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# Quantification of bacterial mRNA involved in degradation of 1,2,4-trichlorobenzene by *Pseudomonas* sp. strain P51 from liquid culture and from river sediment by reverse transcriptase PCR (RT/PCR)

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**Abstract**

Competitive reverse transcriptase polymerase chain reaction (RT/PCR) was used to quantify the mRNA of the *tcbC* gene of *Pseudomonas* sp. strain P51. The *tcbC* gene encodes the enzyme chlorocatechol-1,2-dioxygenase involved in 1,2,4-trichlorobenzene (TCB) degradation. The mRNA content per cell was monitored in a batch culture growing on 1,2,4-TCB. No mRNA could be detected in the first 2 days of the lag phase. mRNA production became maximal with 20 molecules per cell in the early exponential growth phase but then decreased to less than 10 molecules per cell. When TCB was depleted and the cells entered the stationary phase, the mRNA content decreased slowly below the detection limit within 4 days.
In order to compare detection of *tcbC* mRNA in pure culture and in river sediment, cells of strain P51 pregrown on TCB were added to sediment and RNAs extracted. In sediment samples containing $5\times10^8$ cells per gram the *tcbC* mRNA was quantifiable by RT/PCR. The mRNA recovery was about 3% as compared to the inoculum. The detection limit of the RT/PCR method was about $10^7$ mRNA molecules per gram sediment or $10^6$ copies per ml culture medium which corresponded in our case to $10^5$ molecules per reaction vial. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

*Keywords:* Biodegradation; Quantitative PCR; Bioremediation; Xenobiotic; Chlorinated hydrocarbon

## 1. Introduction

The detection of bacterial activity in the environment is an important topic in microbial ecology and especially in bioremediation. If strains with specific degradation capacities are to be used for bioaugmentation one would like to know if the strain survives in the environment and if it is actively degrading the contaminant. Determination of the population size of the respective organism in the environment is hereto not sufficient since the specific bacterium might grow on other more easily available substrates or even lose its degradative capacity.

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An elegant method to monitor in situ activity would be the detection of specific bacterial mRNAs. This method could even distinguish between isoenzymes or different enzymes catalyzing identical reaction but originating from different organisms. The main difficulties, however, for mRNA detection are the instability of bacterial mRNAs (i.e. half life times of 1–3 min), the poor sensitivity of established detection methods and the problem of selective preparation of mRNA from very complex systems like soil. Nevertheless, some attempts have been at least partially successful [1,2]. Here we show the application of competitive reverse transcriptase PCR for the detection and quantification of a specific bacterial mRNA in a river sediment.

The 1,2,4-TCB degrading organism *Pseudomonas* sp. strain P51 was taken as a model organism [3]. In this strain the catabolic enzymes for TCB degradation are encoded by two gene clusters, *tcbAB* and *tcbCDEF* [4]. The *tcbAB* operon codes for both chlorobenzene dioxygenase and chlorobenzene dihydrodiol dehydrogenase which catalyze the conversion of 1,2,4-TCB to 3,4,6-trichlorocatechol. The *tcbCDEF* genes encode the enzymes for subsequent chlorocatechol degradation, i.e. chlorocatechol-1,2-dioxygenase (product of the *tcbC* gene), chloromuconate cycloisomerase, dienelactone hydrolase and maleylacetate reductase. The *tcbC* gene was taken as the target sequence for the detection of specific mRNA for TCB degradation because it is the first gene on the *tcbCDEF* operon and the first part of the mRNA which is degraded by RNase. Thus, the detection of *tcbC* mRNA provides information on the abundance of intact mRNA rather than partially degraded fragments.

## 2. Materials and methods

### 2.1. Growth of bacteria

*Pseudomonas* sp. strain P51 was taken from the laboratory culture collection [3]. Strain P51 was grown in a 300 ml batch culture on mineral medium Z3 with 250 mg $l^{-1}$ 1,2,4-TCB as sole carbon and energy source in a 3 l gas-tight Erlenmeyer flask [5]. Growth was followed spectrophotometrically at 578 nm and by plate counts of colony forming units on nutrient broth (NB) medium (Biolife, Italy).

If not otherwise stated, nucleic acid techniques were performed according to standard protocols. As far as possible, all solutions were treated with diethylpyrocarbonate (DEPC) in order to inactivate contaminating RNAses.

### 2.2. Plasmids

The *tcbC* gene of strain P51 was chosen as the target for competitive RT/PCR. Plasmid pTCB82 [6] contained the *tcbC* gene in a pT7-5 expression vector [7]. For competitive PCR, plasmid pRMTC1 was constructed which carried a deletion of 23 basepairs between a *Sal*I and a *Bam*HI site present in the *tcbC* gene of pTCB82 (Fig. 1). The deletion was located in the middle of the DNA fragment to be amplified by PCR with primers 940207 (5′-GAC TTC TGG ATG CTC CTA CTG AGC GT-3′) and 940206 (5′-GC GTC GGC TAC ATG ATG AAA CTG GC-3′). pTCB82 was partially digested with *Sal*I and completely with *Bam*HI. The overhangs were made blunt ended by using Klenow DNA polymerase and both ends were religated. Transformation in *E. coli* DH5α produced the plasmid pRMTC1. The deletion was checked by DNA sequencing. In vitro mRNA was produced from plasmids pRMTC1 and pTCB82 by using T7 RNA polymerase according to the instructions by the supplier (Boehringer, Mannheim, Germany), purified with acidic phenol plus chloroform extraction and precipitated in aliquots with ethanol at −20°C.

### 2.3. RNA isolation

For purification of mRNA from liquid bacterial cultures 1.5 ml aliquots were centrifuged at $10\,000 \times g$ in an Eppendorf centrifuge. Supernatant was discarded, and the cells were suspended in 200 μl lysis buffer I (20 mM Na-acetate, pH 5.5, 1 mM EDTA, 0.5% SDS). The cell suspension was extracted three times with 200 μl acidic phenol (pH 5.5), once with neutral phenol plus chloroform (pH 7.5) and once with chloroform. The purified RNA was precipitated from the water phase with ethanol and Na-acetate (100 mM, pH 5.5) and stored at −20°C.

Concentrations of mRNA were determined spectrophotometrically.

For preparation of mRNA from a sediment sample, 0.4 g sediment was mixed with 0.4 g glass beads (0.1 mm diameter), 75 mg polyvinylpolypyrolidon (PVPP) [8] and 1 ml of lysis buffer II (200 mM Tris/HCl, pH 8.5, 1.5% SDS, 10 mM EDTA, 1% Na-desoxycholate, 1% Nonidet-P40, 5 mm thiourea, 10 mM DTT) in an Eppendorf vial. Samples were treated twice in a cell homogenizer (type 853022/0, Braun, Melsungen, Germany) at 4000 rpm for 1 min with 1 min intermittent cooling on ice. After breaking the cells, the sample was centrifuged at $15\,000 \times g$ for 2 min at 4°C and the supernatant was transferred to an equal volume of acidic phenol. The sample was centrifuged at $10\,000 \times g$ for 2 min after a 5 min incubation at 65°C and the RNA was purified as described above for the isolation from liquid cultures.

All RNA samples were treated with RNase free DNaseI (Boehringer, Mannheim, Germany) for 30 min at 37°C, again purified by extractions with phenol plus chloroform (once) and chloroform only, and finally precipitated with ethanol and Na-acetate as described before. Integrity of the RNA samples was routinely checked on agarose gels and the appearance of the three bands of ribosomal RNA were taken as an indication of sufficiently intact RNA.

### 2.4. RT/PCR

Competitive RT/PCR was performed according to the standard protocol described in the Perkin Elmer Tth DNA polymerase EZ-kit with the two primers 940206 and 940207, where primer 940207 served for cDNA generation as well. Depending on the target *tcbC* mRNA concentration, a constant amount of 0.75 μl in vitro produced mRNA of pRMTC1 containing either $10^6$ or $10^7$ copies was used as internal competitive standard in the RT/PCR to which 0.75 μl of a dilution series of the isolated sample mRNA was added. RT/PCR was run with the following temperature protocol: (RT reaction) 65°C, 1 min; 42°C, 5 min; and 65°C, 30 min; then 40 cycles PCR amplification of: 85°C, 0.1 min; 93.5°C, 1 min; 62°C, 1 min; and 72°C, 1 min. As a final step the reactions were incubated at 72°C for 6 min. Amplification products were separated and analyzed by agarose gel electrophoresis after PCR. The target mRNA concentration present in the sample was estimated from the dilution step at which the amplification products of the standard mRNA and the target mRNA were present in equal amounts. Quantification was always performed on three parallel extractions of sample aliquots.

Dot blot analysis was carried out according to standard protocols on Nylon membranes (Qiagene). RNA loaded membranes were hybridized for 4 h at 54°C with $^{32}P$ end-labeled oligonucleotide 940207 and exposed to Kodak X-OMAT films. Intensities of the hybridization signal of each spot were quantified on a laser densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

### 2.5. Analysis of TCB concentrations

1,2,4-TCB was extracted from 2 ml culture aliquots by shaking with an equal volume of hexane for 3 min. The hexane phase was analyzed on a Varian, Star 3400 CX gaschromatograph/electron-capture-detector (GC/ECD) with a DB5 column. Carrier gas was helium operated at a velocity of 43 cm $s^{-1}$.

Chloride concentrations were measured with a Chlor-o-counter (Flohr Instruments B.V., Nieuwegein, The Netherlands).

River sediment was taken from River Glatt near Dübendorf, Switzerland. The dry weight consisted of 2% coarse sand ($>0.5$ mm), 24% middle sand (0.5–0.25 mm), 67% fine sand fraction (0.25–0.1 mm diameter), 4% very fine sand (0.1–0.05 mm) and 2% silt ($<0.05$ mm) with an organic content of 2%.

## 3. Results

The applicability of competitive RT/PCR for quantification of *tcbC* mRNA was first tested on in vitro synthesized mRNA. The amplification efficiencies of known amounts of in vitro mRNA from plasmid pTCB82, representing the native sequence, were compared to those of the shorter internal standard derived from plasmid pRMTC1. In a competitive assay with both RNAs present in the same reaction vial, equal amounts of amplification products were observed at concentrations of both

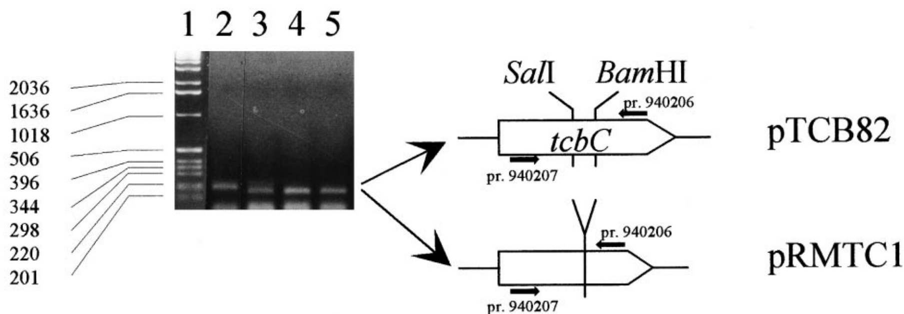


Fig. 1. Agarose gel electrophoresis of the amplification products from a competitive RT/PCR of pRMTC1 in vitro mRNA and *tcbC* target mRNA prepared from a liquid culture of strain P51. The pRMTC1 standard mRNA was kept constant at $10^6$ copies per vial. Extracted mRNA was added in different amounts to the reaction vials. Lane 1, DNA molecular mass marker (Boehringer, Mannheim); lanes 2–5, $10^0$–$10^{-3}$ diluted mRNA respectively. The cartoon shows a schematic view of the target and standard DNA used in this work.

the shorter standard mRNA and the pTCB82-derived mRNA of $10^6$ molecules per reaction vial (data not shown). This indicated there were no strong differences in the amplification efficiency of the two RNA sequences.

Next, mRNA was extracted from a culture of strain P51 and dilutions were prepared to which the standard RNA was added. At ratios of standard mRNA to sample mRNA of 3:1 and 1:3 (with constantly $10^6$ molecules of standard mRNA present) amplification products clearly differed in band intensity in the agarose gel electrophoresis. When the sample mRNA was present in excess as compared to the standard RNA only the larger amplification product of the normal mRNA sequence was visible (Fig. 1, lane 2). When both mRNAs were present in identical amounts, both bands were visible (Fig. 1, lane 3) and when the standard mRNA was present in excess to the sample mRNA only the shorter amplification product was visible (Fig. 1, lane 4). These data were verified independently with dot blot hybridization of the isolated mRNAs to a *tcbC* antisense directed oligonucleotide (not shown).

We then tried to determine the *tcbC* mRNA content of a growing batch culture of strain P51 on 1,2,4-TCB. After a short lag phase of 2 days the cells grew exponentially until they reached the stationary phase on day 4 (Fig. 2A). The culture medium was constantly saturated with 1,2,4-TCB (45 μM) during the first 3 days and excess 1,2,4-TCB was present as a second liquid phase. On day 4, 1,2,4-TCB concentration decreased drastically to below the detection limit of 50 nM and the cells entered the stationary phase as the carbon substrate became limiting. Measurements of the chloride release indicated that the most active degradation of TCB took place be-

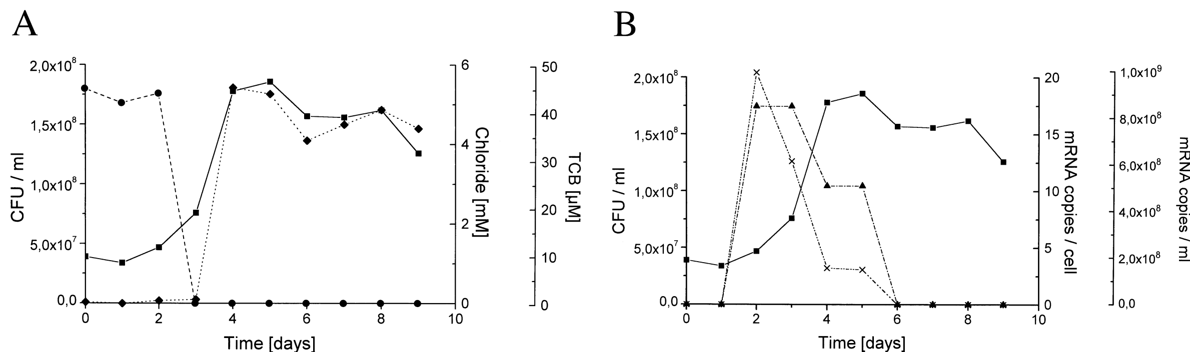


Fig. 2. Growth curve of *Pseudomonas* sp. strain P51 on TCB. A: Solid line, cell density as colony forming units (cfu); dashed line, TCB concentration; and dotted line, chloride concentration. B: Solid line, cell density; dashed and dotted line, mRNA concentration; and dashed and double dotted line, mRNA molecules per cell.

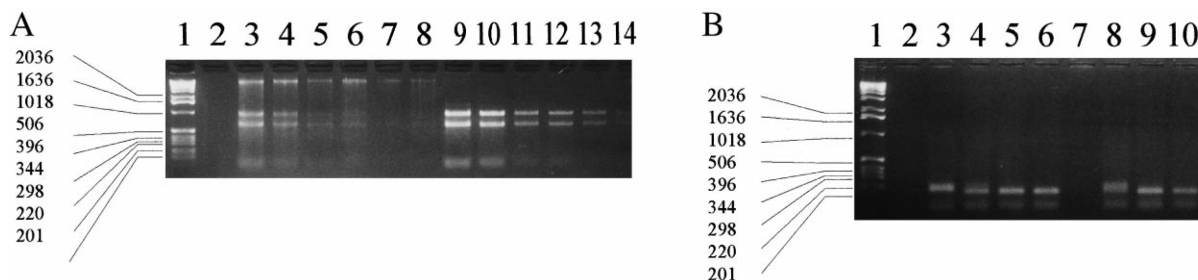


Fig. 3. A: Agarose gel electrophoresis of RNA before DNaseI digestion isolated from river sediment which was spiked with strain P51 cells. Lane 1, DNA molecular mass marker; lanes 3 and 4, $10^8$ cells per g sediment; lanes 5 and 6, $3\times10^7$ cells per g sediment; lanes 7 and 8, $10^7$ cells per g sediment. Lanes 9–14 show the RNA extracts of the same amount of cells from the liquid culture inoculum. Lanes 9 and 10, $10^8$ cells per ml; lanes 11 and 12, $3\times10^7$ cells per ml; lanes 13 and 14, $10^7$ cells per ml. B: Agarose gel electrophoresis of a competitive RT/PCR amplification of *tcbC* mRNA purified from a liquid culture grown on 1,2,4-TCB and from the same amount of cells inoculated to sediment. Lane 1, molecular mass marker; lanes 3–6, amplification products of diluted mRNA extracted from a liquid culture containing $10^8$ cells ($10^0$–$10^3$-fold diluted respectively). Lanes 8–10, dilution series of mRNA extracted from $5\times10^8$ cells added to sediment ($10^0$–$10^2$-fold respectively). All PCR reactions were performed in the presence of $10^7$ copies of in vitro synthesized standard RNA.

tween day 3 and 4. On each day of the batch experiment, aliquots were taken for mRNA quantification by competitive RT/PCR and dot blot hybridization (Fig. 2B). No mRNA production was detectable in the first two days of the lag phase until on day 3 maximal induction was observed. The amount of *tcbC* mRNA was constantly high during day 3 and 4 with about $10^9$ molecules per ml culture and decreased slowly on day 5 and 6 to $3\times10^8$ molecules per ml when 1,2,4-TCB became limiting. When analyzed on a per cell basis the mRNA copies decreased from 20 per cell on day 3 to 13 on day 4 and to approximately 3 *tcbC* mRNA molecules per cell on day 5 and 6. There was no *tcbC* mRNA production detectable on day 7 or later. Dot blot hybridization of the mRNA samples also indicated highest amounts of *tcbC* mRNAs during day 3 and 4 (not shown).

In order to see whether *tcbC* mRNA could be extracted from soil and quantified, we introduced different amounts of strain P51 cells (pregrown on 1,2,4-TCB) to a river sediment. RNA of sufficient intactness could be recovered from the seeded river sediment as indicated by the rRNA bands (Fig. 3, lanes 3–8). Simultaneously, mRNA was purified from the same amounts of cells as used for the inoculum (Fig. 3, lanes 9–14). rRNA bands from sediment samples were less intensive indicating an unspecific loss or degradation of RNA.

The efficiency of mRNA recovery from soil was analyzed with a river sediment to which $5\times10^8$ $g^{-1}$ sediment TCB-grown strain P51 cells were added. After DNaseI digestion mRNA concentrations were quantified with competitive RT/PCR (Fig. 3B). $4.5\times10^7$ copies *tcbC* mRNA per g sediment were recovered from $5\times10^8$ cells seeded per g sediment. In contrast, $3.5\times10^8$ copies *tcbC* mRNA were quantified from $10^8$ cells per ml of the liquid culture inoculum which corresponds to 3.5 copies per cell. Thus, only about 3% of the *tcbC* mRNA was measured in the sediment samples to which strain P51 cells were added as compared to the extracts of the pure culture inoculum. This reduced the detection limit for the sediment samples to about $10^7$ copies per gram sediment and corresponds to $10^5$ molecules per reaction vial.

## 4. Discussion

mRNA of the *tcbC* gene of *Pseudomonas* sp. strain P51 was quantified in batch cultures growing on 1,2,4-TCB and in a river sediment to which strain P51 was added. The project was initiated to reveal if mRNA quantification with competitive RT/PCR can be used as a tool to estimate bacterial activity in the environment. Although it is rather difficult to obtain quantitative measurements of initial target

copy numbers with PCR more or less accurate determinations can be performed by using competitive PCR with an internal standard [9]. The internal standard at best consists of the same RNA sequence as the target RNA but carries for example a small insertion or deletion which allows to distinguish the amplification product of the standard from that of the target. Competitive PCR is especially advantageous with environmental samples because these often contain contaminants like humic acids which could inhibit the reaction. In the competitive assay both the target and the standard RNA face identical conditions for amplification.

RT/PCR has been used in the past to detect mRNA of viruses and fungi from environmental samples [10]. We were able to quantify mRNA with a detection limit of about $10^5$ mRNA copies per RT/PCR vial which, due to the different recoveries from either a pure culture or sediment, corresponded to $10^6$ copies per ml culture medium or $10^7$ copies per gram sediment, respectively. Although the method in principle detects mRNA to a relatively low level, the question remains what a particular number of target mRNA copies will mean in terms of activity of the population. In a batch culture experiment, the mRNA induction pattern showed several phases of mRNA abundance. A first uninduced lag phase with undetectable low mRNA numbers was followed by a second phase of highest net mRNA synthesis in the start of the exponential growth phase and a third phase of decreasing mRNA synthesis comprising the late exponential and the stationary growth phase. Surprisingly, mRNA production per cell was not maximal in the exponential growth phase with the highest substrate turnover but in the first part of the log phase. Similar results have been obtained for quantification of manganese peroxidase mRNA levels of the white rot fungus *Phanerochaete chrysosporium* [10]. In the batch experiment TCB was present as a non-aqueous phase liquid and the water phase remained constantly saturated as long as TCB was detectable. Nevertheless, the induction of the *tcbC* gene, as measured by the mRNA content per cell, was not constant. In analogy to the 3-chlorocatechol pathway of *Pseudomonas putida* (pAC27) the transcription inducer of the *tcbC* gene is very likely to be 2,3,5-trichloro-cis,cis-muconate which is formed by the activity of the TcbC protein (the chlorocatechol-1,2-dioxygenase) from 3,4,6-trichlorocatechol [11–13]. It might be that the inducer accumulated in the early exponential growth phase because the activity of TcbC was higher than the activity of the chloromuconate-converting enzyme chloromuconate cycloisomerase encoded by the *tcbD* gene of the same operon. In a later growth phase the activity of the chloromuconate cycloisomerase might have increased as compared to the chlorocatechol dioxygenase activity and the chloromuconate concentration was reduced.

In the environment many habitats are oligotrophic with little substrate turnover and especially in bioremediation degradation processes are very slow due to limited substrate availability or lack of suitable electron acceptors [14]. Thus, induction of the catabolic genes and mRNA production will be low as well. mRNA content per cell will be less than one molecule per cell in an oligotrophic environment as indicated by the batch culture. When induced, it is possible to recover and quantify specific mRNAs from sediment samples by RT/PCR in reasonable amounts as it was shown by our inoculation experiment. The efficiency of mRNA recovery might depend on the type of soil sample and the target bacterial species of interest as humic acid content, physiological state of the cells or gram type can influence the extraction procedure significantly. Environmental applications of RT/PCR are at present restricted by the problem of the detection limit as long as no better methods are available which increase the sensitivity by several orders of magnitude.

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