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Abstract: 4-Chlorosalicylate (4-CS) can be degraded completely by a bacterial consortium consisting of *Pseudomonas reinekei* (MT1), *Achromobacter spanius* (MT3) and *Pseudomonas veronii* (MT4). The fourth species *Wautersiella falsenii* (MT2) is thought to act as a 'necrotizer' of the community. Single cell approaches were used to follow every species' degradation activity within the community by assuming that growth and proliferation are activity markers for the utilization of 4-CS and its degradation pathway intermediates as carbon and energy sources.

A primary/secondary antibody staining technique for species differentiation was applied and a species-resolved determination of proliferation activity by flow cytometry undertaken. Degradation was followed by quantifying 4-CS and the resulting intermediates by HPLC. A good correlation of HPLC bulk data with the proliferation activity states of every species within the community was found. It was also assumed that

reduced activity of strain MT4 and increased proliferation of strain MT2 might have caused an observed breakdown of the consortium grown in the bioreactor.

The double staining technique provided the chance to follow bacterial cell states and their roles in mixed cultures without applying labelled substrates. It is therefore in line with single cell techniques already successfully applied in biotechnology for developing strategies to optimize microbially catalyzed production processes.

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Ms. Ref. No.: MIMET-D-08-00166

Dear Prof. Trevors,

Leipzig, 05/15/08

enclosed you will find our revised paper:

**Community-based degradation of 4-chorosalicylate tracked on
the single cell level**

by Sonja Pawelczyk, Wolf-Rainer Abraham, Hauke Harms and Susann Müller for publication in the
Journal of Microbiological Methods.

The manuscript has been thoroughly revised according to the suggestions given by the referee. For
convenience any modifications done in the manuscript are highlighted in blue colour in the text.
As recommended by the referee, Figure 3 and the Supplemental Information 1, 2, 3 and 4 were
modified.

We thank you and the referee for the very helpful comments and we hope that all changes in the
manuscript have been to your full satisfaction.

Sincerely yours

Susann Müller

Enclosures: Detailed answers to referee

Susann Müller

Answers to the referee

General comments

This paper describes an original approach combining flow cytometry and strain specific antibodies to monitor the biodegradation of 4-Chlorosalicylate (4-CS) by a consortium of 4 bacterial species. It is clearly written and well documented. Results are presented in a very convincing way. The addressed questions are pertinent to the Journal of Microbiological Methods and the article deserves to be published with minor revisions.

Specific comments

All the single specific comments were involved in the text and are highlighted with blue characters.

There were two questions:

Pg 11, l 19: (rel. Fl): make this more specific

We explained this in the text as suggested by the reviewer:

The gates for the DNA distribution patterns (grey circles) are set according to the mean values of the DNA subpopulations (determined by the blue fluorescence intensity of the labelled chromosome equivalents):...

Pg 12, l 12: do you mean concentration decreased (from 5 mM?) to 3.42 mM, or did it rise from 1.76 mM to 3.42 mM. How can 4-CS be restored in that case. This point is unclear to me.

The concentration did rise from 1.76 mM to 3.42 mM. This was written correctly within the text and is also shown in Figure 4.

The reason for this is that we did use a chemostat. 4-CS is constantly pumped into the balanced bioreactor system at a low dilution rate (D of 0.06 h^{-1} , please, see also materials and methods).

The initial residual concentration of 4-CS as sole carbon and energy source was 5mM. Due to reasons of stability the chemostat culture was build up strain by strain beginning with strain MT1, which degraded the 4-CS to 1.76 mM, followed by strain MT3, which application disturbed the equilibration within the bioreactor for only a short time (preventing successful further degradation shortly) until the residual concentration dropped conveniently to 0.5- 0.02 mM 4-CS.

Figure 3:

We changed the Y axis accordingly, thanks for the hint and corrected this also at Pg 17, l 34.

Supplemental Information 1-3:

We changed the unit as suggested in all Figures. Additionally we changed the description in the Figure legend page 18, line 6.

Supplemental Information 4:

The referee is right. Again, we changed the lettering of the figure and in the text: Pg 6, l 39; Pg 7, l 3; Pg 18, l 35.

Community-based degradation of 4-chorosalicylate tracked on the single cell level

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Running title: Community-dependent degradation of 4-CS

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Abstract

4-Chlorosalicylate (4-CS) can be degraded completely by a bacterial consortium consisting of *Pseudomonas reinekei* (MT1), *Achromobacter spanius* (MT3) and *Pseudomonas veronii* (MT4). The fourth species *Wautersiella falsenii* (MT2) is thought to act as a 'necrotizer' of the community. Single cell approaches were used to follow every species' degradation activity within the community by assuming that growth and proliferation are activity markers for the utilization of 4-CS and its degradation pathway intermediates as carbon and energy sources.

A primary/secondary antibody staining technique for species differentiation was applied and a species-resolved determination of proliferation activity by flow cytometry undertaken. Degradation was followed by quantifying 4-CS and the resulting intermediates by HPLC. A good correlation of HPLC bulk data with the proliferation activity states of every species within the community was found. It was also assumed that reduced activity of strain MT4 and increased proliferation of strain MT2 might have caused an observed breakdown of the consortium grown in the bioreactor.

The double staining technique provided the chance to follow bacterial cell states and their roles in mixed cultures without applying labelled substrates. It is therefore in line with single cell techniques already successfully applied in biotechnology for developing strategies to optimize microbially catalyzed production processes.

Introduction

Microbial degradation processes are restricted by limits of the indigenous microbial cells and the microenvironmental conditions. The cells' performances depend on their intrinsic metabolic abilities mirrored by the activities and types of the involved degradation pathways and enzymes, which determine the flux rates through the metabolic sequences. These rates might vary depending on the concentrations and bioavailability of the contaminants and also on possible abiotic conversion of metabolites and pathway intermediates, as e.g. for 4-chlorosalicylate (4-CS) (Nikodem et al. 2003), the target chemical in this paper. Accompanying microorganisms will have important influence on flux rates since they might scavenge intermediates with high or low affinity thus changing the concentration of the available intermediates to be further metabolized (Camara et al. 2007a). Therefore dynamic metabolic intra- and interspecies cross-degradations are very complex.

This is the main reason why it is difficult to develop strategies to improve the degradation capacities of microbial communities in contaminated sites. The complex and constantly changing mixture of substrates and intermediates makes it nearly impossible to follow the action of a distinct species, let alone the action of individuals. There are some approaches to overcome those limitations. PCR screening for functional genes and expression profiling based on mRNA using reverse transcriptase or microarrays are options when the degradation pathways are known (for review, see Watanabe and Hamamura 2003). Other methods linking degradation activities with identities of community members are based on the uptake of isotope-labelled substrates. Examples are a combination of fluorescence *in-situ* hybridisation and microautoradiography (Rosello-Mora et al. 2003) as well as stable isotope probing of DNA or RNA (DNA-SIP/RNA-SIP, for review, see Radajewski et al. 2003). Multiparametric flow cytometry has the potential to identify the active community members without requiring knowledge of metabolic genes or application of labeled substrates. It provides information on the physiological states and activities of individuals and populations within communities, while allowing identifying individuals by specific fluorescence labeling (Fuchs et al. 2005) or by rRNA-based methods after cell sorting (Kleinstüber et al. 2006). Proliferation patterns are among those structural parameters of an individual which give reliable and robust information on growth (Müller 2007), therefore providing information of actual degradation. Flow cytometrically derived bacterial DNA distributions contain information about the duration of cell cycle phases and thus of cellular growth (Cooper 1991). Especially, if pure cultures are available and their specific DNA proliferation can be characterized beforehand, this approach provides a successful and time saving technique to get knowledge of the action of the individuals within a community and enables to develop control strategies of degradation processes.

In this study the activity of the members of a 4-CS-degrading consortium was analysed by attributing characteristic DNA patterns to each member of the community. The bacterial consortium consisted of four bacterial strains: *Pseudomonas reinekei* (MT1, Camara et al. 2007b), *Wautersiella falsenii* (MT2),

1 *Achromobacter spanius* (MT3) and *Pseudomonas veronii* (MT4) (Pawelczyk 2007). The consortium
2 was originally isolated from the sediment of the river Spittelwasser, a tributary stream of the river Elbe
3 near Bitterfeld, Germany (Faude and Höfle, 1997). The community is able to degrade 4-CS by sharing
4 metabolites of the degradation pathway as substrates within the consortium (Pelz et al., 1999).
5 Immunohistochemical analysis using strain specific antibodies revealed that MT1 is the most abundant
6 strain within the consortium with 84% (± 3), MT3 and MT4 with abundances of 8% (± 4), each and
7 MT2 with only 1% (Pelz et al., 1999). Here, the community was grown as a mixed chemostat culture
8 and samples were taken on a daily base. Cells were then fluorescence-optically separated using strain
9 specific antibodies and growth of every single species was determined by analysing proliferation
10 activity via individual chromosome content analysis and multiparametric flow cytometry.

11
12 The combination of the two approaches (differentiation vs. proliferation) enabled us to determine time
13 periods and rates of active growth, and by this, the catabolic activity of each species during the
14 concerted mineralization of 4-CS. We provide a high speed detection tool capable of determining the
15 degradation potential of distinct and known species useful to support bioremediation processes in
16 fresh- or groundwater systems of contaminated regions.

Materials and Methods

Isolation of strains and genotypic identification

The bacterial community used to degrade 4-CS consists of four strains, MT1, MT2, MT3 and MT4. It was isolated as described by Faude and Höfle (1997). The sequencing of the 16S rRNA genes revealing high homologies to known organisms led to the following affiliations: *Pseudomonas reinekei* MT1, *Wautersiella falsenii* MT2, *Achromobacter spanius* MT3 and *Pseudomonas veronii* MT4 (Pawelczyk 2007; Camara et al. 2007b).

Cell cultivation

P. reinekei MT1, *W. falsenii* MT2, *A. spanius* MT3 and *P. veronii* MT4 were grown as pure cultures aerobically at 30°C and pH 7.5 in 500-ml shake flasks on 300 ml peptone media (L⁻¹): 5 g peptone from meat (pancreatic), 3 g NaCl, 2 g K₂HPO₄, 10 g meat extract, 10 g yeast extract, 5 g glucose or on the minimal medium PMS at 110 rpm for batch experiments (see supplemental information SI 1-3). The composition of the minimal medium was as follows (mg L⁻¹): Na₂HPO₄ x 2H₂O: 7800; KH₂PO₄: 6800; MgSO₄ x 7H₂O: 410; NH₄-Fe-citrat: 10; Ca(NO₃)₂ x H₂O: 50; NaNO₃: 85; ZnCl₂: 0.7; MnCl₂ x 4H₂O: 1; H₃BO₃: 0.62; CoCl₂ x 6H₂O: 1.9; CuCl₂ x 2H₂O: 0.17; NiCl₂ x 6H₂O: 0.24; NaMoO₄ x 2H₂O: 0.36; conc. HCl 13 µl. The carbon and energy sources were acetate (0.2 %) or 4-CS (0.8 mM, dissolved in DMSO). Additionally, the strains were regularly cultivated on LB-agar (Lennox, Carl Roth GmbH; Agar from Difco) or on minimal-medium-agar to control purity. All strains were stored as stock cultures frozen in liquid medium containing 15% glycerol at -70°C.

The consortium was grown as a chemostat culture over a time period of maximal 90 days in a fermenter with a working volume of 490 ml. The cells were grown in minimal medium with 5 mM 4-CS as sole carbon and energy source (mg L⁻¹): Na₂HPO₄x2H₂O: 8778; KH₂PO₄: 3000; (NH₄)₂SO₄: 1237; MgSO₄: 164.8; FeSO₄ x 7 H₂O: 13.4; EDTA: 49.5; MgO: 14.3; CaCO₃: 2.7; ZnSO₄ x 7 H₂O: 1.9; MnSO₄ x 2 H₂O: 1.2; CoSO₄ x 7 H₂O: 0.4; CuSO₄ x 5 H₂O: 0.3; 79.8 µg H₃BO₃; 0.0682 ml conc. HCl. For the chemostat culture strains MT1, MT3 and MT4 were grown as pure pre-cultures on 30 ml M9-medium with 5 mM sodium acetate as sole carbon source. Strain MT2 was grown as a pure pre-culture on 30 ml minimal medium supplied with 1 g/L yeast extract.

Pre-cultures were incubated over night at 30°C with continuous shaking at 110 rpm. After incubation, the pure culture cells were washed with PBS and resuspended in minimal medium with 5 mM 4-CS as sole carbon source to an optical density of 1 measured at 600 nm (OD₆₀₀). Due to reasons of stability the chemostat culture was build up strain by strain beginning with strain MT1, followed by strains MT3, MT4 and MT2. The chemostat culture was run at a dilution rate *D* of 0.06 h⁻¹ at 20° C on a reservoir concentration of *c*₀ = 0.01 mM 4-CS. The continuous flow of the medium was controlled by a peristaltic pump. A magnetic stirrer ensured oxygen supply (300 rpm). After a five-fold volume change, samples of 10 ml were taken on a daily base. This experiment was conducted several times and analysed flow cytometrically twice.

Analysis of 4-CS and its metabolites

To analyse the concentrations of 4-chlorosalicylate, 4-chlorocatechol, protoanemonin and cis-dienlacton in the supernatant of the chemostat, samples were taken and centrifuged at 4,600 x g for 10 min. The supernatant was frozen at -20°C until analysis. One ml of the supernatant was used for HPLC analysis on a Waters Alliance 2695 Separation Module with a Waters 996 UV/VIS Photodiode-Array-Detector (Eschborn/Germany). The system was equipped with a Nucleosil 100-3 C18 reversed phase column (Macherey-Nagel, Düren/Germany). Methanol-H₂O containing 0.1% (vol/vol) H₃PO₄ was used as eluent at a flow rate of 0.25 ml min⁻¹. The concentration of methanol increased from initially 20% to 80%. The column effluent was monitored at 260 nm.

Cell preparation and staining procedures for flow cytometry

The harvested cells were centrifuged at 3,200 x g for 5 min, fixed with 10 % NaN₃, and stored at 4°C. This procedure was found to preserve the cells for at least 3 months. For flow cytometry measurements, preserved cells were centrifuged again, washed in NaCl-phosphate buffer (0.4 M Na₂HPO₄/NaH₂PO₄, 150 mM NaCl, pH 7.2) and resuspended at a concentration of 3x10⁸ cells ml⁻¹.

DAPI staining:

Two ml of diluted cell suspension were treated with 1 ml solution A (2.1 g citric acid/0.5 g Tween 20 in 100 ml bidistilled water) for 10 min, washed and resuspended in 2 ml solution B (1.5 µM 4',6-diamidino-2'-phenylindole (DAPI, SIGMA), 400 mM Na₂HPO₄, pH 7.0) for at least 20 min in the dark at room temperature using a modification of a standard procedure (Meistrich et al., 1978).

Antibody-staining:

For the immunodetection of individual members of the bacterial community consisting of strains MT1, MT2, MT3 and MT4 indirect immunofluorescence was employed. Polyclonal sera against outer membrane proteins of individual members of the community were produced as in Pelz et al. (1999) and used as strain specific primary antibodies. The fluorescence immunodetection resulted from the binding of the fluorescence-conjugated secondary antibody which binds to the IgG heavy chains and all classes of immunoglobulin light chains of the primary antibody.

Flow cytometric differentiation of the species within the community was done after using the above described DNA staining technique: 3 x 10⁸ DAPI stained cells/ml PBS were incubated with 2.5 µl of the first unlabelled strain-specific polyclonal antibody from rabbit (MT1: #200; MT2: #201; MT3: #202; MT4: #170, Tesar et al., 1996) as well as from mouse (MT3: #1A3, Tesar et al., 1996) for 60 min at 20°C. Afterwards, for flow cytometric analysis, 1.5 µl of a secondary antibody (anti-rabbit coupled with AlexaFluor635 (Invitrogen, Karlsruhe) or Cy3 (Sigma-Aldrich, Steinheim) as well as anti-mouse coupled with DTAF (dichloro-triazinylaminofluoresceine; Dianova, Hamburg) were added for another 60 min at 20°C in the dark. Cross-reactivity of the primary and secondary antibodies against the respective strains of the consortium was tested by the method of Pelz et al. (1999) and by flow cytometry but was not detected (not shown).

The calibration of the antibody staining procedure was done with batch-grown as well as chemostat-grown cells on either peptone (MT3) or 4-CS (MT1, D = 0.06 h⁻¹). Time and quantity dependencies of

the fluorescent secondary antibody binding were determined flow cytometrically with MT1 (#200) as well as MT3 (#1A3) and by applying the respective secondary fluorescent antibodies (MT1: anti-rabbit AlexaFluor635; MT3: anti-mouse DTAF) at different quantities and different times (see SI 4). A stabilisation of the secondary antibody binding started at about 60 min although no saturation was observed with regard to concentration, therefore 1.5 µl of secondary antibody were applied and found to be sufficient for fluorescence detection.

Single cell analysis requires planktonic cells. Unfortunately, if the two strains were cultivated together on 4-CS and stained in a concerted action with #200 and #1A3 as well as anti-rabbit AlexaFluor635 and anti-mouse DTAF, respectively, insufficient differentiation of the strains by flow cytometric analysis was achieved. The reason for this malfunction was the appearance of double positive events since about a quarter of all cells had both red (anti-rabbit AlexaFluor635) and green fluorescence (anti-mouse DTAF, not shown). Obviously, this procedure resulted in unspecific surface protein interactions of the labelled strains, therefore causing clogging, which prevented reliable differentiation. This behaviour was not observed when both species were grown on peptone. However, as the targeted community had to be grown on 4-CS and aggregation to be prevented in any case the strains were differentiated in separate schemes one after the other in all experiments. Therefore, one by one species quantities were analysed and their proportions within the community determined had no problems to reliably distinguish the species. Also, we decided to use only the red labelled (AlexaFluor635) anti-rabbit antibody since this colour was conveniently far away from the DAPI fluorescence of bacterial DNA.

Flow cytometry

Multiparametric flow cytometry: Flow cytometric measurements were carried out using a MoFlo cell sorter (DakoCytomation, Fort Collins, CO) equipped with two water-cooled argon-ion lasers (Innova 90C and Innova 70C from Coherent, Santa Clara, CA). Excitation of 580 mW at 488 nm was used to analyze the forward scatter (FSC) and side scatter (SSC) as trigger signal at the first observation point. DAPI was excited by 180 mW of ML-UV (333-365 nm) at the second observation point. The orthogonal signal was first reflected by a beam-splitter and then recorded after reflection by a 555 nm long-pass dichroic mirror, passage by a 505 nm short-pass dichroic mirror and a BP 488/10. DAPI fluorescence was passed through a 450/65 band pass filter, green fluorescence through BP 520/15 and, red fluorescence through BP620/45. Photomultiplier tubes were obtained from Hamamatsu Photonics (models R 928 and R 3896; Hamamatsu City). Amplification was carried out at linear or logarithmic scales, depending on the application. Fluorescent beads (Polybead Microspheres: diameter, 0.483 µm; flow check BB/Green compensation Kit, Polyscience, USA) were used to align the MoFlo. Also, an internal DAPI-stained bacterial cell standard was introduced for tuning the device up to a CV value not higher than 6%.

Each species was labelled apart from the others by using the double antibody staining technique. The resulting dot plots were gated with regard to the fluorescence intensities of the labelled subpopulations and the total cell number was determined by SSC vs. FSC gate setting. The

1 proportions of stained and unstained cells were calculated with the Summit software
2 (DakoCytomation, Fort Collins, CO).

3 Proliferation activity was determined by evaluating the flow cytometric DNA histograms using both
4 Summit (DakoCytomation, Fort Collins, CO) and WinList 2.01 (Verity Software House, Maine)
5 software. The percentage of cells of any sub-population with a distinct DNA content was recorded.

6 7 **Microscopy**

8 To verify the quality and quantity of staining for flow cytometry, the cells were subjected to image
9 analysis (camera: DXC-9100P; software: Openlab 3.1.4., Improvision, Lexington, MA, USA) using
10 ultraviolet light from a 100-W mercury arc lamp. Magnification was x 1,000. Blue fluorescence was
11 observed using an excitation filter 20 UG1, IF 17L420; green fluorescence by an excitation filter 450-
12 490, DC 510, LP 520; red fluorescence by an excitation filter 20BP545, DC 580-800, IF 17O590.
13 Microphotographs were taken by another microscope with an attached camera system (Axioplan, Carl
14 Zeiss Jena, Germany; camera: CCD-3 Focus Imager 4000 System, Intas, Goettingen, Germany).

15
16 To obtain microscopic pictures a 1 ml sample of the chemostat culture needed to be centrifuged at
17 16,000 x g. Cells were resuspended in 1 ml phosphate buffered saline (mg L⁻¹) (NaH₂PO₄: 540;
18 Na₂HPO₄: 2850; NaCl: 8770). 5 µl of #200 (MT1) and #1A3 (MT3) were added. After 1 h of
19 incubation at 20°C, 5 µl of the secondary antibodies Cy3 and DTAF were applied and incubated in the
20 dark at 20°C. Subsequently, cells were stained by adding 50 µl DAPI (1 µg/ ml). The stained cells
21 were concentrated by filtration onto hydrophilic Durapore membrane filters (pore size 0.22 µm,
22 Millipore, Schwalbach). The filters were then attached to a glass slide and covered with a cover slip
23 (Figure 1).

Results

Activity states of the pure strains obtained from proliferation patterns

The proportion of cells in the different stages of the cell cycle is a measure of the duration of these phases and thus a useful tool to estimate the proliferation activity of a bacterial population. In this paper, we used the DNA pattern as a snapshot fingerprint of the proliferation activities of every species present in the community. The DNA subpopulations were named according to the observed DNA contents as C_n , whereas the subpopulation with the lowest DNA content was defined as C_1 (one assumed chromosome equivalent), although we were aware that even this lowest DNA content might represent multiple genomes in the respective individuals.

To follow proliferation activity of each strain within the consortium, the DNA distribution pattern of every of the four species needed first to be analysed when cultivated in pure cultures. The strains were grown axenically on different substrates like MT1 on 0.8 mM 4-CS, 0.2 % acetate, or peptone medium; MT4 and MT3 on 0.2 % acetate or peptone, respectively; and MT2 on peptone. To enhance the reliability and ease of the DNA pattern attribution, analyses of proliferation activities were performed upon growth on different substrates and by choosing a linear scale to represent DNA fluorescence intensities in the histograms. Patterns for exponential growth and stationary phase cultivation are shown in Figure 2 for each strain. The subpopulations which characteristically increased in abundance under stationary growth conditions are marked with a grey background and used as markers for restricted growth or absence of growth. The subpopulations which typically increased in abundance during the exponential growth phase are marked with a striped background. The proportions of cells in these subpopulations report on proliferation and growth. The complete results for every strain are provided as supplemental information (SI 1 for MT1 on 4-CS, acetate, and peptone, SI 2 for MT3 on acetate and peptone, SI 3 for MT4 on acetate and peptone as well as MT2 on peptone); the subpopulations used as tool for the strains activity determination are listed in Table 1.

The C_2 subpopulation was the most stable subpopulation in MT1 (SI 1). A clear relative increase of this subpopulation was observed during growth on all three substrates (A: 4-CS, B: acetate, C: peptone) going along with the increase in OD until C_2 dominated the stationary phase of growth. The early and mid-exponential growth phases, in contrast, were characterized by increased C_1 and the C_4 subpopulations. Also, bigger cell sizes (FSC) were clearly detected in the early exponential growth phase during growth on acetate and peptone, yet hardly observed for growth on 4-CS, which might be caused by the very slow growth on the latter substrate ($\mu = 0.07 \text{ h}^{-1}$). Therefore, the sum of the C_1 and C_4 cells of this species were considered as the marker to represent proliferation activity on all substrates (between 43.15 % (± 4.82) and 63.3 % (± 1.93); Table 1). Contrarily and as expected, the proportions of cells exhibiting a C_2 -chromosome content were high in the stationary phase and

1 therefore suited as a marker subpopulation for the stationary state of this species (between 50.03 %
2 (± 1.86) and 86.96 % (± 5.82); Table 1).

3
4 The shapes of the DNA distributions of MT3 were found to be much more variable during growth on
5 peptone and acetate than those of MT1. The MT3 individuals gave fluorescence intensities correlating
6 to 1, 2, 3, 4 and more chromosome equivalents per cell (SI 2). Subpopulations containing C_2 , C_3 , and
7 C_4 chromosome equivalents dominated the exponential growth phase with 95.57 % (± 1.63 , peptone)
8 and 99.42 % (acetate, for only a short moment; otherwise around 47% (± 35.16)) of these cells. The
9 corresponding mean values of all subpopulations clearly pointed to asynchronous cell cycle behaviour.
10 At the end of the cultivation, under substrate depletion, the cells always stayed mainly within the first
11 subset of subpopulations (70.19% (± 5.54) on acetate and 79.49% (± 1.42) on peptone). Similar to
12 MT1 a large increase in cell size (FSC) was observed during early exponential growth.

13 MT4 (SI 3, A and B) showed increasing proportions of C_2 , C_4 and even C_8 cells during exponential
14 growth on peptone (B: 80.25 % (± 8.90)) and of C_2 cells when grown on acetate (A: 39.63 (± 5.33)):
15 Both were therefore used as marker subpopulations for growth. The stationary phase was dominated
16 by C_1 cells (73.39 and 84.06 (± 2.84), respectively, Table 1). Similar to the behaviour of MT1 and MT3,
17 an increase of the cell size (FSC) was observed during the early and mid-exponential growth phase.

18 In comparison to MT4 there were lower percentages of cells in the C_1 -subpopulation (33.8 % ($\pm$
19 0.86)) in MT2 during stationary cultivations (SI 3, C). Large variations of cells with the fourfold
20 chromosome equivalents were observed. This subpopulation primarily indicated the proliferation
21 activity of this strain, although the proportion of C_2 cells also increased somewhat during early
22 exponential growth. As before, an increase in cell size (FSC) was observed during the early
23 exponential growth phase.

24 Summarizing, to describe the state of high proliferation activities the proportions of cells (given in
25 percent) with the following chromosome equivalents were used as the subpopulation markers: C_1+C_4
26 for MT1, $C_2+C_3+C_4$ for MT3, $C_2+C_4+C_8$ for MT4, and C_2+C_4 for MT2, respectively. The subpopulation
27 markers of the species staying under stationary conditions are C_2 (MT1) and C_1 (MT3, MT4, MT2; see
28 also Table 1).

29 The proliferation patterns of MT1, MT3, MT4 and MT2 show the same overall DAPI (A/T) fluorescence
30 intensities and scatter characteristics (not shown). Hence, the strains cannot be distinguished by only
31 analysing DNA, cell size (FSC), and cell granularity (SSC). Consequently, differentiation by antibody
32 binding was used to determine their proportions when growing as a mixed bioreactor community (for
33 antibody labelling see Materials and Methods). During the first days of bioreactor cultivation negligible
34 quantities of double stained cells showed no or dim DNA labels, meaning that those cells may have
35 had a restricted ability to take up DAPI through the cell wall or the DNA dye may have been quenched
36 by the antibody application. With chemostat equilibration this problem ceased and full DAPI labelling
37 of the antibody labelled cells was achieved (see SI 5 A).

Proliferation states of the community members during growth on 4-CS

The four strains were grown together on 5 mM 4-CS, since the compound **cannot** be mineralized as a sole substrate by any of the individual species. Only MT1 was able to grow on the substrate as the sole source of carbon and energy, yet only transiently and at low growth rates. Therefore, the only way to mineralize 4-CS is to combine all four strains thereby enabling them to achieve the degradation in concerted action. The proportions of the community members shown in SI 5B were very similar to those reported earlier (Pelz et al., 1999). The dominant strain is MT1 with around 80 %, followed by MT3 which decreased from nearly 20 % to 10 % during the first 40 days. Species MT4 was stably represented at around 5 %, MT2 levelled to maximally 5% after 60 days of mixed cultivation. Although there is some **fluctuation, the whole** picture indicates very stable contributions to the degradation process of 4-CS even after laboratory cultivation over weeks.

To get information about strain proliferation activities during combined cultivation each species was followed by analysing the chromosome contents every few hours while monitoring changes in community composition by antibody differentiation. In particular proliferating and stationary parts of the populations were assessed. Proliferation patterns of the consortium members are represented in Figure 3. The signals in the lower left corner of the **cytograms** are due to electronic noise, partly or unstained cells and cell debris. The individual population patterns are evident; also the chromosome equivalents are clearly distinguished. The gates for the DNA distribution patterns (grey circles) are set according to the mean values of the DNA subpopulations (**determined by the blue fluorescence intensity of the labelled chromosome equivalents**): MT1: 38.82 for C₁, 79.06 for C₂, 123.11 for C₄; MT2: 43.92 for C₁, 84.92 for C₂, 243.43 for C₄; MT3: 35.24 for C₁, 72.35 for C₂, 109.82 for C₃, 148.64 for C₄; MT4: 51.50 for C₁, 97.73 for C₂, 220.18 for C₄. Since all the data are comparable due to the fluorescent bead alignment of the MoFlo, similar relative intensities of DNA labelling are obvious. As expected, the genome patterns lie within the same order of magnitude and could not be used for differentiation of the species without applying the double antibody labelling approach.

For the sake of stability the bacterial community in the chemostat was build up strain by strain. The bioreactor started as a batch culture of the MT1 species able to degrade the 4-CS. Afterwards the chemostat-mode was started at $D = 0.06 \text{ h}^{-1}$. High proliferation activity of MT1 was seen at the start of the cultivation which continued in the presence of MT3 and MT4. MT3 was added to the chemostat as the second species 12 days later and remained in a proliferation phase until all species were present in the bioreactor. Afterwards, this species as well showed a slowly yet steadily increase in the proportion of cells with stationary phase characteristics to nearly 50 %. MT 4 as the third species was added after 25 days of cultivation. The fraction of MT4 cells with stationary phase characteristics rose up to the end of cultivation, the part of strains' cells in proliferation phase decreased continuously. until the forth was added. The application of species MT2 after 38 days apparently caused the proliferation of MT1 to decrease until the culture broke down at day 82 (Figure 4, above). The DNA pattern of strain MT2 showed a high proportion of cells in proliferation phase right from the start at day 38 up to the breakdown of the culture after 82 days. The fraction of stationary cells was low and even decreased until the end of the cultivation. This may support the assumption that *W. falsenii* MT2

is not able to degrade 4-CS or any of its metabolites but is supported by a high amount of dead or injured cells of the other during the last third of the cultivation. During the last 15 days of cultivation the OD₆₀₀ decreased from 0.394 to 0.286.

Metabolic versatility of the community

The residual metabolite concentrations within the bioreactor were determined by HPLC. During initial batch cultivation, the concentration of 4-CS stayed high at up to 5 mM (Figure 4, middle). Thereafter, the chemostat mode was started and degradation of 4-CS by strain MT1 to 1.76 mM was observed. This was accompanied by high proliferation activity, which was determined to be about 74.04 %. As expected this is similar to the activity determined for growth of this strain in batch culture on 4-CS (Table 1).

Addition of the second species MT3 on day 12 resulted in about 50% biomass loss. The residual 4-CS concentration rose to 3.42 mM on day 22 before decreasing to a final concentration of 0.5- 0.02 mM. The concentrations of the MT1 dead-end metabolites protoanemonin and cis-dienlacton decreased from 0.378 mM to 0.007 mM and from 0.018 mM to 0.0006 mM, respectively. The 4-chlorocatechol (4-CC) concentration remained nearly zero since MT3 is described to show a high affinity to this substrate (Pelz et al., 1999). However, on day 19 the concentration of this first intermediate increased to 0.017 mM. The binary community of MT1 and MT3 present at this time then decreased the 4-CC concentration to 0.0005 mM. This all shows that the system was able to cope with the increased concentration of highly toxic intermediates. The proliferation activities of the MT1 and the MT3 increased to high levels (94.06 % for MT1 and 69.63 % for MT3) and a nearly balanced growth of the two species was observed.

The addition of the third species MT4 on day 25 resulted in another disturbance, but to a lower degree, which may be due to imbalances of the system by the augmentation of this species. Addition of this strain led to an immediate decrease of 4-CS from 0.471 mM to 0.068 mM, which was accompanied by an increase of 4-CC to 0.014 mM on day 30. This upshift of 4-CC was again followed by its return to 0.003 mM during the next 20 days of incubation and further reduction to below the detection level afterwards.

Additionally, the concentrations of cis-dienlacton and protoanemonin decreased rapidly in the presence of strain MT4 and remained on a low but fluctuating levels between 0.001 - 0.002 mM for cis-dienlacton and 0.013 - 0.008 mM for protoanemonin. In summary, in the presence of the three species 4-CS was degraded reliably throughout. Also, proliferation activity of all three species went on, pointing at the absence of toxicity in the bioreactor system. We checked for protoanemonin around day 35, but it was always nearly zero. The reason may lie in the high affinity of MT4 for protoanemonin ensuring the quick degradation of this potent toxicant (Pelz et al., 1999).

Surprisingly we found that the addition of the fourth member of the consortium decreased the proliferation activity of MT1 (from 75.6 to 42.4 %), MT3 (from 58.9 to 49.7 %) and MT4 (from 30.1 to 23.7%). In the same cultivation interval the number of cells representing stationary growth increased to 57.5 % (MT1), 49.9 % (MT3), and 73.4% (MT4). Invariably, the 4-CS and the 4-CC concentrations

1 ceased to zero but residual cis-dienlacton and protoanemonin concentrations rose in between days 44
2 and 45. This rapid increase in the concentrations of those intermediates was again buffered by the
3 bacterial consortium. Within approximately the next seven days of incubation the concentrations
4 decreased to 0.001 mM for cis-dienlacton and 0.01 mM for proteanemonin. The species MT2 showed
5 proliferation activities similar to those observed during growth on peptone (88.4 % and Table 1). This
6 activity remained stable for more than 30 days until the culture break down preceded by a decrease in
7 the OD.

Discussion

The success of the degradation of organic compounds obviously depends on the bacterial species present and the metabolic pathways they catalyze, but also on how potentially toxic substrates or intermediates are transferred or shared within a community. Since there is the a need to develop reliable degradation processes, it is helpful to obtain a maximum of information on the involved microbial species. In this respect, obtaining information about the activity states of populations and individuals that make up a bacterial consortium is still challenging. Therefore, the basic intention of this study was to develop and test a technique suited to investigate interspecies relationships in a bacterial consortium without applying [substance markers during](#) the degradation process. A single cell approach was used to follow the degradation activity of [all populations](#) within a four-membered bacterial consortium. The technique was based on the assumption that growth and proliferation can be used as an activity marker for the metabolization and utilization of a contaminant and its degradation intermediates.

Although 4-CS is utilized by one of the community members *P. reinekei* MT1 at very low growth rates (Table 1, $\mu = 0.07 \text{ h}^{-1}$, also see Pelz et al. 1999), substantial mineralization was described to be obtained only by a consortium that was originally isolated from a polluted habitat (Faude and Höfle 1997). Besides strain MT1, it comprises *A. spanius* MT3 and *P. veronii* MT4, which use intermediates of the 4-CS degradation for growth thus preventing accumulation of toxic intermediates such as 4-CC and protoanemonin (Petz et al. 1999). The fourth species *W. falsenii* MT2 is considered as a 'necrotizer' living on constituents of the other community members.

If from an application perspective the members of the community are regarded as catalysts which are supposed to mineralize 4-CS as completely and quickly as possible, it is obvious that tools are needed to monitor the process. It is thus desirable to monitor the proportions of the community members during the process. Our approach relies on the determination of proliferation activity by multiparameter flow cytometry for the identification of active populations/subpopulations, a method which has been used previously in biotechnology to optimize microbially catalyzed processes (for review see Müller 2007). It quickly estimates the proportions of community members by accounting for at least 1000 cells per second and it has the additional power that it identifies periods at which the consortium members use the substrate contaminant or its intermediates for growth.

To develop the method we used the fact that every species of our consortium was cultivable as pure culture. Individual cultivation was used to obtain information on growth activity states correlated to growth velocity on different substrates and media. The degree of proliferation activity was quantified flow cytometrically by DNA staining and fluorescence intensity analysis as a sensitive measure of the bacterial ability to grow under a variety of micro-environmental conditions. Changes were followed by quantifying subpopulations characterized by various chromosome equivalents designated C_1 to C_{xn}

1 which are defined to evolve as a consequence of the metabolic capabilities of their individuals. These
2 DNA distribution patterns provided valuable information. Specific sub-distributions are dominant when
3 the cells are starving; others are dominant if the cells are growing. The dominance pattern within a
4 population is thus a fingerprint of the actual growth velocity (proliferation activity) of a population and
5 its dynamics within the community is witness of the population's contribution to the community-based
6 degradation of 4-CS. Such an analysis based on DNA contents can be regarded as highly reliable and
7 quantitative since fixed cells will not pump out the DNA label such as viable organisms do with other
8 types of activity stains (Nocker et al. 2007, also for this problem see e.g. review on bacterial pumps:
9 Saier 2000).

10
11 The DAPI fluorescence intensities of all four species in the blue PMT of the flow cytometer were
12 similar. Despite the appearance of different subpopulations during the growth curve, no differentiation
13 was possible according to the overall A/T content of the DNA of the four species although differences
14 in the chromosome G/C content are described (for MT2: 34%, Kämpfer et al., 2006 as well as for
15 MT3: G+C 65%, Coeyne et al., 2003). As SSC and FSC characteristics of the species were also not
16 suited for differentiation, an alternate approach based on a combination of species specific surface
17 staining via a double antibody staining technique and quantitative DNA labelling was successfully
18 applied. A similar approach based on flow cytometry and lectin labeling was already successful to
19 follow the dynamics of a herbicide-degrading binary bacterial community (Müller et al. 2002).

20
21 Using the new technique the specific role of each member of the consortium with regard to the
22 degradation of 4-CS was followed. We found increased proliferation activity of *P. reinekei* MT1 in
23 presence of MT3 and MT4. This confirmed earlier findings of the stimulation of MT1 by MT3 and MT4
24 during the degradation process of 4-CS. Earlier results had been obtained after stable isotope probing
25 using labelled metabolites and separation of the cells by immunocapture (Pelz et al., 1999). In the
26 second half of cultivation in the bioreactor proliferation activity decreased slowly until day 82 as seen
27 from the increased proportion of cells in stationary phase. This could be due to formation of the highly
28 toxic dead-end metabolite protoanemonin during the degradation of 4-CS by strain MT1. This
29 intermediate can be mineralized by strain *P. veronii* MT4. Since the main proportion of this population
30 seemed to stay in stationary phase of growth after its addition to the chemostat culture on day 25,
31 protoanemonin may have been utilized only by the small proportion of MT4 cells in the proliferation
32 phase. The supernatant of chemostat samples indeed showed initially an elevated concentration of
33 protoanemonin. Addition of MT4 decreased the concentration to a relatively constant value of 0.02
34 mM. Only on day 45 a sudden increase to 0.38 mM was observed which was difficult to interpret.
35 Strain *W. falsenii* MT2 behaved differently. The predominant proportion of this population remained in
36 the proliferation state throughout and even increased during the cultivation. This corroborates the
37 findings of Pelz and co-workers (1999) who described strain MT2 as the 'necrotizer' of the consortium,
38 referring to the fact that this strain is living on the remains of dead or compromised cells of the
39 community. Strain MT2 cannot be cultivated on mineral media containing only single aromatic carbon

sources and is therefore thought not to grow on 4-CS or its degradation intermediates. It belongs to the *Flavobacteriaceae* which have been found to grow aerobically on sugars and alcohols (Kämpfer et al., 2006). Whereas the proliferation activity of the other three species dropped towards the end of the chemostat cultivation the MT2 activity remained high.

A strength of the approach reported here is that after initial calibrations the user disposes of a quick tool for activity estimation of the species involved in a production or degradation process. Flow cytometry is presently about to become a standard tool for biotechnological process optimization (Hewitt et al. 2004). In environment research, however, most applications have focused on oceanic samples, in particular on phytoplankton (Rutten *et al.* 2005, Zubkov and Burkhill 2006). There are some recent studies into the composition and stability of microbial consortia which specifically address mutualistic, commensalistic or parasitic interactions (Vogt et al. 2005). However, studies monitoring the proliferation dynamics within enrichment cultures or natural communities remain scarce until now and will still remain a considerable challenge in the near future. They have also considerable potential to be combined with phylogenetic and metaproteome analyses directed towards the identification of distinct functions (Vaulot *et al.* 1995, Fuchs *et al.* 2005, Kleinsteuber *et al.* 2006). Increasing and physiological knowledge as well as improved data acquisition, data handling and the development of reliable phylogenetic and transcriptomic/proteomic data bases will further strengthen the basis of such approaches in microbiology.

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Tables and Figures

Table 1:

Proliferation activity and growth rates of MT1, MT2, MT3, and MT4 on peptone medium or PSM-medium with either acetate or 4-CS as the carbon and energy source.

Only MT 1 was grown on 4-CS since none of the other strains used this compound. MT 4 was grown on peptone. The numbers of chromosome equivalents per cell and the changes in cell size were recorded. Proliferation and stationary phase states of every strain differ from each other and become evident from upcoming subpopulations with distinct DNA contents.

Figure 1:

P. reinekei MT1 (red) and *A. spanius* MT3 (green) were fluorescently labelled according to DNA and differentiated by specific antibody binding.

Cells were incubated with 5 μ l of the primary antibodies #200 and #1A3, respectively, as well as with the secondary antibodies Cy3 and DTAF, respectively. Following, cells were stained by adding 50 μ l DAPI (1 μ g/ ml). The stained cells were concentrated by filtration onto hydrophilic Durapore membrane filters and visualized by fluorescent microscopy.

Figure 2:

Typical proliferation pattern for MT1 (on peptone), MT3 (on acetate), MT4 (on peptone), and MT2 (on peptone) which were grown in batch culture and harvested during the early exponential (expp) and the stationary growth phases (sp). The cells were stained with DAPI for the chromosome equivalents and evaluated with Summit software to quantify the cell numbers in the different subpopulations. Cells summed up in various subpopulations steadily depending on growth states. The representative phase related subpopulations are highlighted by underlying quadrangles (exponential phase: grey stripes, stationary phase: grey).

Figure 3:

Overview of the species growth activities during degradation of 4-CS in the bioreactor. The data are presented as cytograms. Some typical time points were chosen for every species during growth on 4-CS. All species were stained towards their chromosome equivalents and they were separated one after the other by the application of the respective antibody combination (ab₁-mt_x + ab₂-AlexaFluor635). The cells were flow cytometrically analysed at a logarithmic scale for both fluorescence intensities (arbitrary units). In the lower left corner of every cytogram always the contents of debris and electronic noise are recorded to show the sensitivity of the system and the distance to the quantified bacterial samples. Specific proliferation patterns for every species are gated individually after antibody staining and named according to the expected chromosome equivalents C₁ to C₄. The data obtained by this gating are presented in figure 4 within the bulk diagram above.

Figure 4:

Results of a chemostat cultivation of the 4 species consortium on 4-CS. Upper bar diagram: Proliferation phase related (upper bars) and stationary phase related subpopulations (lower bars) are determined by analysing the respecting subpopulations of every species (bar code: MT1 black, MT3 grey, MT4 white, MT2 stripes). Cells in all subpopulations are set to 100 % for every species. The bioreactor was inoculated with the species one by one. Lower diagrams: residual substrate and intermediate concentrations are analysed by HPLC-analysis: 4-CS (black square), 4-CC (white square), protoanemonin (black triangle) and cis-dienlacton (white triangle). Analysis of the optical density of the bacterial consortium was done at 600nm.

Supplemental Information

Supplemental information 1:

MT1 was grown on 4-CS (0.8 mM, PMS-medium, A), acetate (0.2 %, PMS-medium, B), and peptone (C). OD was analysed at 700 nm (d= 5mm), the cell size was determined by analysing forward scatter (FSC) in [arbitrary units](#). To compare the changes in cell size during growth on the different substrates, the starting mean values of FSC on the respective substrates were set to channel number 70. MT1 showed three different DNA subpopulations (C_1 , C_2 , C_4) during growth on the different substrates, which containing cell numbers changed depending on growth velocity on either substrate. The marker populations for ongoing proliferation activity were found to be the C_1 and the C_4 subpopulations, whereas the C_2 subpopulation dominated the stationary phase.

Supplemental information 2:

MT3 was grown on acetate (0.2 %, PMS-medium, A) and peptone (B). OD and FSC were determined as explained in SI 1. MT3 showed various DNA subpopulations (C_1 , C_2 , C_3 , C_4 and more) during growth on the two substrates. The subpopulations with the higher chromosome contents are not shown, yet C_3+C_4 cells contributed up to 70 % to proliferation activity. The marker populations for proliferation activity were found to be C_2 , C_3 , and C_4 , whereas the C_1 subpopulation dominated the stationary phase.

Supplemental information 3:

MT4 was grown on acetate (0.2 %, PMS-medium, A) and peptone (B). OD and FSC were determined as explained in SI 1 (also for MT2, see below). MT4 showed four different DNA subpopulations on peptone (C_1 , C_2 , C_4 , C_8) and three on acetone (C_1 , C_2 , C_4). The C_8 subpopulation contributed to maximum 4 % to the events, so its behaviour was not shown. The marker populations for proliferation activity were found to be C_2 , C_4 , and C_8 , whereas the C_1 subpopulation dominated the stationary phase. MT2 was only grown on peptone (C). MT4 showed three DNA subpopulations on peptone (C_1 , C_2 , and C_4). The marker populations for proliferation activity were found to be C_4 cells, mainly, yet C_2 cells were also considered. The C_1 subpopulation was set as marker for the stationary phase.

Supplemental information 4:

Calibration curves of the 4-CS grown cells' (MT1 ● and MT3○, 20°C) incubation with the primary unlabelled strain-specific antibodies (MT1: #200; MT3: 1A3; 60 min) and the following secondary anti-rabbit fluorescent antibodies (MT1: anti-rabbit coupled with AlexaFluor635; MT3: anti-mouse coupled with DTAF) according to time and [antibody quantities](#). After about 60 min a stabilisation of the staining equilibrium was achieved, however, no saturation was observed for the protein concentrations applied.

Supplemental information 5:

Overview of the species quantities during degradation of 4-CS in a bioreactor. Above: Quantities of the four species MT1 (●), MT3 (○), MT4 (○) and MT2 (□) after staining them towards their DNA contents and with the respective fluorescent ab_1 -mt_x + ab_2 -fluorescence combination. Below: Equalized (to 100%) quantities of the four species MT1, MT3, MT4, MT2 (symbols the same as above) after autarchic preceded staining with the respective ab_1 -mt_x + ab_2 -fluorescence combination, only.

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Figure 1

Figure(s)

Figure 2

Figure(s)

Figure 3

Table 1

	MT1	MT3	MT4	MT2
Cells in proliferation state	(% C1+ %C4)	(% C2+C3+C4)	(% C2+C4+C8)	(% C2+C4)
peptone	57.90 (± 8.13)	95.57 (± 1.63)	80.25 (± 8.90)	83.41 (± 6.35)
acetate	43.15 (± 4.82)	47.38 (± 35.16)	39.63 (± 5.33)	
4-CS	63.30 (± 1.93)			
Cells in stationary state	(% C2)	(% C1)	(% C1)	(% C1)
peptone	86.04 (± 5.93)	74.49 (± 1.42)	73.39 (n.d.)	33.8 (± 0.86)
acetate	86.96 (± 5.82)	70.19 (± 5.54)	84.06 (± 2.84)	
4-CS	50.03 (± 1.86)			
μmax				
peptone	0.46 h ⁻¹	0.26 h ⁻¹	0.48 h ⁻¹	0.38 h ⁻¹
acetate	0.25 h ⁻¹	0.22 h ⁻¹	0.27 h ⁻¹	
4-CS	0.07 h ⁻¹			

Supplemental information 1

Supplemental information 2

Supplemental information 3

Supplemental information 4

Figure(s)