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Population dynamics of an introduced bacterium degrading chlorinated benzenes in a soil column and in sewage sludge

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Abstract

The capacity of the β-Proteobacterium *Pseudomonas* sp. strain P51, which degrades chlorinated benzenes, to metabolize 1,2,4-trichlorobenzene (TCB) under environmental conditions was tested by its release into two experimental systems. The first system consisted of laboratory scale microcosms which were operated with and without the addition of TCB and which were inoculated with sludge from a wastewater treatment plant. The second system consisted of a non sterile, water saturated soil column. We determined survival of strain P51 after its introduction and its ability to degrade TCB. The population dynamics was followed by selective plating and applying the polymerase chain reaction (PCR) to detect strain P51 and the chlorobenzene (*tcb*) genes on catabolic plasmid pP51. The results showed a completely different behaviour of strain P51 in the two habitats under the applied conditions. In the soil column the P51 bacteria inoculated the entire area and their population reached 2×10^6 cells/g soil. The population remained active since TCB was degraded to concentrations below the detection limit of 30 μg/l. In the sludge microcosms, the number of strain P51 cells immediately decreased from 4×10^7 cells/ml to 10^5 cells/ml over a period of 2 days after inoculation, and then the strain disappeared to levels below our detection limit (10^3–10^4 cells/ml). In the reactor without TCB the population of P51 maintained a stable value of 10^5 cells/ml during 8 days but then also decreased to levels below the detection limit. In addition, no significant TCB degradation was found in the sludge reactors. The influence of presence of TCB on maintenance of strain P51 in the two habitats is discussed. This work demonstrates the possibility to successfully apply preselected strains to degrade otherwise poorly degradable substances in complex mixed microbial communities. However, survival and activity may depend strongly on the type of system into which the strain is introduced.

Introduction

Spontaneous biological removal of aromatic pollutants in the environment may proceed relatively slow due to insufficient degradation capacity and slow adaptation of the indigenous microorganisms. By introducing specifically enriched or even genetically modified bacteria (i.e., bioaugmentation), biodegradation rates of such pollutants in soils, sediments or waste water treatment plants could in principle be enhanced (Pipke et al. 1992; Wagner et al. 1992; Jansson 1995; Megharaj et al. 1997; Selvaratnam et al. 1997; Watanabe et al. 1998). However, there are several points for consideration when applying such strains. Inoculated strains often survive poorly and may loose their activity in mixed microbial ecosystems (McClure et al. 1989; Postma & van Veen 1990; McClure et al. 1991; Wagner et al. 1992; Recorbet et al. 1993; Watanabe et al. 1998). Several factors have been implicated in the survival, activity and maintenance of introduced strains. Some factors are of physicochemical nature, such as presence or absence of oxygen, pH or temperature.

Other factors reflect the physiological adaptability of the bacterium, such as kinetics of substrate utilization, nutrients and trace elements scavenging (Postma & van Veen 1990; Fujita et al. 1991; Megharaj et al. 1997; Watanabe et al. 1998). Some bacteria may be particularly prone to predation by protozoa when they maintain a freely suspended state rather than attach themselves easily to surfaces or form sticky material (McClure et al. 1991). Finally, the availability of a specific substrate for the introduced strain and the relative concentration of the specific substrate compared to other metabolizable substrates will be important for survival and maintainance (Pipke et al. 1992; Sobecky et al. 1992; Wagner et al. 1992; El Fantroussi et al. 1997; Ravatn et al. 1998). Some concern also exists that under some conditions the genetic information for degradation of the pollutants may not be stable in the introduced organism and be lost or altered after some time (Kumar & Schugerl 1990; Proctor 1994; Arturo et al. 1996).

To obtain more information on the applicability of selected bacterial strains to perform biodegradation under real-life conditions, we performed several experiments to study the population dynamics and activity of an introduced bacterium. The bacterium we used was a specifically selected strain for degradation of chlorinated benzenes. This strain, previously described as *Pseudomonas* sp. strain P51, is able to use monochlorobenzene, all isomers of dichlorobenzene and 1,2,4-trichlorobenzene (1,2,4-TCB), as sole source of carbon and energy (Van der Meer et al. 1991a). Chlorinated benzenes are compounds which are resistant to microbial attack and not generally degraded by indigenous, non-adapted microorganisms. Therefore, they are more easily removed by specifically enriched strains, such as strain P51, and the catabolic activity of the strain can be addressed exclusively by measuring chlorobenzene disappearance.

The strain was inoculated in two different systems, which were both operated on a laboratory scale for periods up to one month. One of these consisted of fermentors which were pre-inoculated with sludge from a wastewater treatment plant. The other system consisted of soil columns filled with river bed sediments which were percolated under water-saturated conditions. To be able to monitor the population density of the introduced strain at a sufficiently sensitive level, we applied polymerase chain reaction (PCR)-based quantification methods in combination with selective plate countings.

Materials and methods

Bacterial strains and growth conditions

The strain we used in the inoculation experiments was a spontaneous rifampin resistant mutant of *Pseudomonas* sp. strain P51. The strain was routinely grown at 30 °C and maintained on Z3 minimal medium (Van der Meer et al. 1987), saturated with 10 mg of 1,2,4-TCB per 40 ml medium.

Escherichia coli DH5α and TG1 (Sambrook et al. 1989) were used as hosts for cloning purposes and were grown on Luria Broth (Sambrook et al. 1989) supplemented with ampicillin at a final concentration of 50 μg/ml when necessary.

Cloning and sequencing of a region of the 16S rDNA of Pseudomonas *sp. strain P51*

A 1,440 base-pair region of the 16S rDNA of *Pseudomonas* sp. strain P51 was amplified in the PCR by using primers for conserved regions of the Bacterial 16S rDNA (Weisburg et al. 1991), modified as follows (modifications as small caps), 6F (5′ggAGAGTTaGATCcTGGCTCAG) and 1510R (5′gtgctgcAgGGTTACCTTGTTACGACT). The amplified fragment was ligated with the vector pGEM-T Easy (Promega, Wallisellen, Switzerland) and transformed to *E. coli* DH5α, according to established procedures (Sambrook et al. 1989). The nucleotide sequence of the 16S rDNA fragment of strain P51 was determined on both strands by using a Thermosequenase Kit (Amersham, Little Chalfont, UK) with IRD-800 labeled primers (MWG Biotech, Ebersberg, Germany), and analysis on a LiCOR DNA sequence analyzer (model 4000L, Lincoln, Ne, USA). The DNA sequence of the 16S rDNA of strain P51 can be retrieved from GenBank under accession number AF015487.

Set-up of the sludge reactors

Sludge reactors consisted of one-liter fermentors operated with 500 ml solution under continuous cultivation with automated temperature and pH control. Where applicable 1,2,4-TCB was added by connecting part of the air flow through a vial containing liquid 1,2,4-TCB. The air flows were adjusted to achieve an approximate concentration of 1,2,4-TCB in the reactor medium of 1 mg/l.

As a basic complex medium we used a synthetic waste water (based on DIN 38412 T24, German In-

dustry Norm, 1981), which contained per liter: 1.28 g peptone from casein, 0.88 g meat extract, 240 mg urea, 14 mg NaCl, 8 mg $CaCl_2 \cdot 2H_2O$, 0.06 g $MgSO_4 \cdot 6H_2O$ and 11.5 ml phosphoric acid (85%). Final pH of the medium was 3.0; in the reactor the pH was adjusted to pH 7.4. The total DOC of the medium, added to each reactor at 20 ml/min, was 1 g/l. Dilution rate of the system was 0.04 h^{-1} (yielding a residence time of 25 h). Both reactors were kept aerobic under all conditions by bubbling air through at a flow rate of 120 ml/min, and maintained at 30 °C. The reactors were pre-inoculated with activated sludge from a municipal aerobic wastewater treatment plant (ARA Neugut, Zürich, Switzerland) and operated for two weeks in continuous flow mode to establish a steady-state bacterial population. The optical density (OD) at 600 nm of this stabilized population after two weeks was 1.25. After this period we inoculated cells of strain P51. These cells had been pregrown on Z3 medium with 1,2,4-TCB and were harvested at a cell density of 3×10^8 cells/ml. After washing the cells, we resuspended them in Z3 medium to a density of 5×10^9 cells/ml and inoculated each reactor with 4 ml of this suspension. The final amount of P51 in each reactor was thus 4×10^7 cells/ml.

Set-up of the soil column

A soil column was constructed from a 23 cm long PVC cylinder with an inner diameter of 5.25 cm, and total volume of 500 cm^3. On the side of the column, sampling ports were constructed at 2, 5, 8, 13 and 18 cm distance from the column inlet (hereafter named ports A, B, C, D, and E, respectively). The ports consisted of teflon tubes with an inner diameter of 2 mm which ended in the middle of the column. Samples of soil and soil liquid could be taken from the column by attaching a syringe to the sampling ports. The column was percolated in an up-flow mode at a flow rate of 40 ml/h (0.08 column volumes per h) with a mineral medium as described previously (Van der Meer et al. 1987). The medium was kept saturated with oxygen and was directed through a continuously stirred flask containing liquid 1,2,4-TCB. In this way a constant concentration of 8.3 μM 1,2,4-TCB (1.5 mg/l) could be maintained in the influent.

The column was filled with a sandy sediment from the Aare river, Switzerland, which was sieved through a 0.5 mm sieve, filled into the PVC cylinder and then restored to water-saturated conditions. The column was run for 8 days without the addition of strain P51. Respiration of indigenous microorganisms in the sediment during this period reduced the inflow oxygen concentration from 10 mg/l to 7 mg/l at the outlet. Strain P51 was pregrown on 1,2,4-TCB as described above, washed and inoculated at an amount of 5×10^9 cells at port C.

Viable counts

Total viable counts of heretrophic bacteria and of strain P51 were performed by plating tenfold serial dilutions on NB (Nutrient Broth, Biolife, Milan, Italy) agar or on NB agar supplemented with 20 μg/ml rifampin, respectively. The number of viable P51 bacteria was further addressed by plating serial dilutions on Z3 mineral agar plates, which were incubated in the presence of vapourized 1,2,4-TCB. All plates were incubated at 30 °C, and counted after 2 to 5 days.

DNA isolations from the sludge reactors and soil columns

DNA was extracted from 0.4 g soil samples, which were taken from the side ports of the column with a syringe and transferred to an eppendorf tube. To the samples we added 0.4 g glass beads (0.10 mm average diameter; B. Braun Melsungen AG, Inotech AG, Dottikon, Switzerland) and 1 ml of an extraction buffer (containing 50 mM Tris-HCl pH 8.5, 20 mM EDTA, 1% [w/v] polyvinylpolypyrrolidone and 100 mM NaCl) (Hallier-Soulier et al. 1996). The soil-glass bead mixtures were then blended twice for 1 min with 1 min intermittant cooling on ice in a Cell Homogeniser (B. Braun Melsungen AG). The suspension was subsequently centrifuged for 2 min at 15,000 $\times$ *g* and the supernatant recovered. The DNA from the supernatant was purified by two passages over Sephadex spin columns (BioRad, Glattbrugg, Switzerland), and once by Elutip-D treatment (Schleicher and Schuell, Dassel, Germany).

From sludge samples and liquid cultures of strain P51 we isolated the DNA by taking 100 μl solution into an eppendorf tube, covering the solution with 2 drops of mineral oil (Sigma Chemical Co., St. Louis, USA) and heating it for 10 min at 98 °C in a Crocodile II thermocycler (Appligene, Illkirch, France). Cell debris was removed by centrifugation at 15,000 $\times$ *g* for 3 min, the supernatant was transferred to a new tube and stored at −20 °C. This solution could be used directly in the PCR.

DNA amplification and semi-quantification of initial DNA concentrations by using the PCR

The PCR was performed with *Taq*-polymerase in a reaction mixture of 50 μl as suggested by the supplier (Life Technologies, Basel, Switzerland). In general, 0.2 mM of each primer was used in the reaction. Reactions were run on a Crocodile II thermocycler. Before use, tubes were heated up to 80 ° C for 2 min after which 2 μl of template DNA were added. The samples were initially denatured at 93.5 ° C for 1.5 min and then run for a total of 35 cycles under the following conditions: denaturation at 93.5 ° C for 1 min, primer annealing at 55 or 60 ° C for 1 min and DNA extension at 72 ° C for 1 min. After amplification, the DNA products were analyzed on agarose gel stained with ethidium bromide.

The initial amount of DNA in the samples was determined in a limiting dilution-PCR (Sykes et al. 1992). First, each sample was serially diluted. All dilutions were then amplified in the PCR individually. The maximal dilution which gave rise to a photographically detectable product on ethidium bromide stained gels was taken as the detection limit for that sample. This dilution was then compared with the detection limit obtained similarly in the PCR with a dilution series containing known amounts of cells of strain P51. For each set of primers a separate dilution series was made. Specific primers for strain P51 and the *tcb*-genes were: P1.1 (5′ATAACGGAGCGAAAGCTTTGC) and P1.2 (5′GGACCCCAGGTATTACCAGAGT) for the 16S rDNA, A1.1 (5′CGTATGGAGGCAATAGTGCCCTG CG) and A1.2 (5′CGATCTGGTACAGGTCTTCATC GG) for *tcbAa* (Werlen et al. 1996), C3.1 (5′GCGTCGGCTACATGATGAAACTGG) and C3.2 (TGCGAGTCATGGAGCATCCAGAAGT) for *tcbC* (Van der Meer et al. 1991a).

To determine the error in the quantification procedure we first estimated the error arising from calculating DNA amounts when comparing detection limits. Hereto DNA was isolated from liquid samples with known amounts of strain P51 cells (5×10^8 cells/ml), amplified in the PCR and compared with the standard series described above. The DNA isolation and PCR amplifications were repeated two times for each sample. The determination of the amount of cells appeared accurate within a plus or minus three-fold dilution with 99.5% confidence. Secondly, the error occurring in the extraction procedures was estimated. For this purpose, we added a standard amount of strain P51 cells (5×10^7 cells) to soil or sludge samples, extracted the DNA and run the PCR. Recovery and detection of strain P51 again appeared accurate within a plus or minus threefold dilution of the sample with 99.5% confidence.

Analytical measurements

1,2,4-TCB concentrations were measured in 1.5 ml of liquid sample, which was centrifuged in an eppendorf tube for 2 min to precipitate large particles. Supernatants were transferred to a sealed glass tube and extracted with an equal volume of hexane. The hexane phase was analyzed on a Varian GC3400 instrument (Varian International AG, Basel, Switzerland) equipped with a DB-5 column (30×0.25 mm) and an ECD detector. TCB concentrations were derived from comparison with a 1,2,4-TCB standard curve. The detection limit for 1,2,4-TCB in this manner was 30 μg/l (165 nM).

The oxygen concentration in the soil pore water was measured with a flowthrough electrode (type 5356/5300 YSI, Yellow Springs, Ohio) which was connected to the effluent of the column.

Statistical analysis

Trend analyses of the different ratios of viable counts to PCR counts, or 16S rDNA counts to *tcbC* counts, with respect to column length and incubation time, were performed according to Sachs (1984). Since the distribution of the standard error was unknown, the Wilcoxon signed rank test was used to analyze pairwise differences of the numbers obtained by viable counts, 16S rDNA or *tcbC* counts rather than the Student's *t* test (Sachs 1984).

Results

Detection of strain P51 by amplification in the PCR

To be able to specifically detect *Pseudomonas* sp. strain P51 in the two mixed microbial communities, we sequenced one copy of its 16S rDNA. A FastA alignment (Pearson & Lipman 1988) of the sequence with other 16S rDNA sequences in GenBank revealed highest similarities with *Variovorax paradoxus* (94.7%), *Rhodoferax fermentans* (94.1%) and *Comamonas testosteroni* (93.8%). Phylogenetic positioning performed on a subalignment of the 20 most similar 16S rDNA sequences retrieved

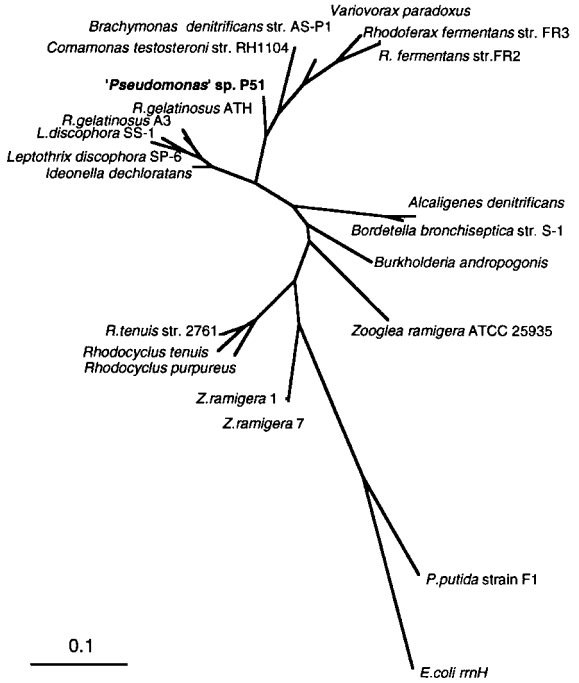

Figure 1. Phylogenetic tree based on maximum-parsimony analysis of 1,469 positions in the 16S rDNA sequences from *Pseudomonas* sp. strain P51 and 20 neighbouring sequences from β-Proteobacteria retrieved from the Ribosomal Database Project. *Escherichia coli* and *Pseudomonas putida* F1 were used as outgroup. This tree was obtained from 100 resamplings in bootstrap analysis. The relative branching between strain P51, *C. testosteroni* and *B. denitrificans* was not 100% supported by bootstrapping. The bar indicates the distance corresponding to 0.1 nucleotide changes per position.

from the Ribosomal Database Project (Maidak et al. 1996) by maximum-parsimony Analysis (DNAPARS) and Maximum Likelihood Analysis (DNAML) (all programs part of the Phylogenetic inference package PHYLIP 3.52c (Felsenstein 1993)), indicated a branching of strain P51's 16S rDNA sequence within the Rubrivivax subgroup of the β-Proteobacteria nearby *Comamonas testosteroni* and *Brachymonas denitrificans* (Figure 1). Alignment revealed that only three regions within the 16S rDNA of strain P51 contained sufficient variability to distinguish the P51 sequence from its closest relatives (a small part of this alignment is shown in Figure 2).

Within these three regions, sequences that could be used as primers for the PCR were identified and tested for their specificity and their limit of detection. The primer set P1.1 and P1.2 appeared the most suitable combination, since with this set we could amplify a small region of the P51 16S rDNA at an annealing temperature of 60 °C. The smallest initial amount of P51 genomic DNA resulting in a visible product after amplification in the PCR with this primer combination was 0.025 pg. Such an amount corresponds to between 1 and 10 bacteria depending on the number of gene copies assumed per cell. DNA extracted from serial dilutions of a culture of strain P51 amplified in the PCR with primers P1.1 and P1.2 gave a detectable product with as little as ten bacteria (not shown). This means that we could detect the presence of approximately 10^3 cells/ml in unknown samples (due to adding 2 μl of DNA sample to each reaction only). To test the specificity of the amplification reaction with these primers for P51 DNA, we added 1 μg of DNA isolated from the same soil and sludge used in the inoculation experiments. Both environments contained no previous history of distribution of strain P51, which was imported from Wageningen (The Netherlands). No amplification products were detected on ethidium bromide stained agarose gels after 35 cycles with DNAs isolated from the soil sample (data not shown). The amount of background amplifiable DNA in the sludge corresponded to approximately 10^4 indigenous cells/ml (Figure 3A, lane 1), probably arising from related β-proteobacteria which are abundant in sewage sludge (Snaidr et al. 1997). This indicated that we could not specifically detect cells of strain P51 below 10^4 cells/ml in the sludge reactor with the 16S rDNA primers.

Two other primer sets were directed against regions on the *tcbA* and *tcbC* genes, which we applied as marker for the presence of the plasmid-located genes for chlorobenzene degradation. For this purpose, we compared the DNA sequence of the *tcbAa* gene with various other related genes, such as *benA*, *xylX*, *nahAc*, *ndoB*, *todC1*, and *bphA1* (overview shown in: (Werlen et al. 1996)). The *tcbC* gene sequence was aligned with those of *catA*, *clcA* and *tfdC* (Van der Meer et al. 1991a), all encoding catechol 1,2-dioxygenases. On the basis of these alignments, regions with poor conservation were identified and used to define two primers (sense and antisense) specific for the *tcbAa* gene and two for the *tcbC* gene. By applying the *tcbA* and *tcbC*-primers in the PCR we could detect an amplification product with an initial amount of 0.025 pg of strain P51 purified total DNA. Again this corresponds to an amount of 1–10 copies of *tcbA* or *tcbC*, or an equivalent of 1–10 pP51 plasmids. Similarly, when we added DNA isolated from serial dilutions of a growing culture of strain P51, the minimal amount of cells in the PCR which gave rise to a detectable amplification product was between 1–10 (not shown). This cell

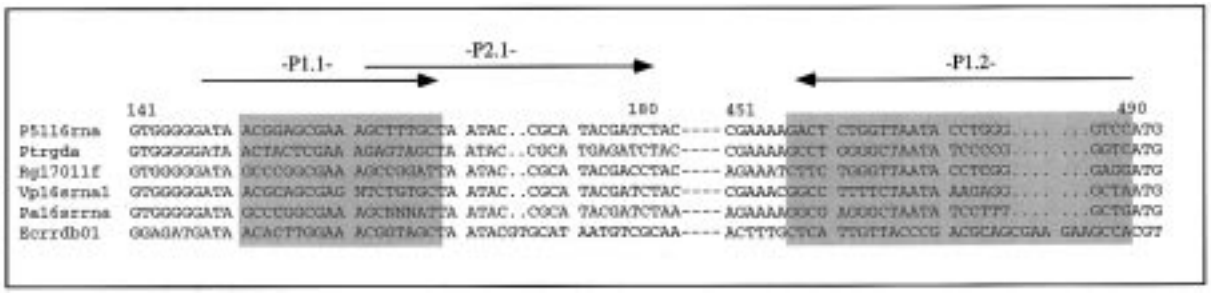

Figure 2. Partial alignment of the 16S rDNA of *Pseudomonas* sp. strain P51 with the most related sequences of *Comamonas testosteroni* (Ptrgda), *Rubrivivax gelatinosus* (Rg17011f), *Variovorax paradoxus*, (Vp16srna1), *Pseudomonas andropogonis* (Pa16srrna), and with *Escherichia coli* (Ecrrdb01). Only the variable relevant regions between nucleotides 141–180 and 451–490 of the sequence alignment are shown. Shaded regions represent variable sequences. The arrows indicate the regions in which specific primers for strain P51 were selected.

number corresponds to a minimal detection level of approximately 10^3 cells/ml in unknown samples.

Survival and activity of Pseudomonas *sp. strain P51 in the soil column*

Before strain P51 was inoculated into the soil column, the system was stabilized for 8 days. No degradation of 1,2,4-TCB was found during this period, indicating that the activity of strain P51 could specifically be followed by analyzing 1,2,4-TCB degradation (Figure 4A). We found approximately 5×10^5 rifampin resistant cells/g of soil on NB agar plates supplemented with 50 μg/ml rifampin (Figure 5A). No amplification products were found with DNA isolated from soil samples at ports C, D and E in the PCR using primers for the 16S rDNA of P51 and the *tcbC*-gene. However, with DNA from samples at ports A and B, amplification products could be detected (corresponding to between 3 and 6×10^5 cells/g soil) (Figures 5B and C). Since no 1,2,4-TCB was being degraded throughout the column up to the time point of P51 inoculation (Day 0, Figure 4A), the positive reaction obtained in the PCR at samples from ports A and B at Day -3 cannot have been due to active cells of strain P51. After 9 days of continuous operation we inoculated 5×10^9 cells of strain P51 pregrown on 1,2,4-TCB at port C (i.e., day 0 in Figures 4 and 5). The next day the TCB-concentration had dropped from 8 μM at the inlet to 2 μM at the outlet (Day +1, Figure 4A), indicating that the cells were actively degrading 1,2,4-TCB. Profile measurements along the length of the column showed a clear decrease in 1,2,4-TCB concentration around the port where the P51 cells were introduced (Figure 4A). In addition, measurements of dissolved oxygen concentrations in inlet and outlet column solution showed a clear decrease in oxygen level (Figure 4B). We could detect the introduced P51 bacteria on NB plates with rifampin, since the number of rifampin resistant bacteria had increased to 3×10^6 cells/g soil at port C at day +1, and 5×10^6 cells/g soil at day +4 (Figure 5A). The establishment of the P51 population in the column could be observed in the period afterwards. The number of cells appearing on NB plates with rifampin increased mainly at ports A and B. At the other ports, the increase was less pronounced. This suggested that the cells migrated towards the column inlet, and distributed according to the gradient of 1,2,4-TCB along the column. Throughout the remaining period (up to 16 days after inoculation), no big changes could be observed in the distribution of the P51 population along the column and the 1,2,4-TCB concentration profile.

The data obtained from PCR quantification for the 16S rDNA and *tcbC* marker of strain P51 more or less followed the trends observed from counting colony forming units. Unfortunately, the variations between individual measurements by PCR exceeded by far those of determining plate counts. At port A, 16S rDNA numbers showed an increase after inoculation to about a level of 10^6 cells/g soil (Figure 5B). Apart from two aberrant measurements at days +7 and +10, the 16S rDNA numbers remained at this level of 10^6 cells/g soil. Measurement of P51 cell numbers on the basis of determining the *tcbC* target resulted in a comparable pattern as those derived from 16S rDNA. Astonishingly, a value of 6×10^5 cells/g soil was measured from tcbC gene copies at day -3 in port A (Figure 5B). However, since no 1,2,4-TCB was being degraded at this moment, this measurement doesn't seem to indicate the presence of active cells of P51 and should be interpreted in the context of all other PCR measurements. Trends for population

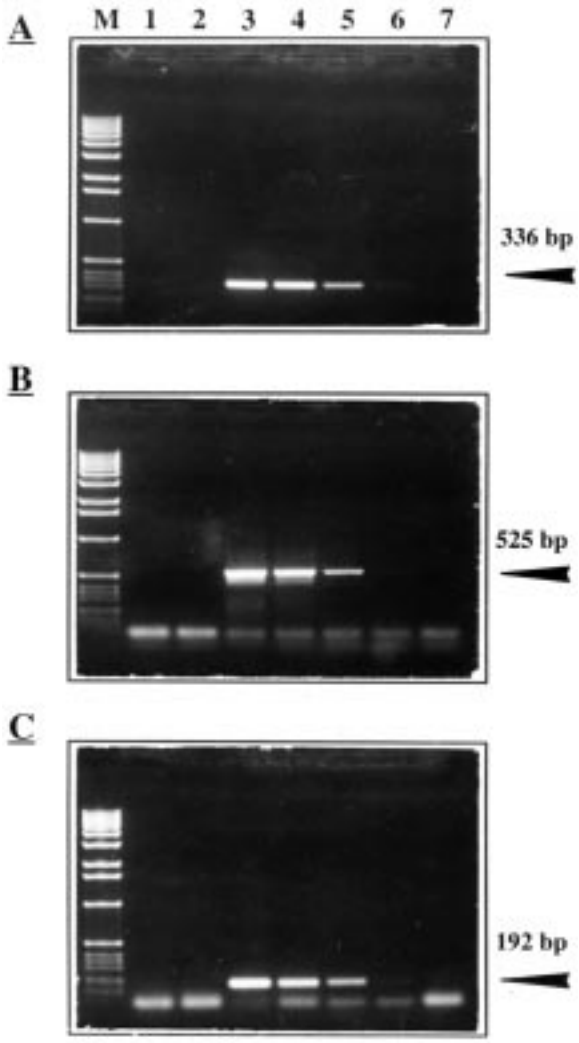

Figure 3. Examples of limiting dilution-PCR detection of strain P51 in sludge. The figure shows ethidium bromide stained agarose gels of PCR products obtained from tenfold serial dilutions of sludge DNA with the different primers for strain P51. (A) PCR product obtained with the 16S rDNA primers, (B) with the *tcbAa* primers and (C) with the *tcbC* primers. Lanes: 1–2; undiluted and 1/10 dilution of sludge DNA taken before inoculation of strain P51. Lanes: 3–7; 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions of DNA samples taken after inoculation of strain P51, respectively.

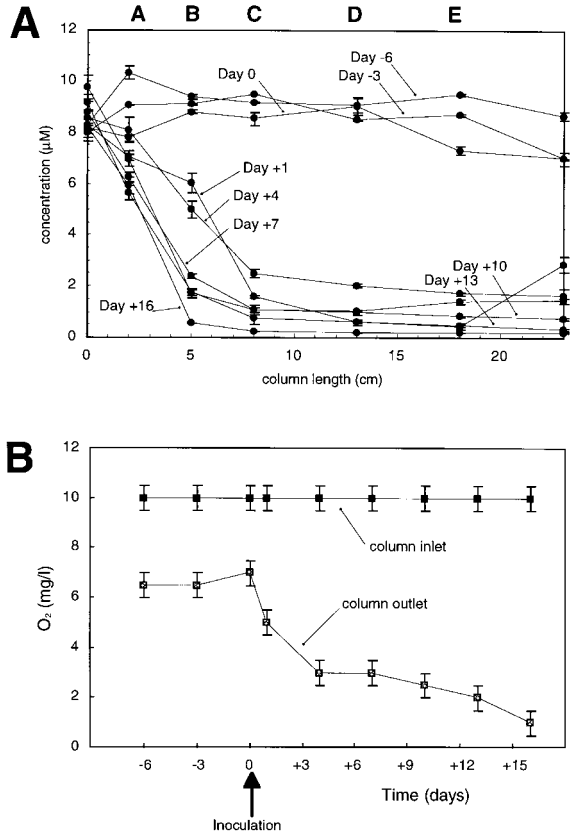

Figure 4. (A) Concentration profiles of 1,2,4-TCB concentrations along the length of the column during the inoculation experiment. (B) Dissolved oxygen concentrations at the column inlet and outlet. Days of incubation before inoculation are indicated as 'minus' days; those after inoculation as 'plus'. Sampling port lettering used in the text is indicated above panel A.

development observed at other ports throughout the experiment showed mostly similar behaviour as those obtained from plate counting (Figures 5C through F). PCR measurements at some ports (e.g., B and D, Figures 5C and E, resp.) turned out better than at other ports.

Overall, although PCR quantification showed the increase in P51 population after inoculation, and their distribution along the whole column, it could not discriminate the differences in active P51 population along the column length between days +7 through 16 observed by plate counting (Figure 5A). On these occasions the PCR-derived numbers were more or less constant along the column at values around 2×10^6 cells/g soil. The data were then analysed by statistical methods for their significance in the following hypotheses. This included only the data of the days after inoculation of strain P51. Are the numbers of P51 cells obtained by amplification of its 16S rDNA or *tcbC* gene in the PCR significantly higher than the numbers obtained by counting colony forming units on NB plates with rifampin? Are the numbers obtained by amplification of 16S rDNA and those of the *tcbC*-gene significantly different, and did the differences between the viable counts and numbers obtained by 16S rDNA and *tcbC* gene amplification change along the column or during the time course of the experiment? The

signed rank test indicated that for all data points during the whole experiment the numbers of strain P51 obtained by amplification of the 16S rDNA were significantly higher than those obtained by amplifying the *tcbC* target sequence (at P = 0.01). The numbers obtained by 16S rDNA or *tcbC* amplification were only weakly significantly different from those obtained by counting colony forming units on rifampin plates (at P = 0.1). Trend analyses substantiated the following observations. The ratio of numbers obtained in the PCR (for both markers) to those obtained by viable countings increased significantly in samples taken at the end of the column as determined by regression analysis (correlations of 0.894 and 0.978, respectively), with a probability of $p<0.05$ that the regression curve is equal to zero. A visual comparison of Figure 5B (data of port A) and Figure 5F (data of port E at the end of the column) illustrate this effect. We found no significant trend in a change of the ratios during the time course of the experiment. In addition, there was no trend in the ratios of the numbers determined by 16S rDNA amplification to those of *tcbC*, neither during time nor along the length of the column.

Survival and activity of Pseudomonas *sp. strain P51 in the sludge reactors*

In our second experimental system, we introduced strain P51 into sludge reactors, and followed its survival and activity during time in the presence or absence of 1,2,4-TCB. Before inoculating strain P51 to the reactor, we let the sludge bacteria adapt for two weeks in the two reactors to the operating conditions. The OD_{600} of the bacterial suspension in the reactions fluctuated around a value of 1.3, which corresponds to approximately 10^9 cells/ml. We could not detect any amplification products from sludge DNA in the PCR with the *tcbA* and the *tcbC* primers, indicating that these genes were not present above a level of 10^3 copies/ml in both reactors at the time before inoculation of strain P51. We did, however, detect amplifiable DNA in the PCR with the primers for the 16S rDNA of strain P51 (see above). Counting colony forming units on NB plates with rifampin indicated that around 10^6 cells/ml in the sludge were rifampin resistant. On Z3 mineral plates with 1,2,4-TCB no colonies appeared.

After two weeks of continuous operation, we added 4×10^7 cells/ml of strain P51 into the sludge reactors (Figure 6). This number was determined by counting the inoculum in a Buerker counting chamber, and by counting the number of rifampin resistant colonies in samples taken from both reactors directly after inoculation. In DNA isolated from samples which were taken directly after inoculation of P51, we estimated a P51 population size of 10^7 cells/ml by the limiting dilution-PCR method (with the *tcbA* and *tcbC* primers) and 10^8 cells/ml (with the 16S rDNA primers). Inoculation of strain P51 into the sludge microcosms did not induce any major changes in the total bacterial density. The OD_{600} remained more or less the same during the experimental period. One day after inoculation, the number of colony forming units on NB plates with rifampin and on Z3 plates with 1,2,4-TCB dropped below 10^5 cells/ml. We could, therefore, no longer use this method to assess the survival of strain P51 in the sludge reactors and had to rely solely on quantifications obtained by the PCR. PCR quantification results with all three primer sets also indicated that the cell numbers of strain P51 decreased dramatically within one day from 10^8 cells/ml to 10^6 cells/ml in reactor A (with 1,2,4-TCB) and to 10^5 cells/ml in reactor B (without) (Figure 6). We observed such a decrease repeatedly in independently performed inoculation experiments in these reactors.

In reactor B to which no 1,2,4-TCB was added, the population of P51 remained at a stable value of 10^5 cells/ml for the following 7 days, but then declined to an undetectable low number as determined in the PCR with the 16S rDNA primers (Figure 6). Quantification of the number of *tcbC* gene copies, however, gave consistently lower values, indicating that the bacteria perhaps did not retain the P51 plasmid. Two days after inoculation the number of *tcbC* gene copies had decreased to 10^4 per ml and declined even further to 10^3 per ml after 8 days. Quantification of the number of *tcbA* gene copies from day 1 until day 6 revealed a similar trend as observed for the number of *tcbC* gene copies. From day 8 on until the end of the experiment at day 15, the amount of *tcbA* gene copies increased again to 10^4 per ml.

In reactor A to which 1,2,4-TCB was added, the decrease in cell numbers of strain P51 was even faster (except for the first two days) than in reactor B (Fig. 6). During the first eight days after inoculation the numbers of gene copies for *tcbA*, *tcbC* and 16S rDNA decreased steadily to below their detection limits. The cells of strain P51 did not measurably degrade 1,2,4-TCB in the reactor, except perhaps for the day directly after the strain was inoculated. On this instance we measured a slight decrease in TCB-concentration (Figure 6A).

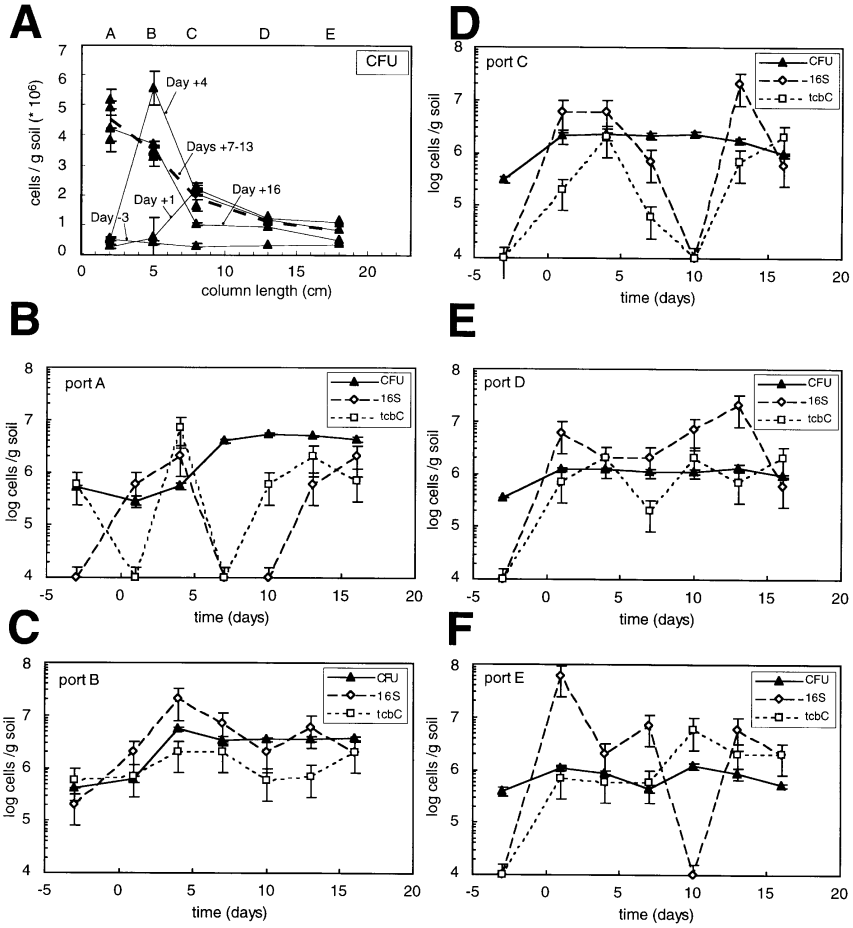

Figure 5. Population dynamics of *Pseudomonas* sp. strain P51 in the soil column during time and along the length of the column. (A) CFUs of strain P51 on NB plates plus rifampin. Days before inoculation are indicated as 'minus' days; those after inoculation as 'plus'. Sampling port lettering used in the text is indicated above panel A. (B) through (F) P51 Population development during time at the different sampling ports of the column. Data points show mean values plus standard error from triplicate measurements (for CFUs) or duplicate measurements (for PCR-derived numbers on the basis of 16S rDNA of *tcbC* target amplification). Individual PCR measurements were accurate within a plus- or minus threefold dilution with 99.5% confidence.

Discussion

Effective assessment of the capabilities of bacteria introduced into environmental systems requires the study of their maintenance and activity. A first necessity is then to be able to monitor population dynamics of the inoculated strain and, ideally, follow its metabolic activity. Different physiological and molecular genetic methods are available for this purpose. However, not one single method probably can give definitive answers on population dynamics and activity. In this study we attempted to use PCR quantification in combination with specific plating and concentration measurements to determine the presence and activity of an introduced chlorobenzene degrading microorganism. Very few reports actually address the use of the PCR for quantifying bacterial populations (Leser et al. 1995; Watanabe et al. 1998); mostly it is used for sensitive detection only. For example, Lévesque et al. (1997) used the PCR to detect survival of inoculated *Desulfitobacterium frappieri* PCP-1, a pentachlorophenol degrader in soil. El Fantroussi et al. (1997) detected survival of *Desulfomonile tiedjei*, inoculated with the purpose of enhancing anaerobic 3-chlorobenzoate degradation, by PCR. Recently Watanabe et al. (1998) demonstrated use of a quantitative PCR method to monitor the populations of two phenol degraders with distinct kinetic differences for phenol utilization inoculated to aerobic sludge.

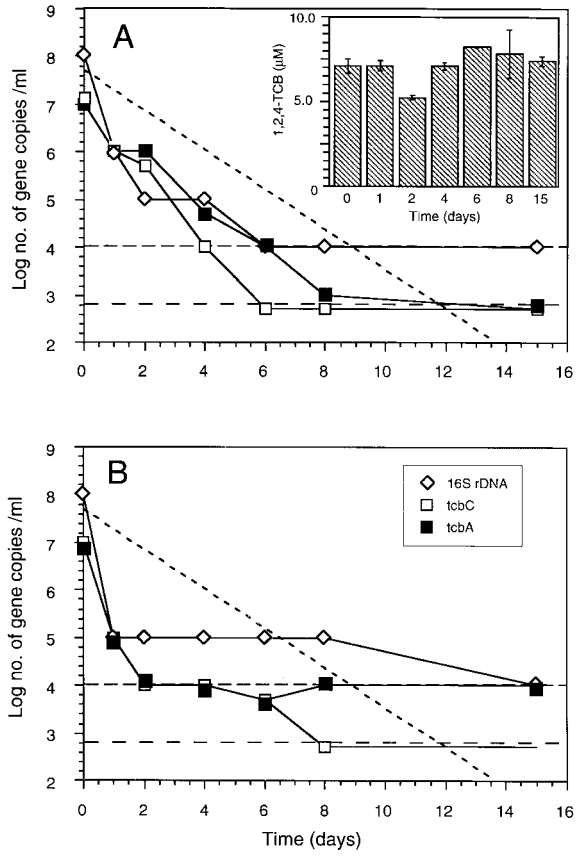

Figure 6. Survival of *Pseudomonas* sp. strain P51 in the sludge reactors. (A) Reactor with 1,2,4-TCB added, (B) without 1,2,4-TCB. PCR-derived numbers for strain P51 in sludge samples by using different primer sets are shown, for *tcbAa* for *tcbC*, and for the 16S rDNA. The horizontal dashed lines indicate the specific detection limits for the *tcb* (at log 3) and the 16S rDNA (at log 4) target genes of strain P51. The dotted line represents the theoretical washing-out curve for a non-growing population of strain P51 at the applied dilution rate. The inset displays the measured dissolved concentrations of 1,2,4-TCB in reactor A. All measurements were performed in duplicate and were accurate within a plus- or minus threefold dilution with 99.5% confidence. The figures show typical results of experiments repeated three times.

In our study it was indeed possible to detect strain P51 by using the PCR among a very high number of other bacteria (e.g., 10^3–10^4 cells/ml among 10^9 indigenous bacteria/ml of sludge), whereas quantification with selective plating became impossible as soon as the population size decreased below 10^6 cells/ml. This was possible by using very specific primers for the *tcbC*- and *tcbA*-targets of strain P51. The use of the 16S rDNA as target in the PCR was not completely specific for strain P51. Especially in the sludge environment, a background level of around 10^4 cells/ml was measured even without inoculation of the strain, probably due to the presence of related β-Proteobacteria (Snaidr et al. 1997). Larger problems for the routine use of the PCR to quantify strain P51's population were the occurrence of false positive and false negative results, and considerable sample to sample variations. For example, some samples of the soil column resulted in a clear *tcbC*-derived number before inoculation of strain P51 (Figure 5B). This was unexpected and may indicate either a laboratory contamination during the PCR or a contamination of the soil in the column. In addition, on some occasions unexpectedly no PCR signals were obtained (Figures 5B, 5D). These results indicate that PCR measurements on samples taken from complex communities involving various handling steps, or from environments where exact reproduction of a single sample is impossible, are prone to false positive and false negative results. For a proper data interpretation one will therefore have to rely on simultaneous complementary techniques (classical platings, activity measurements), and on analyzing many samples distributed spatially and timely. Similar difficulties were recently described by one of us in a groundwater study (Van der Meer et al. 1998). Finally, the use of limited dilutions to quantify target numbers in the PCR involved many laborious steps, which in this study may have resulted in considerably sample-to-sample variations, further complicating the exact interpretation of microbial population dynamics.

Despite all the limitations of the PCR quantification on complex systems, and taken together the complementary analyses which we performed in our studies, we are confident to have shown the strong effect of inoculating P51 cells on 1,2,4-TCB degradation in a soil environment. Other aspects, like loss of culturability of the introduced cells, loss or transfer of the plasmid pP51, could not clearly be discerned. Some trends were supported by statistical analyses, such as consistently higher P51 population size at the end of the column determined by PCR than by platings. However, the interpretation of these trends remains presently speculative. It became clear that strain P51 was able to colonize the entire column 15 days after its inoculation. In addition, the cells degraded 1,2,4-TCB to undetectable amounts, which was ample indication for their in situ activity. The final distribution of the bacteria in the column corresponded rather well to the TCB concentration profile. In contrast, strain P51 was not able to maintain a detectable population size in the sludge reactors, and no apparent degradation of 1,2,4-TCB could be observed.

In the sludge microcosm we observed two main phenomena; i) apparently the conditions were not favourable for survival of strain P51, and ii) the presence of 1,2,4-TCB posed no selective advantage for maintenance and growth. Since the inoculum of P51 was grown under the same conditions for both the soil column and the sludge reactors, the reasons for the differences in survival and activity must lay in the ecosystem to which the cells were introduced. One day after inoculation into sludge less than one per cent of the introduced cells had remained. An active process, such as predation by existing protozoa in the sludge (McClure et al. 1989) must be responsible for this decline, since the disappearance of strain P51 was much faster than the calculated wash-out from the reactor (modelled wash-out behaviour indicated by the dotted line in Figure 6). After the initial decline, the cells could more or less establish a small population of 10^4–10^5 per ml in the reactor without 1,2,4-TCB added, similar to trends observed in other inoculation studies in activated sludge (McClure et al. 1989; Watanabe et al. 1998). Contrary to what we expected, the cells did not establish themselves in the reactor to which 1,2,4-TCB was added, and declined even sharper than in reactor B. This may indicate that a toxic metabolite is formed by the cells. The toxic metabolite might be chlorocatechol itself, which is transiently formed because the genes for oxidation of chlorobenzene to chlorocatechol are more or less constitutively expressed, whereas those for chlorocatechol degradation are regulated (Van der Meer et al. 1991b). Once P51's population declined too much, it apparently did not regrow to detectable size, indicating that the presence of 1,2,4-TCB in the sludge reactors posed no selective advantage for development of a detectable population.

Although the concentrations of 1,2,4-TCB added to both soil column and sludge reactor were approximately the same (8 μM), the cells in the sludge did not seem to take advantage of their specific substrate. When one assumes that 8 μM 1,2,4-TCB was available to strain P51 in the reactor at all times, this can sustain a population size of approximately 3×10^5 cells (Van der Meer, unpublished data). Interestingly, we did not find such a population size, but at most around 10^3 cells per ml. This large difference between population size expected from growth kinetic considerations and measured was also observed in other inoculation studies performed with *Pseudomonas* sp. strain B13 (Ravatn et al. 1998), and seemed to indicate that the bacteria inoculated to sludge waste considerable part of the carbon substrate. An interesting hypothesis for this phenomenon is that the inoculated bacteria need to compete for essential trace elements which are limiting in mixed culture systems with relatively high density of microorganisms (such as sludge), and excrete part of their assimilated carbon as iron chelators (Egli 1995). In the soil columns, the total density of bacteria was tenfold lower than in sludge and more iron may have been available from soil particles, enabling strain P51 to convert more incoming 1,2,4-TCB to biomass.

For many years inoculation of microorganisms (bioaugmentation) has been considered an important method to stimulate cleaning up of specific pollutants in natural environments and semi-technical systems (Goldstein et al. 1985; McClure et al. 1989; Fujita et al. 1991; Pipke et al. 1992; Wagner et al. 1992). Our report can add that inoculation of preselected strains from the environment is possible in order to achieve enhanced degradation of difficult compounds such as chlorobenzenes. However, the use of this specific strain seemed limited to a soil environment. Considerable discussion still exists on the reasons governing survival and in situ activity of inoculated strains (Wagner et al. 1992; Recorbet et al. 1993; Blasco et al. 1995; Megharaj et al. 1997), and thus their applicability. Most inoculation studies have shown that the population size of introduced strains decline strongly in mixed microbial ecosystems (McClure et al. 1989; Postma & van Veen 1990; Watanabe et al. 1998). Nevertheless, inoculated strains often maintain themselves, and a relatively low population level may still be sufficient for stable removal of the target compound (Pipke et al. 1992; Brunsbach & Reineke 1993; Ravatn et al. 1998). On the other hand, some strains were shown to be particularly effective in colonizing or maintaining themselves (McClure et al. 1991; Megharaj et al. 1997), which was attributed to the fact that the strains were 'preadapted' to the prevailing conditions or originating from the same environment. This would also be an explanation for the supreme survival of strain P51 in the soil column, since the strain was originally isolated from sediments. Unfortunately, such factors are ill-defined and may mask underlying principles, which need further detailed study.

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