrinsic physiological and genetic differences of bacteria significantly influence the persistence behaviour of the introduced microbial strains which can vary, not only at the genera level, but also at the species and strain level.
In the present study, functional AFLP-derived strain-specific DNA markers were developed for 10 bacterial strains that were high priority microbial substances on the Canadian Domestic Substances List (DSL, in reference 5) as identified under the Canadian Environmental Protection Act. To further monitor the fate of introduced strain populations in nature environments, we continued to investigate the persistence of 10 high priority risk group 2 bacterial strains in soil microcosms and attempted to provide interpretations of persistence behavior of the DSL strain populations in soil.

MATERIALS AND METHODS

Soil and DSL strain inoculation set up. The soil used in this study was collected from Alberta, Canada in April, 2007. The soil has a clay loam texture and a granular structure with 50.3% sand, 41.6% silt and 7.9 % clay, and contains 9.5% organic matter, 5.25% C and 1.70% N. It has a pH of 5.8 and a water holding capacity (WHC) of 70% (Environment Canada report, 2005†). The soil was passed through a 4-mm-pore-size sieve, homogenized, then adjusted to a final 60% WHC, distributed (140 gram oven dry soil) into separate 1000-ml plastic jars with vent holes in the lids. The number of microcosms prepared per study included 27 independent replicates plus 9 independent replicates for an un-inoculated control which allowed for soil sampling 9 times in triplicate after inoculation. The soil was separately inoculated with aqueous suspensions of live or autoclaved (60 minutes) microorganisms from each strain. The harvested cell culture of each DSL strain was inoculated by spreading 5 mL of cell suspension containing $10^8 - 10^{10}$ CFU/mL inoculum over the top ~ 1 cm of soil. Following inoculation, each microcosm received an additional 3.0 mL of sterile water which allowed the inoculum to slowly distribute in the soil. The soil was incubated at 22°C for 182 days in an incubator with a daily cycle of 16 hours light and 8 hours dark (to mimic concurrent single species toxicity tests being performed). Soil WHC was kept at a consistent 60% by using a syringe to add water to the jars at regular periods (3 to 6 days) during the whole incubation period. Soil was sampled by sacrificing the replicates on days 0, 2, 7, 14, 28, 42, 63, 136, and 180 for each strain (soil sampled within 3 hours after inoculation served as day 0, and soil sampled before inoculation as

† Environment Canada report, EPS 1/RM/45, 2005
negative control). Soil sampled from the autoclaved inoculum microcosms occurred on days 0 and throughout the study to serve as another negative control (non-infection control). The collected soil samples were stored at –20°C until processing.

**DSL Strain growth conditions.** The 10 DSL strains and 14 reference strains used in this study are listed in Table 1. The 10 DSL bacterial strains used in this study were all obtained from ATCC (American Type Culture Collection, Manassas, USA) and cultured by following the ATCC guidelines. Two strains *Bacillus subtilis* NRRL 941 and *Bacillus amyloliquefaciens* NRRL 942 were obtained from the United States Department of Agriculture. They and other reference strains were cultured on Luria-Bertani (LB) agar plates or in LB broth at 30°C.

DNA extraction from bacteria strains and spiked soil persistence tests. The extraction and purification of genomic DNA from pure cultures of each DSL strain were performed using lysozyme (Sigma, Catalog no. L6876) with sodium dodecyl sulfate (SDS) lysis buffer (Promega Wizard® Genomic DNA Purification Kit, Promega, Madison, WI, Catalog no. A1120) as recommended by the manufacturer. Soil DNA (0.25 g) was obtained by bead beating in SDS buffer and heat incubation (PowerMax Soil DNA Kit, MoBio, CA, Catalog no. 12888-100) as recommended by the manufacturer. Briefly, 0.25 g soil was added to 2-ml PowerBead tubes. Then 60 µl of solution C1 (a lysis buffer containing SDS) provided in the PowerSoil kit was added to the mixture, and the tube placed horizontally on the flat-bed of a vortex adaptor (MoBio, Catalog No. 13000-V1) and vortexed for 10 minutes at maximum speed. The mixture was incubated at 70°C for 30 minutes. After incubation, the genomic DNA was extracted by following the manufacturer’s instructions of the PowerSoil DNA extraction kit. The recovered soil DNA and pure genomic DNA were quantified using a NanoDrop 1000 (ThermoFisher Scientific) and stored at -20°C until further analysis.
**Development and specificity test of the AFLP markers.** AFLP fragments were generated by a modified method of Vos et al. (47). MseI and EcoRI digestion of genomic DNA and ligation of double-stranded adaptors were completed in one-step using AFLP® Core reaction reagents (Invitrogen, catalog No. 10482016, Ontario, Canada) with a total 25 µl reaction mixture consisting of 5 µl of 5× reaction buffer, 6 µl of each adaptor (MseI adaptor: 5′-GACGATGAGTCCTGAG-3′; EcoRI adaptor: 5′-CTCGTAGACTGCCTACC-3′), 1 µl MseI, 1 µl EcoRI, 0.5 µl T4 DNA ligase, ~0.4 µg of DNA, and double deionized (dd) H2O (sterile). The resultant adaptor ligation reaction was then diluted 10-fold for use in the pre-selective PCR reaction (1.0 µl DNA solution, 10 µl 10× Ready Mix Taq (Sigma-Aldrich, catalog No. P4600), 1 µl 10 µmol/L EcoRI pre-selective primer (E: 5′-GTAGACTGCCTACAAATTC-3′, containing sequences complementary to the core of the adaptor as well as the restriction site of EcoRI: G^AATTC), 1 µl 10 µmol/L MseI pre-selective primer (M: 5′-GACGATGAGTCCTGAGTAA-3′, containing sequences complementary to the core of the adaptor as well as the restriction site of MseI: T^TAA) and sterile dd-H2O in a total volume of 20 µl of PCR mixture). The pre-selective PCR reaction conditions included an initial denaturation of 95°C for 15 minutes followed by 30 cycles consisting of 30 seconds at 94°C, 45 seconds at 56°C, and 2 minutes at 72°C, and then a final extension of 5 minutes at 72°C. The pre-selective PCR products were diluted 10-fold for use in the selective PCR. A total of 64 selective primer pairs were used for the initial screening of the AFLP-derived sequences. The selective primers were M-xyz (M representing MseI pre-selective primer as above, xyz representing two or three extra bases: CG, CA, CC, CT, CTG, CTA, CTC, and CAC) and E- xyz (E representing EcoRI pre-selective primer as above, xyz representing two or three extra bases: TA, TC, TG, TGA, TGG, TGC, TGT, and ACG). The PCR mix was the same as the pre-selective PCR. The selective PCR included an initial denaturation of 94°C for 15 min, which was followed by 38 cycles including 30 seconds at 94°C, 50 seconds at 56°C, and 2 minutes at 72°C, and then a final extension of 5 minutes at 72°C. PCR products were visualized and compared to other available strains at the genera level using ethidium bromide staining followed by electrophoresis in a 1.5% (m/v) agarose gel. The distinguishable AFLP bands were recovered from the gel and purified with an UltraClean™ GelSpin™ Kit (Mobiol, catalog No. 12400-100). The purified AFLP products were stored at -20°C and used for analysis.
The purified distinguishable AFLP bands generated in the selective PCRs from the 10 DSL strains were cloned into pCR4-TOPO (Invitrogen), according to the manufacturer’s instructions, and 8 randomly selected cloned inserts from each AFLP band sequenced using the vector-specific M13F and M13R primers (Agencourt Bioscience Corporation, Beverly MA, USA). The obtained DNA sequences were compared with the known sequences by BLAST (1) at the National Centre for Biotechnology Information (NCBI, Bethesda, Maryland, USA; http://www.ncbi.nlm.nih.gov). After trimming to remove vector sequences, the AFLP sequences were used to design strain-specific primers. The primers targeting the internal sequences of the AFLPs were designed using a web-based primer design tool called Primer Design Assistant (PDA available at http://dbb.nhri.org.tw/primer/, 6). Primer specificity was initially tested by the PCR technique with the total genome DNA from target DSL strain and other nontarget relatives and uninoculated soil DNA samples. The PCR conditions included 15 minutes at 95°C, and 40 cycles consisting of 30 seconds at 94°C, 50 seconds at the annealing temperature set for each specific primer pair, and 2 minutes at 72 °C, and then a final extension of 5 minutes at 72°C.

The AFLP sequences used to design DSL strain specific primers were deposited in GenBank with accession numbers GQ473167 to GQ473177.

**Quantitative analysis of the DSL strain persistence in the soil microcosms.** A real-time PCR assay, based on the Sybr-green chemistry-primer PCR method, was developed for the DSL strain-specific AFLP marker in order to quantify the DSL strain populations in the soil microcosms. The specific AFLP primers for the 10 DSL strains were listed in Table 1.

The AFLP-based DNA marker qPCR was carried out in triplicate per soil treatment and in duplicate for each DNA sub-sample when performing the PCR in a 96-well polypropylene plate on a PTC-200 Peltier Thermal Cycler with a Chromo 4 Continuous Fluorescence Detector (Bio-Rad). The 20 µl reaction mixture contained the following: 10 µl of Platinum® SYBR® Green qPCR SuperMix (Invitrogen, cat number 11733-
0.5 µl of each AFLP sequence-based primer set for the target strain (10 µM), 0.5 µl bovine serum albumin (10 mg ml⁻¹), 1.0 µl ROX dye (50 fold dilution in TE buffer solution), 5.5 µl H₂O, and 1 µl template DNA (0.2 - 100 ng µl⁻¹). PCR conditions were optimized for each AFLP-derived primer set for each DSL strain, including 10 minutes at 95°C, and then 40 cycles of reactions consisting of 10 seconds at 94°C (denaturation), 15 seconds at the annealing temperature set for each specific primer pair, and 15 seconds at 72°C (elongation); and a final 5 minutes at 72°C.

To examine the persistence of the target DSL strains in soil microcosms, a standard curve was prepared comparing the amount of target-strain DNA extracted from soil, previously inoculated with a serial diluted known quantity (CFU/g dry soil) of the test organism, against the cycle threshold of the qPCR reaction. To avoid possible discrepancy in efficiency of DNA extraction and amplification inhibition due to the presence of humic acids, DNA extraction from the spiked soil for the preparation of standard curves was conducted by following the same protocols described as above for the strain persistence test. Briefly, overnight cultures of each DSL strain grown in LB broth was harvested by centrifugation at 2500 × g for 10 minutes. The obtained cell culture pellets were re-suspended in sterile water and diluted in increments of a 10-fold dilution series. The 10 µl of each 10-fold dilution was dropped on an agarose plate in triplicate, and then counts of total bacteria in each 10-fold serial solution was determined by counting the average number of single colonies in each drop. The 300 µl of 10-fold serial cell culture solution was added to a 2-ml PowerBead tube (provided by the PowerSoil kit) containing wet blank soil (unspiked control soil) corresponding to 0.3 g oven dried soil. Then 100 µl of solution C1 provided in the PowerSoil kit was added to the mixture, and the genomic DNA was extracted from the spiked soil with the known quantity of the target DSL strain as determined above. The inoculum of the 10 DSL strains spiked in soil was prepared separately in a dilution series ranging from 1.33 ×10⁹ to 1.3 CFU/g dry soils. qPCR was performed with the functional AFLP-derived strain-specific primer sets for each DSL strain from the serially diluted cell cultures and from the DNA from the persistence test soils. The abundance (CFU/g dry soil) of the target strain in the test soil was calculated by the established standard curves relating the cycle threshold value (Ct) to the abundance of the target strain in soil microcosms and applying linear regression analysis to the standard series plot (R² ≥ 0.97). Following each sample analysis, both agarose gel electrophoresis and qPCR
melting curve analysis were conducted to confirm that the size of the amplicon was correct and that the fluorescence signal originated from a single PCR product.

Analysis of variance (ANOVA) was used to determine whether the persistence data was significantly between the different sampling dates. All statistical analyses were performed using SAS (36).

RESULTS

Generation of AFLP-Derived Strain-Specific DNA markers for the DSL Microorganisms

The AFLP fragments were generated using different pairs of selective primers for 10 DSL strains (Fig. 1). Among the 64 AFLP primer combinations screened, about 93% of the primer combinations produced polymorphic fragments among the 10 strains. Nine primer pair combinations were selected for screening of the strain specific fragments. The obtained AFLP profiles by the same primer set were compared among the close relatives by using 2.5% agarose gel electrophoresis. Only the distinguishable fragments for the individual strains were excised from the gel (as labeled with small letters in Figs. 1A, 1B and 1C), and purified for the following cloning reaction. A total of 203 distinguishable sequences were obtained for the 10 DSL strains.

Figs. 1A, 1B and 1C

Specificity assessment of the AFLP-derived Strain-Specific DNA markers

The AFLP sequences at a range of ~200 to ~900 bp were analyzed by via a BLAST search. They were divided into four categories: (1) completely novel; (2) <95% similar to known sequences; (3) carrying distinguishable regions of ~100 to 250 bp adjacent to, or flanked by, a segment that was similar (with ≥98% identity) to known sequences; and (4) ≥98% identical to reported sequences. Most of the obtained sequences were similar to the known species. *P. stutzeri* ATCC 17587 was similar (with <99% maximum similarity) to *P. stutzeri* A1501 (CP000304); Strains *P. aeruginosa* ATCC 31480, ATCC 700370, and ATCC 700371 were similar (with <99% similarity) to *P. aeruginosa* PAO1 (AE004091); AFLP-based sequences from the *P. denitrificans* ATCC 13867 strain were below 94% similarity to *Pseudomonas* sp.; *B. subtilis* ATCC 6051 and ATCC 13933 were below 99% and 96% similar to *B. subtilis* strain W168 (EF191547), respectively; while *E. hermannii* ATCC 700368 and *E. aerogenes* ATCC 13048 were below 81% and 79% similarity to *Enterobacter* sp. strain 638 (CP000653).
respectively. Only the sequences that meet the first three criteria were selected for further primer design because those sequences carried a distinguishable region or were <95% similar to the known species, hence facilitating the design of a specific primer. The variable regions of some sequences with 98% similarity to the reported strains were also used for screening of the functional specific primer sets for the strain *B. subtilis* ATCC 6051.

The specificity of the obtained AFLP-derived strain-specific DNA markers were assessed using purified genomic DNA from the related species as listed in Table 2. Among the 120 pairs of AFLP-derived primers screened, only 13 primer pairs (Table 2) produced the distinguishable expected amplicon for the target DSL strains. Eleven of the primer sets were derived from the AFLP sequences that had a distinguishable segment or partial sequence with <98% similarity to the known strains, and thus had relatively high specificity at the strain level. An additional two primer pairs, P31480 6F/6R and B6051 VI 3F/R, were developed at the beginning of this study which produced no amplicons from the negative control soil and where thus used for further qPCR analysis, although their amplicons were 99% and 98% similar to *B. subtilis* stain 168 and *P. aeruginosa* PAO1, respectively. The two primer pairs, P31480 6F/6R and B6051 VI 3F/R, for *P. aeruginosa* ATCC 31480 and *B. subtilis* ATCC 6051 strain were considered specific at the functional strain level (Table 2).

**Evaluation of the qPCR assay for the 10 DSL strains in the soil microcosms**

Standard curves for the 10 DSL strains were developed (Fig. 2A and 2B). Quantitative amplification parameters for the DSL strains were optimal with linearity over a 5 to 6 log-unit dynamic range (R^2 ≥ 0.97 for the 10 AFLP-qPCR assays) with the overall PCR amplification efficiencies between 0.97 to 1.03. The limit of detection was found to be different among the different strains. The four strains, *B. subtilis* ATCC 14579 and ATCC 13933, *P. aeruginosa* ATCC 31480 and *P. denitrificans* ATCC 13867 had a low detection limit at a range from 7.31 × 10^1 to 1.4 × 10^3 CFU/g soil, while the other 6 strains had a relatively high detection limit ranging from 1.4 × 10^4 to 3.25 × 10^5 CFU/g soil (Table 3). The persistence duration is significantly distinct among different genera (Fig. 3A), species and strains (p<0.001 at the different sampling date for each strain, Figs. 3A and 3B). Although all of the ten tested DSL strains showed a trend towards a declining persistence following inoculation into a natural soil (Figs. 3A and 3B), they showed a different persistence patterns and could be categorized into three pattern types. The first was long term persistence over the incubation period. For example, *P. stutzeri* ATCC
17587 and *P. dinitrificant* ATCC 13867 remained at a high concentration, $3.4 \times 10^5$ and $1.4 \times 10^7$ CFU/g dry soil, respectively, for 180 days (Fig. 3B). In the second pattern, the inoculated strains (e.g., *E. hermannii* ATCC 700368 and *B. subtilis* ATCC 6051) dropped dramatically below the detection threshold after 10 to 21 days (Fig. 3A), while the third pattern resulted in a gradually decrease falling below a detectable level within a 62 to 126-days incubation period (e.g., *B. subtilis* ATCC 13933, *B. cereus* ATCC 14579, and *E. aerogenes* ATCC 13048 in Fig. 3A and *P. aerugonosa* strains ATCC 31480, ATCC 700370 and ATCC 700371 in Fig. 3B.).

### DISCUSSION

#### Specificity and detection limits of the AFLP-derived primers for qPCR analysis

In the current study, we considered the AFLP-derived sequences as distinguishable based on two criteria: they were distinguishable among the close relative strains in the agarose gel (Figs 1A, 1B, and 1C) and in the sequence database as well. Additionally, they were also tested for specificity by a comparison of PCR products with the AFLP-derived primers among the relative strains. For four DSL strains *Enterobacter aerogenes* ATCC 13048, *Escherichia hermannii* ATCC 700368, *Pseudomonas stutzeri* ATCC 17587 and *P. denitrificans* ATCC 13867 without relative strains available at our laboratory, the specificity of the obtained AFLP-sequences were tested by PCR analysis among the relatives at the genera level, and tested by a comparison of sequences among the close relative strains that registered in the DNA DataBank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL), and GenBank at the United States National Center for Biotechnology Information (NCBI). The primer pair B14579 P4CF/P4CR set for *Bacillus cereus* ATCC 14579 strain were based on the complete genome DNA sequences (Accession number: AE016877) and plasmid pBClin15 sequences (AE016878). The specificity of the corresponding PCR generated sequences with primer B14579 P4CF/P4CR were compared with the close relative strains in the database and also tested among the available strains at the genera level.
The results showed that the $C_T$ values obtained for the non-spiked soil (negative control) were similar to the $C_T$ values obtained for sterile water using the AFLP-derived primers. Only the expected DNA fragment was detected from the AFLP sequence-based marker of the target strain in the inoculated soil, but not detected from the un-inoculated soil or the soil samples inoculated with the autoclaved strain through agarose gel electrophoresis. This indicated that the specificity of the AFLP sequence-based DNA marker was correct for the proper strain, suggesting that the PCR amplification was uncontaminated.

Only seven AFLP-derived primer sets were determined to be highly specific at the functional strain level; others were relatively functionally strain specific (Table 2), although most of these primers were derived from sequences that contained distinguishable regions with no relatives detected through a BLAST search. The specificity limitation of the AFLP-derived primers is partly attributed to the availability of limited sequence data. Moreover, the reference strains have not been exhaustively tested; with more strains evaluated, there is a greater possibility to find positive results from the non-target strains. For example, the primer pair B14579 P4CF/P4CR based on the complete genome DNA sequences (Accession number: AE016877) and plasmid pBClin15 sequences (AE016878) of strain *B. cereus* ATCC 14579 produced a distinguishable 109-bp fragment in which the sequence had no relatives in the sequence database using the BLAST tool (1), and thus were distinguishable.

However, after the specificity evaluation of the strains in the NCBI database and comparison against the relative strains in our laboratory, the primer pair B14579 P4CF/P4CR generated the expected size of PCR products from the non-target strains such as *B. subtilis* 941 and ATCC 55405, and *B. thuringiensis* ATCC 13367. The primer pair P700371 S3F/S3R for *P. aeruginosa* ATCC 700371 generated a 209-bp fragment only from the target strain, in which the sequence had no relatives by the BLAST search in early 2008, but had one close (with 99% similarity) relative, *P. aeruginosa* LESB58 (FM209186), reported in 2009 (51). Additionally, the limitation of specificity of AFLP-derived sequences may be attributed to the potential contamination by the similar size of AFLP fragments and low PCR efficiency of the specific regions within the genome DNA of the close relatives, where the generated AFLP bands might be very weak and fail to be visualized in the agarose gel during the screen of initial AFLP sequences. For example, the primer pair B13933 Q1CF/Q1CR for *B. subtilis* ATCC 13933 was generated from the distinguishable AFLP fragments among the close relatives, but generated a 142-
bp fragment in which the sequence was 92% similar to *B. subtilis* (Z99111), and also produced the expected PCR products from the non-target strain *B. licheniformis* ATCC 55406. This may be improved by using polyacrylamide instead of agarose gels during the AFLP marker development as similar size DNA bands may be more clearly distinguishable (55).

Several strategies were used to eliminate the potential influence of environmental factors, such as soil humic acids and PCR efficiency, on the detection limits of each strain in soil (25, 29, 30, 35). In choosing a suitable method to extract DNA from the soil used in this study, five different commercial kits for soil DNA extraction were initially compared in our parallel study in our laboratory: UltraClean™ soil isolation kit (Mo Bio, CA, Catalog no. 12800-50), PowerSoil™ DNA Isolation Kit (Catalog no. 12888-50), FastDNA® SPIN Kit for Soil (Qbiogene, Montreal, CA, Catalog no. 6560-200), SoilMaster™ DNA extraction kit (Epicentre Biotechnologies, Madison, WI, Catalog no. SM02050), and E.Z.N.A.TM stool DNA isolation kit (Omega Bio-Tek Inc., Doraville, CA, Catalog no. D56251-01). Based on our preliminary results (data unpublished), DNA extraction using a combination of bead beating and the SDS buffer method (PowerSoil™ DNA Isolation Kit) resulted in a substantial DNA yield and less inhibition from soil humic acids as compared to the other kits. This was consistent with previous preliminary conclusions that bead-mill homogenization and the SDS buffer method had a higher DNA yield (15, 29, 30, 35) which made the current results comparable with our earlier data under the same project for DSL microorganisms (24, 40, 41). Previous comparisons of qPCR assays with DNA extracted from pure strain cell cultures spiked in both soil and sterile water showed a consistent result on DNA amplification efficiency, suggesting a high quality of DNA extracted by using the improved protocol of PowerSoil DNA isolation kit (35). In this study, therefore, a combination of bead beating, SDS buffer and hot incubation method was used for DNA extraction from the spiked soil. In a previous study using a similar DNA extraction protocol of UltraClean™ soil isolation kit, a 100-fold lower sensitivity was determined suggesting a possible influence of soil inhibitors on the PCR amplification efficiency (29). In order to rule out any potential inhibitors from the soil DNA, we usually used a 10-fold-diluted DNA extract in our research. qPCR annealing temperature and amplification cycles were also optimized for each specific AFLP-derived primer set. For strains with short persistence periods such as *E. hermannii* ATCC 700368 and *B. subtilis* ATCC 6051 (Table 1 and...
Figs.), the strain persistence type was confirmed by re-running the qPCR with a lower annealing temperature, more amplification cycles, and 1 or 10-fold diluted soil DNA templates. Previous studies have indicated that the limit of detection by qPCR could result from a number of factors. These factors include the length of PCR products, secondary structure of extracellular polymeric substance (EPS) (8, 19), and DNA GC content (25, 50) of the target nucleic acid molecule. In this study, all of the qPCR amplification parameters were optimized for all of the ten strains tested. In the case of the AFLP-derived P. stutzeri ATCC 17587 sequence, its high GC content (~60.6%) and secondary structure may have reduced the PCR efficiency. Moreover, the differences in concentrations of extracellular polymeric substance (EPS) in the extracted soil DNA from the different Pseudomonas sp. strains (19, 23) and the affinity of the specific seeded strain DNA to soil particles (7) may also influence the efficiency of the DNA extraction following the PCR amplification reaction for the different target strains. Decay of naked DNA in soil during storage may also influence the detection and persistence assessment of the microbial strains in soil. To reduce the fluorescence interference from the soil background and primer dimers, gel electrophoresis tests were also conducted for each replicated PCR product. It was also realized that qPCR analysis of the specific DSL strains can also be improved by using Taqman probes (38). The limitations of PCR efficiency might be enhanced by nested qPCR, particularly in cases where no amplicons or gel electrophoresis bands were detected after long incubation times.

PCR-based assays have been demonstrated as a useful genetic approach to quantitatively monitor pathogenic microorganisms, at the genera level, in the environment (32), and at the strain level (15, 29). Providenti et al. (29) determined the detection limit of Trichoderma reesei QM6A#4 to be in the range of ~10^6 CFU/g soil. In a subsequent publication, the detection limit of Bacillus and Paenibacillus species were determined to be 10^2 CFU/g soil (30). In the present study, it was determined that the target strains were detectable at levels from 10^2 to 10^3 CFU/g soil, which exhibited a sensitivity similar to or lower than aforementioned Bacillus and Paenibacillus study (30), but higher than the T. reesei study (29). This confirmed that our qPCR sensitivity was equivalent to other studies and thus sufficient for our study.

The data showed a significant difference of persistence between strains within a similar detection limitation (Table 3 and Figs 3a, 3d, 3i and 3j). For example, strain P. aeruginosa ATCC 700370 persisted in soil for 122
days, whereas the population of the *B. subtilis* ATCC 6051 strain were only detected after inoculation for 8 days even though both strain persistence was determined at a similar detection limitation of approximately $10^5$ CFU/g soil (Table 3). The population of strain *P. aeruginosa* ATCC 31480 persisted for 62 days and appeared to drop in a linear gradient upon inoculation (Fig. 3c) although the abundance of the target strain could be detected at a very low level at $1.3 \times 10^2$ CFU/g soil (Table 3); whereas, the populations of *P. stutzeri* ATCC 17587 strain dominated with an abundance of $10^7$ CFU/g soil throughout the soil incubation experiment even though its detection limitation was 200-fold higher than that of *P. aeruginosa* ATCC 31480. This indicated that the significant differences of the 10 DSL strain persistence were not possibly attributed to the discrepancy in efficiency of DNA extraction from soil and DNA amplification between strains as observed in Table 3. Short persistence of strains *B. subtilis* ATCC 6051 and *E. hermannii* ATCC 700368 most likely reflect their low capability of colonization in soil, while the long persistence of strains *P. stutzeri* ATCC 17587 and *P. denitrificans* ATCC 13867 indicated their high capability of colonization in the tested soil. This makes it possible to compare the persistent patterns among the 10 DSL strains tested.

**Difference in the persistence behaviors of the 10 DSL strains and their ecological implications**

The present study showed a general tendency of the introduced strains to decrease in the soil over the incubation time (Figs. 3A and 3B), which is consistent with results in the literature (11, 15, 30, 45) and reinforced the anti-invader repression effect of autochthonal microbes on the introduced strain population (45). To survive and reproduce in soil, the introduced bacterium should be able to rapidly adapt to the soil environment and compete with the autochthonal microorganisms. It is assumed that the population size of most inoculants, such as *Pseudomonas* sp. strains in Figure 3A, and *Enterobacter* sp. and *Bacillus* sp. in Figure 3B, dropped under the detection limit because of its inability to compete for nutrients and further repression by the indigenous microorganisms in the soil. Our result, however, also showed an apparent difference in the persistence patterns of *P. stutzeri* ATCC 17587 and *P. denitrificans* ATCC 13867 from those of the other 8 DSL strains (Figs. 3A and 3B). Following an initial adaptation period after inoculation into soil, the population size of *P. stutzeri* ATCC 17587 and *P. denitrificans* ATCC 13867 remained stable at a relatively higher cell density.
level ranging from $1.44 \times 10^7$ to $3.09 \times 10^7$ cells/g soil and $2.4 \times 10^5$ to $4.9 \times 10^5$ cells/g soil, respectively (Fig. 3A). This indicated that *P. stutzeri* ATCC 17587 and *P. denitrificans* ATCC 13867 were able to find a niche and survive within the soil microbial community and possibly affect the soil ecosystem. The sequence search result from our BLAST search showed that the *P. stutzeri* ATCC 17587 strain was a close relative of the nitrogen-fixing and denitrifying bacterium *P. stutzeri* A1501 strain isolated from rice roots (53, 54). Among the obtained 26 AFLP-based sequences from *P. stutzeri* ATCC 17587, three of them had high similarity (99%) and coverage (88%) to the complete genome sequences of the *P. stutzeri* A1501 strain. The long term persistence of *P. stutzeri* ATCC 17587 and *P. denitrificans* ATCC 13867 may be attributed to the high denitrification ability of their population (27, 28, 54), in which its growth might be favorable for the dissimilatory nitrite reduction to respond to the nitrite accumulation in the moist soil (52). By contrast, *E. hermannii* ATCC 700368 is not a ubiquitous strain, and thus the populations abruptly dropped below the detection level (Fig. 3B). The above results suggest that the intrinsic capability of bacterial colonization in soil strongly influences the persistence of the introduced microorganisms.

This study has shown the different persistent patterns of the introduced 10 DSL strains in soil. This is very helpful for our understanding of the inoculant fate in the environment. The introduction of commercial bacterial strains could theoretically disturbed indigenous microorganisms in the soil microcosms, leading to a change in the carbon and nitrogen cycling and behavior of the microbial community in soil. Further research would be necessary to assess the dynamics and interaction of introduced bacterial strains and indigenous soil microorganisms at the population and functional community level using tools and methods such as denaturant gradient gel electrophoresis. This study provided an estimate of persistence of the DSL strains by relating the qPCR threshold cycle value to predetermined plate-based counts. On the issue of estimation of the live cell count, advanced methods to directly correlate the fluorescent intensity of the target live cells to the CFU in soil by qPCR assay (49) would enhance our understanding of the survival of the introduced DSL strain in the environment. It should be noted, however, that since DNA was not detected in soil where strains were not detected is good evidence that viable strain numbers had fallen below the detectable level (30). Our results have indicated that the AFLP-derived strain-specific markers are functionally-specific, which allows us to
quantitatively track the introduced strains in soil microcosms. The developed DNA markers may also be applied
to track the specific strains in the environment. However, specificity tests are still limited since the reference
strains have not been exhaustively tested, and the reference sequences in the database are very limited. For some
important possible pathogenic strains, the strain specific markers might still need to be developed through
complete genome sequencing and tested among more reference strains. In summary, AFLP is a useful technique
for developing specific DNA markers and tracking specific strains in a soil matrix with high sensitivity and
specificity at the functionally strain level. The findings have shown the distinct persistent patterns of the
different DSL microorganisms among the different genus, species and strains in soil, and strongly support the
idea of the intrinsic differences in competition with the indigenous microorganisms influencing the dynamics of
the introduced DSL strain populations in soil.

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Biotechnology, administered by the Biotechnology Section of the Emerging Priorities Division in Environment
Canada.
REFERENCES


of *Escherichia coli* RNA polymerase alter transcription pausing and termination. Genes Dev. 4:1623–1636.


Table 1. DSL and reference strains used in this study

<table>
<thead>
<tr>
<th>Reference strains*</th>
<th>DSL strains</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus amyloliquefaciens</em> NRRL 942</td>
<td><em>Bacillus subtilis</em> ATCC 6051</td>
</tr>
<tr>
<td><em>Bacillus circulans</em> ATCC 9500</td>
<td><em>Bacillus subtilis</em> ATCC 13933</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em> ATCC 12713</td>
<td><em>Bacillus cereus</em> ATCC 14579</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em> ATCC 55406</td>
<td><em>Escherichia hermannii</em> ATCC 700368</td>
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<td><em>Bacillus megaterium</em> ATCC 14581</td>
<td><em>Enterobacter aerogenes</em> ATCC 13048</td>
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<td><em>Bacillus subtilis</em> ATCC 55405</td>
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<tr>
<td><em>Bacillus subtilis</em> ATCC 6051a</td>
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<td><em>Bacillus subtilis</em> NRRL 941</td>
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</tr>
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<td><em>Bacillus thuringiensis</em> ATCC 13367</td>
<td><em>Pseudomonas stutzeri</em> ATCC 17587</td>
</tr>
<tr>
<td><em>Escherichia coli</em> OP50</td>
<td><em>Pseudomonas denitrificans</em> ATCC 13867</td>
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<tr>
<td><em>Pseudomonas aeruginosa</em> ATCC 31479</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> ATCC 13525</td>
<td></td>
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<td><em>Pseudomonas fluorescens</em> ATCC 31483</td>
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<td><em>Pseudomonas pudita</em> ATCC 31800</td>
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<tr>
<td><em>Pseudomonas pudita</em> ATCC 700369</td>
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<tr>
<td><em>Pseudomonas syringae</em> ATCC BAA 871</td>
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*, The reference strains were used for the specificity test of the AFLP-derived primers.
Table 2. AFLP-derived primers and summary of specificity test

<table>
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<tr>
<th>Target strain</th>
<th>Primer</th>
<th>AFLP sequence accession no.</th>
<th>Primer sequence (5’→3’)</th>
<th>Annealing temperature (ºC)</th>
<th>PCRs size (bp)</th>
<th>Specificity</th>
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<tr>
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<td>CTGAAAGATGGCCTTGGGTGTCT</td>
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<td>B6051 V2F*</td>
<td>GQ473171</td>
<td>CGGGTGGAAGGATATATTC</td>
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<td>290</td>
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<tr>
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<td>B6051 V2R*</td>
<td></td>
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<td>290</td>
<td>+</td>
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<td>GCACGTTGCTGATAATATTC</td>
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<td>B14579 P4CR</td>
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<td>169</td>
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<td>B14579 Z1R**</td>
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<td>++</td>
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<td></td>
<td>B700368 O1R</td>
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<td>GCACGTTGCTGATAATATTC</td>
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<td></td>
<td>E13048 IV2R</td>
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<td>CGGGTGGAAGGATATATTC</td>
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<td>290</td>
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<td><em>Pseudomonas aeruginosa</em> ATCC 700370</td>
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<td>400</td>
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<tr>
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<td>P700370 A4R</td>
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<td>GCACGTTGCTGATAATATTC</td>
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<td><em>Pseudomonas aeruginosa</em> ATCC 700371</td>
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<td>CGGGTGGAAGGATATATTC</td>
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<td>400</td>
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<td>GAACGTTGCTGATAATATTC</td>
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<td>P17587 F2R</td>
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<td>GCACGTTGCTGATAATATTC</td>
<td>52</td>
<td>400</td>
<td>+++</td>
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<td><em>Pseudomonas denitrificans</em> ATCC 13867</td>
<td>P13867 K1F</td>
<td>GQU31604</td>
<td>GAACGTTGCTGATAATATTC</td>
<td>52</td>
<td>400</td>
<td>+++</td>
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<td></td>
<td>P13867 K1R</td>
<td></td>
<td>GCACGTTGCTGATAATATTC</td>
<td>52</td>
<td>400</td>
<td>+++</td>
</tr>
</tbody>
</table>

*, primers obtained at the end of the study were not used in the qPCR analysis.
**, primers with low PCR amplification efficiency were not used in the qPCR analysis.

"+", functional strain specificity (no expected PCR products from the negative soil control, but expected PCR products were visualized from two or three non-target strains); "++", relatively strain specificity (no expected PCR products from the negative soil control, but expected PCR products were visualized from one or two non-target strains); "+++", highly strain specificity (with expected PCR products only from target strain compared with all tested strains).

Two pairs of primers B14579 Z1F/Z1R and B14579 P4CF/P4CR were targeted to stretches corresponding to positions 909509-910708 of the *B. cereus* 14579 stain complete sequence (Accession number: AE016877) and 7855-7966 of plasmid pBClin15 (AE016878).
### Table 3. Summary of DSL strain persistence in soil and reference strains used in this study

<table>
<thead>
<tr>
<th>Target DSL strains</th>
<th>Persistence in soil (Days)</th>
<th>Detection limitation (CFU/g soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em> 6051</td>
<td>8</td>
<td>$1.46 \times 10^7$</td>
</tr>
<tr>
<td><em>Escherichia hermannii</em> 700368</td>
<td>21</td>
<td>$6.41 \times 10^4$</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> 31480</td>
<td>62</td>
<td>$1.3 \times 10^7^{*}$</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> 700370</td>
<td>122</td>
<td>$3.25 \times 10^5$</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em> 13048</td>
<td>125</td>
<td>$1.8 \times 10^4$</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> 700371</td>
<td>126</td>
<td>$1.0 \times 10^4$</td>
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<tr>
<td><em>Bacillus subtilis</em> 13933</td>
<td>127</td>
<td>$7.92 \times 10^3^{*}$</td>
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<td><em>Bacillus cereus</em> 14579</td>
<td>127</td>
<td>$7.31 \times 10^4^{*}$</td>
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<tr>
<td><em>Pseudomonas stutzeri</em> 17587</td>
<td>180</td>
<td>$2.95 \times 10^4$</td>
</tr>
<tr>
<td><em>Pseudomonas denitrificans</em> 13867</td>
<td>181</td>
<td>$1.37 \times 10^3$</td>
</tr>
</tbody>
</table>

* The qPCR detection is very sensitive.
**FIGURE LEGENDS**

**Figure 1.** Electrophoresis pattern of the AFLP results with the strains *P. stutzeri* 17587 and *P. aeruginosa* 31480, 700370 and 700371. The results were with A) Selective primer pair M-CA/ETA, and B) primer pair M-CG/E-TA; and C) primer pair M-CTG/E-TGC and M-CTG/E-TA. Lane 1 in Fig. 1A, 1B and 1C and lane 11 in Fig. 1C contain DNA size standards (100 bp ladder). Lane 2 in Fig. 1A, 1B and lane 10 in Fig. 1C were the negative controls for PCR amplification. Lanes 3 to 6 in Fig. 1A and B and lanes 2 to 5, and 6 to 9 in Fig. 1C were marked with the representative DSL strains used for the selective AFLP reactions. The select PCR patterns were compared among the closely related species and strains, and the distinguishable fragments for specific strains were labeled with lowercase letters and then recovered from agarose gel. M = *Mse*I pre-selective primer, E = *Eco*RI pre-selective primer.

**Figure 2.** Generation of standard curves showing PCR threshold values versus cell count numbers for the 10 DSL strain DNA from serially diluted culture added to the soil. A) Standard curves for *Bacillus* sp. and *Enterobacter* sp.; B) Standard curves for the *Pseudomonas* sp. Values represent means ± 95% confidence interval.

**Figure 3.** Persistence trends of the indicated strains in soil microcosms, based on qPCR analyses of extractable soil DNA. Persistence trend of DSL strains: A) *Pseudomonas stutzeri* 17587, B) *Pseudomonas denitrificans* 13867, C) *Pseudomonas aeruginosa* 31480, D) *Pseudomonas aeruginosa* 700370, E) *Pseudomonas aeruginosa* 700371, F) *Enterobacter aerogen* 13048, G) *Bacillus subtilis* 13933, H) *Bacillus subtilis* 14579, I) *Escherichia hermannii* 700368, and J) *Bacillus subtilis* 6051. The limit for qPCR-based quantification was confirmed by agarose gel electrophoresis detection. In cases where an amplicon is visible through gel electrophoresis, but qPCR data (CFU/g soil) cannot be determined because it is equal to the value for the negative control, the value was set to the corresponding limited one on the standard curve. If no amplicon was detected by either qPCR or agarose gel electrophoresis, the CFU/g value for that treatment soil was considered as zero for calculation of the mean.
Fig. 2

**A. Bacillus sp. and Enterobacter sp.**

- B6051: $C(t) = -3.64 \pm \log (CFU) + 59.66, R^2 = 0.99$
- B14579: $C(t) = -3.41 \pm \log (CFU) + 50.84, R^2 = 0.98$
- E13048: $C(t) = -3.02 \pm \log (CFU) + 47.54, R^2 = 0.99$
- B13933: $C(t) = -2.88 \pm \log (CFU) + 46.59, R^2 = 0.99$
- E700368: $C(t) = -2.94 \pm \log (CFU) + 46.15, R^2 = 0.97$

**B. Pseudomonas sp.**

- P17567: $C(t) = -3.12 \pm \log (CFU) + 55.75, R^2 = 0.98$
- P700370: $C(t) = -3.35 \pm \log (CFU) + 50.96, R^2 = 0.99$
- P31480: $C(t) = -3.41 \pm \log (CFU) + 50.85, R^2 = 0.99$
- P13867: $C(t) = -2.76 \pm \log (CFU) + 47.58, R^2 = 0.98$
- P700371: $C(t) = -2.41 \pm \log (CFU) + 44.13, R^2 = 0.98$
Figs. 3A to 3J.

A. *P. stutzeri* 17587

B. *P. denitrificans* 13867

C. *P. aeruginosa* 31480

D. *P. aeruginosa* 700370