Competition between two isolates of marine p-hydroxybenzoate degrading bacteria

Dorthe Groth Petersen

Microbial Diversity Course
Marine Biological Laboratory
Woods Hole, MA

Correspondiong address:

National Environmental Research Institute
Department of Marine Ecology
Frederiksborgvej 399
4000 Roskilde
Denmark

e-mail: dgp@dmu.dk

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Introduction

Lignin is the most abundant aromatic polymer in nature and it is contained in the cell walls of higher plants and it compromises about 25% of the land-based biomass on earth and is therefore very important in the global carbon cycling (Harwood and Parales, 1996). The compound *p*-hydroxybenzoate (pOB) is a monomer of lignin and is normally degraded to protocatechuate, which is further degraded by an ortho- or a meta pathway (Fig. 1) (Lengeler et al., 1999).

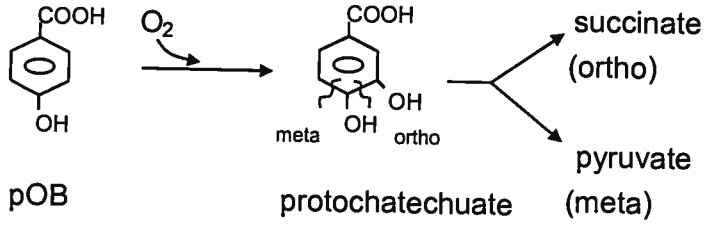

Fig. 1: The aerobic degradation of *p*-hydroxybenzoate to protochatechuate, which is further degraded by ortho- or meta cleavage to succinate or pyruvate.

POB degrading bacteria are distributed throughout many phylogenetic groups and it might be speculated that different pOB degrading strains have different ability to take up and utilise the compound as a substrate. It would therefore be interesting to observe how two different genotypes of pOB degrading bacteria interact with each other in mixed cultures with pOB added as the sole substrate. It has been shown that two different strains of *Escherichia coli* can persist in a stable polymorphism in competition experiments with glucose as the sole carbon source (Turner et al., 1996). Coexistence can be mediated through a cross-feeding between species, where one species take up the substrate e.g. glucose and excrete alternative substrates, e.g. acetate and glycerol that other bacteria can utilise (Rosenzweig et al., 1994). Co-existence is also possible under a serial-transfer regime in an environment, which contains only one carbon source. This co-existence is dependent on that one of the genotypes has a growth-rate advantage when the limiting resource is abundant whereas the other genotype has an advantage when the resource is sparse (Turner et al., 1996).

Material and Methods

Isolation of pOB degraders

Water samples were collected from Eel Pond and Garbage Beach and inoculated in liquid SW-pOB broth in a 30°C shaking incubator. After 1-2 days, 100 µl of the cultures with detected growth was spread onto pOB agar plates and incubated at 30°C, and isolates were restreaked twice within the next week. One liter of Sea Water (SW) base (1x) consisted of 20 g NaCl, 3 g $MgCl_2x6H_20$, 0.15 g $CaCl_2x2H_2O$, 0.2 g KH_2PO_4, 0.5 g KCl, 0.25 g NH_4Cl and MQ water added up to a litre. One liter of SW-pOB broth for pOB degraders consisted of 0.5 ml 1M Na-sulfate, 10 ml 1M MOPS (pH 7.2), 15 g of washed agar and 990 ml 1x SW base. The media was autoclaved, cooled to 60°C and 6 ml 0.5M p-hydroxybenzoate, 1 ml of 1000x SL 10 tracer element solution and 1 ml of 1000x vitamin solution were added. For liquid media, the agar was left out.

Verification of pOB degradation

To check whether the isolates actually were pOB degraders and not agar degraders, liquid shakes with or without pOB were set up for each isolate. The liquid cultures were incubated in a 30°C shaker overnight and the isolates should only grow in the liquids with added pOB.

DNA extraction, PCR and sequencing of 16S rDNA

DNA was extracted from the isolates with pOB degrading ability, a PCR reaction was performed and 16s rRNA gene was amplified and sequenced for phylogeny analysis. DNA was extracted using freeze-thawing. 200 µl of liquid culture from an over-night culture was spun down, the supernatant was removed, 100 µl of 10 mM TRIS buffer (pH 7.5) was added and the cells resuspended. The cells were freeze-thawed (-80°C/60°C) 5 times, popped at 96°C for 10 minutes, followed by a short centrifugation and the template DNA was taken from the supernatant.
The PCR was performed with the general primers 8F (aga gtt tga tcM tgg ctc ag) and 1492R (ggt tac ctt gtt acg act t) that amplify a part of the gene coding for the 16S rRNA. The PCR reaction was performed in a volume of 20 µl with 0.2 µl 8F primer (10 µM), 0.2 µl 1492R primer (10 µM), 18 µl SuperMix and 1.6 µl of template. The thermal cycling conditions were as follows: initial denaturing and activation of

polymerase for 5 minutes at 95°C, followed by 30 cycles consisting of denaturing for 1 min at 95°C, annealing for 1 min at 55°C and extension for 1.5 min at 72°C, with a final 8.5 min 72°C extension period at the end of the PCR. The hold temperature was 4°C.

Prior to sequencing, the PCR product was purified on a Microcon® millipore filter. The PCR product was transferred to the filter, spun for 10 min at 500x*g*, and the PCR product on the filter was transferred to an eppendorf tube in 20 µl of sterile MQ water. The obtained sequences were submitted to BLAST and the bacteria characterised.

ARDRA was performed in order to compare pattern with the achieved sequences. The PCR product was digested with Hha I restriction enzyme, and the reaction consisted of 7 µl PCR product, 1 µl of Hha I (20 U/µl), 1 µl 10x NEBuffer and 1 µl 10x BSA (1 mg/µl). The restriction mixture was incubated for 2 hours at 37°C, and the products were run on a 1% agarose gel and stained with ethidium bromide.

Characterisation of pOB degraders

The obtained 12 pOB degrading isolates were characterised visually under microscope according to cell morphology and motility (Table 1). In order to fix the cells to the slide, the slide containing the sample was heated over a bunsen burner a couple of times.

Furthermore, the 12 isolates were characterised according to their doubling time with pOB as a substrate, the doubling time for growth on succinate for 5 isolates and the ability to utilise glucose as a substrate for 4 isolates (Table 2).

Competition experiment with two pOB degrading isolates

Two isolates were chosen, 5 and 19, based on their different cell- and culture morphology, and similar doubling rates when growing with pOB as the substrate. The doubling rates were 301 and 346 minutes for isolates 5 and 19, respectively. The cell morphology characteristics of isolate 5 were cocci shape and motility, and the cell morphology for isolate 19 was spirillum-like and motility was detected. Visual distinction could be made based on the cell morphology from plate-grown colonies (Fig. 2) and on the culture morphology (Fig. 3). Figures 2 and 3 are found in the back of the paper.

The competition experiment consisted of 2 controls and the competition bottle, (referred to as the comp-bottle) (Fig. 4). The first control was isolate 5 alone; the second control was isolate 19 alone and the comp-bottle was a mixture of isolates 5 + 19.

Overnight cultures of both controls and the comp-bottles were incubated in 250 ml erlemeyer bottles in 100 ml of pOB containing SW media at 30°C starting with an OD of 0.001. Competition was investigated under two different situation, with or without shaking, where the bottles with shaking presumably had a homogeneously oxygen distribution and the non-shaking bottles had a gradient of oxygen with decreasing content towards the bottom. Sampling in the non-shaking tubes was performed after a short shake of the liquid in the erlemeyer bottles.

The first 24 hours of the experiment, samples were taken out for direct counting under microscope and for plating, and afterwards samples were taken out once every 24 hours. At each sampling, OD was measured and direct counts of isolates 5 and 19 in the controls and the comp-bottles was performed, and at every second sampling, samples were also taken out for plating. The samples were diluted up to 10^8 times prior to plating.

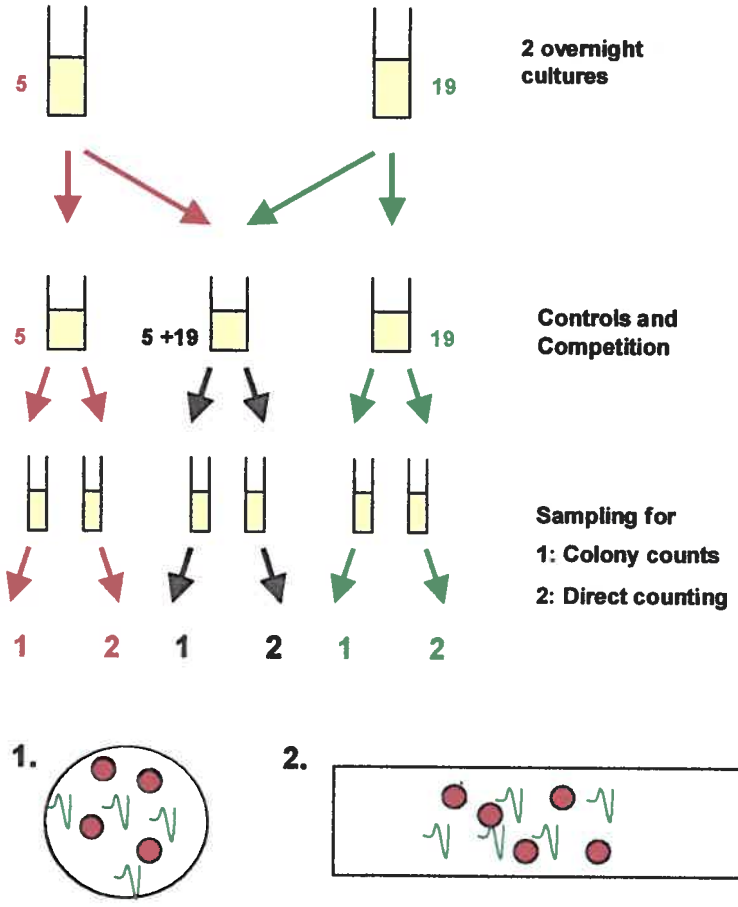

Fig. 4: Flow chart showing the set-up for the competition experiment. Direct counts were performed at every time point, whereas colony counts were only performed at every second time point.

Chromogenic detection of catechol and protocatechuate

In order to investigate whether the degradation of pOB could be detected as catechol or protocatechuate production an assay using *p*-toluidine and iron was performed. Briefly, samples were incubated with *p*-toluidine and ferric chloride which would yield a brownish colour in the presence of either catechol or protocatechuate (Parke, 1992). Both liquid incubations and plating was used for the assay. Controls contained no *p*-toluidine.

Results and Discussion

Characterisation of the pOB degrading strains

The verification experiment with pOB degraders yielded 12 isolates of pOB degrading bacteria and visual characteristics from microscope investigations are found in Table 1. Two species of Halomonas sp., four species of Marinomonas sp. and six unknown species were found based on the 16s rDNA sequencing.
The doubling times for the 12 isolates using pOB as the sole substrate are shown in Table 2 and range from 247 minutes for isolate 23 to 578 minutes for isolate 11.
The doubling time using succinate or glucose as the sole substrate showed that isolate 5 had the fastest doubling rate of the isolates tested.

Table 1: Morphology and motility of the pOB degrading isolates

Species	Shape	Motility
Halomonas sp	cocci	+
Marinomonas sp.	rod, cocci	+
Unknown pOB degraders	rod, cocci, spirillum	+

Table 2: Doubling times (Td) (min) for the isolates with pOB, succinate and glucose as substrate

# Isolate	Species	Td - pOB	Td - succinate	Td - glucose
5	Halomonas sp.	301	165	fast
6	unknown	301	203	
7	Marinomonas sp.	346		
10	Marinomonas sp.	289	239	
11	unknown	578		
12	unknown	346	247	slow
13	unknown	364		
18	unknown	277	277	
19	unknown	346	247	slow
21	Marinomonas sp.	433		
23	Halomonas sp.	247		slow
27	Marinomonas sp.	385		

The ARDRA gels with the pOB degraders were compared to the species that was found based on BLASTing the sequences. It was found that the ARDRA pattern was consistent with the results of the BLAST, meaning that banding patterns for Halomonas sp. were comparable and that banding patterns from Marinomonas sp. were comparable. Three unknowns (marked with an asterisk) from Fig. 5a have comparable banding patterns with the Marinomonas sp. banding pattern and it can be hypothesised that these three unknowns are Marinomonas sp.

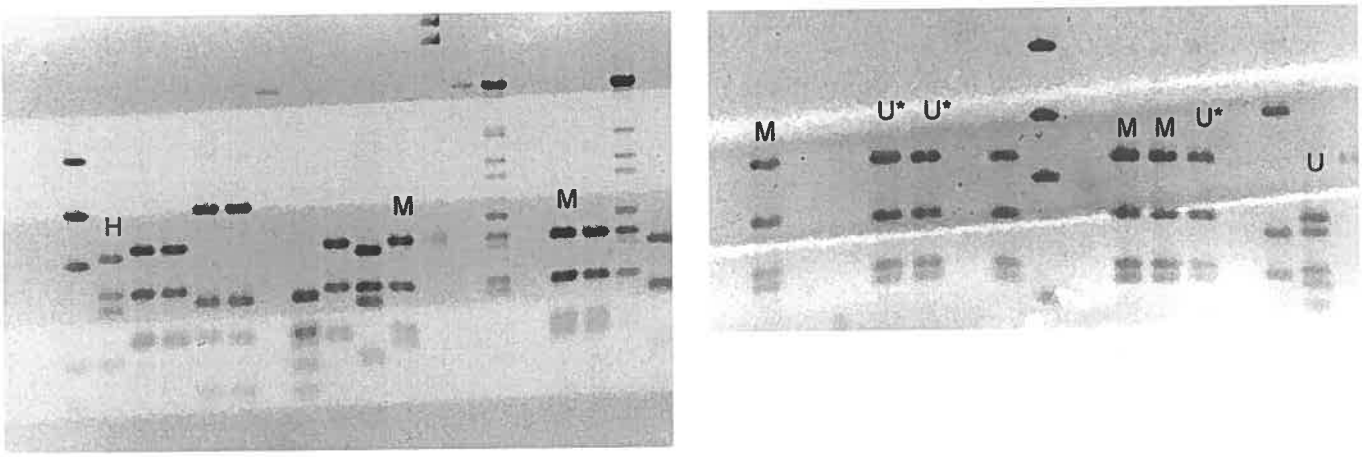

Fig. 5a and b: ARDRA gels showing the pattern from the different marine strains. Lanes with Halomonas sp. are marked with an H, lanes with Marinomonas sp. are marked with an M and lanes with unknowns are marked with U. Only 4 out of 6 unknowns are found on the ARDRA gel.

Competition experiment

Doubling rates (Td) based on the OD measured in the controls during the competition experiment showed that isolate 5 had a Td of 1.5 hours and isolate 19 had a Td of 0.7 hours in the shaking tubes. The Td of isolate 5 was 3.3 hours and Td for isolate 19 was 1.5 hours in the non-shaking tubes (Fig. 6a and b).

Number of Colony Forming Units (CFU) based on plating of the sub-samples from the comp-bottle showed that around 10^9 cells grew at T_{9h} for both colonies in the shaking bottles (Fig. 7a and b). Between T_{9h} and T_{36h} isolate 19 dominated based on CFU, but from T_{49h} to T_{56h}, isolate 5 dominated the comp-bottle. Same pattern was seen for the non-shaking comp-bottle, where isolate 19 dominated until T_{56h} where after isolate 5 was most abundant.

The overall colony formation was higher in the bottles with shake for both controls and comp-bottle compared to the non-shaking bottles. Same patterns are seen if the percentage of colonies 5 and 19 from comp-bottle are calculated from the shaking and non-shaking tubes (Fig. 8a and b).

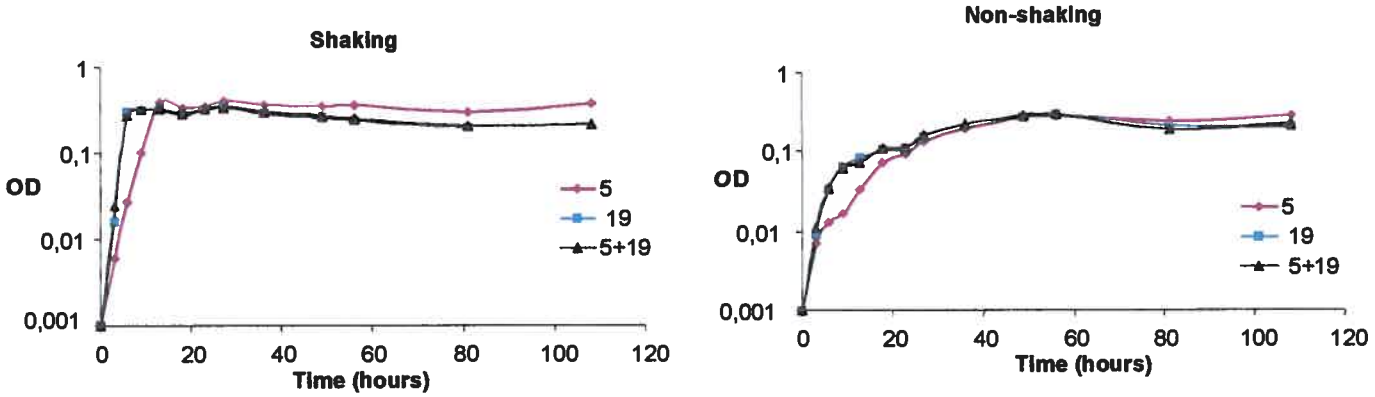

Fig. 6a and b: Growth curve for the control experiment and the comp-bottle. Td is based on the exponential part of the curves.

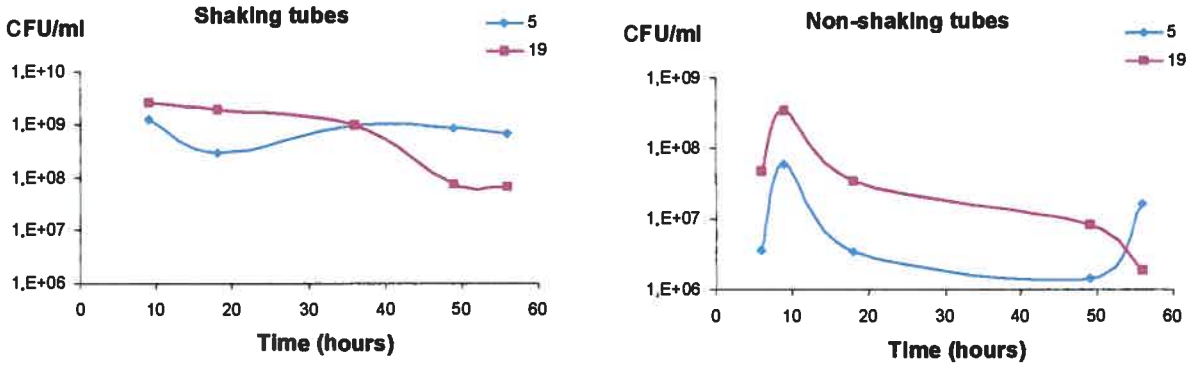

Fig. 7a and b: Colony forming units as a function of time in the comp-bottles. Note the different scales on the y-axis.

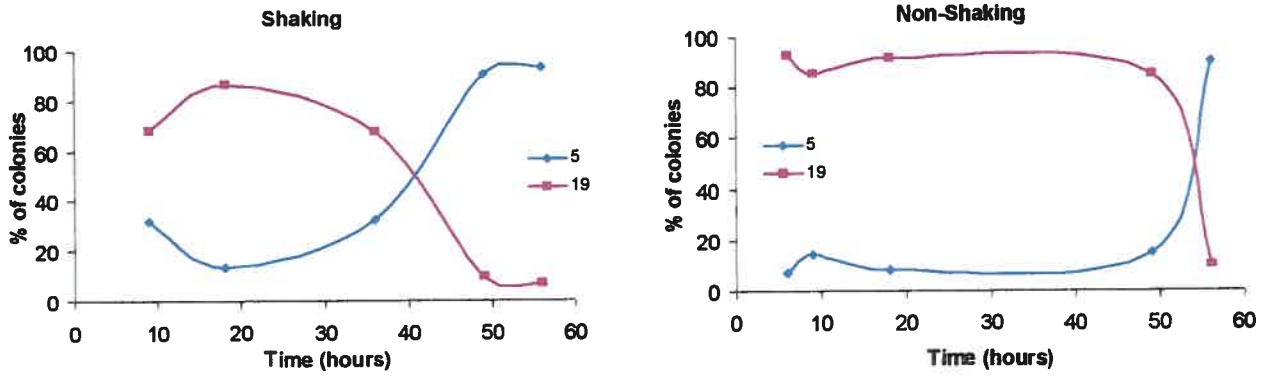

Fig. 8a and b: The % of colonies of isolate 5 and 19 from the comp-bottles as a function of time (hours).

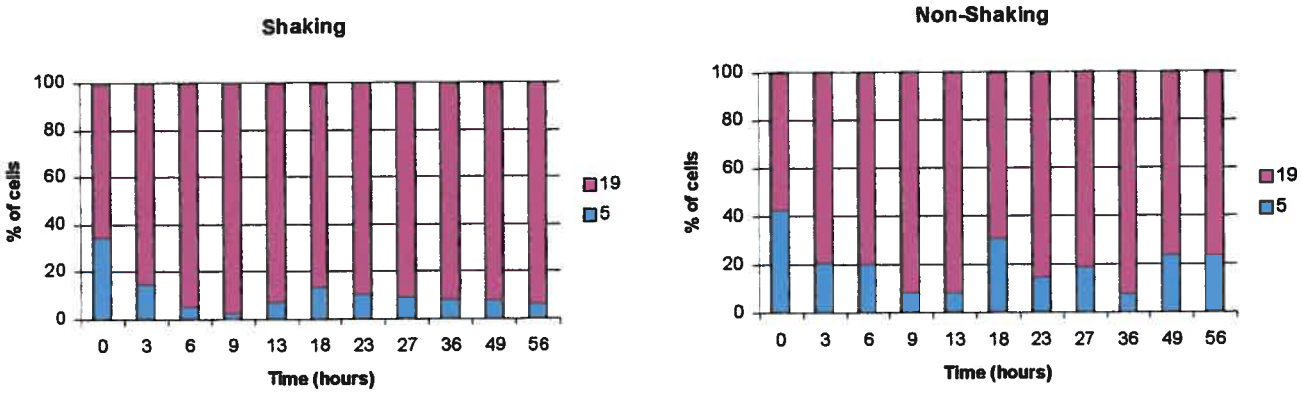

Fig. 9a and b: Distribution of the isolates in the comp-bottle based on direct counts under microscope based on differences in cell morphology

Abundance of isolates 5 and 19 in the comp-bottles based on direct counting and differences in cell morphology were not comparable to the results based on colony morphology (Fig. 9a and b). According to the direct cell counts isolate 19 was always most abundant in both shaking and non-shaking comp-bottles. This inconsistency between the results based on colony morphology and cell morphology can be addressed to several factors. Cells can have varying cell morphology depending on their growth phase, which can make recognition based on cell morphology difficult. Furthermore, the initial cell morphology for isolate 5 and 19 was determined from plate-grown isolates, where morphology can differ compared to the morphology of bacteria grown in liquid. The last important factor in the direct counting is that the heating process to fix the cells affected the shape of the cells. These inconsistencies involved in the direct counting based on cell

morphology makes counting based on colony morphology more appealing and reliable. It should, however, be mentioned that growth on plates also is subjected to biases, e.g. the physiological status of the cells, which can be described with VBNC, viable but non culturable bacteria.

In the competition experiment with isolates 5 and 19, both strains indeed were affected by the presence of the other strain. The growth of isolate 19 was more or less unaffected by the presence of isolate 5 during the first 36 hours of growth, whereas after 36 hours a drop in CFU and percentage of colonies 19 compared to the control was observed. The initial rapid growth of isolate 19 in the comp-bottles was probably due to its rapid growth rate compared to isolate 5 with the slower growth rate.

Isolate 5 was very affected by the presence of isolate 19 in the comp-bottles the first 36 hours, whereas it grew rapidly during the following hours. The explanation of the sudden increase after T_{36h} in the shaking bottles could be that isolate 5 has a higher utilisation rate of the pOB at lower concentrations compared to isolate 19. It can be assumed that the pOB concentration is decreasing with time.

The scenario with competition between isolate 5 and 19 in the comp-bottles can be explained by different evolutionary strategies, which enable different bacteria to survive and maintain themselves within communities. One equation to describe the growth of a population is

$$dX/dt \times 1/X = r - (r/K \times X)$$

where

$dX/dt \times 1/X$ = specific rate of population increase

r = per capita rate of increase of the population

K = carrying capacity of the environment

X = population density as either numbers or biomass

This equation describes population growth in limited environmental conditions. When X is low, the rate of population change is dominated by r, and when X is high the growth rate is limited by the carrying capacity, K of the given environment (Andrews, 1991). Species adapted to high rates of reproduction with high growth rates are called r strategists and species adapted to optimal utilisation of a given

resource and better competition ability are called *K* strategists (Atlas and Bartha, 1998).

When shaking bottles are compared to the non-shaking bottles same patterns are seen, which means, that oxygen availability is not the most important issue in the competition between isolate 5 and 19. It seems that the amount of substrate is more important in the competition.

Chromogenic detection of catechol and protocatechuate

Normally pOB is degraded through protecatechuate, but also catechol can be a degradation product and it could therefore be expected that all pOB degrading isolates would turn out to be positive in the chromogenic test. However, only isolate 18 was positive and developed a brownish precipitate in the shaking bottles and on plates (Fig. 10 and 11). It has been proposed that this test is not very suitable for pOB degrading bacteria, which might explain that only one isolate turned out positive (Parke, 1992).

Conclusion

Competition for substrate seemed to occur in a mixture of the pOB degrading isolate 5 and 19. Isolate 19 had a higher growth rate than isolate 5 and initially took over in the comp-bottles based on colony counting. After appr. 40 hours in the shaking bottles and 55 hours in the non-shaking bottles isolate 5 took over, and this could be explained by a better utilisation of the substrate with low concentrations by isolate 5 compared to isolate 19. No significant effect was found shaking and non-shaking bottles, which means that oxygen is not the most important issue in the competition between isolate 5 and 19. Only one positive isolate was found in the chromogenic detection of catechol and protocatechuate, which could be due to the use of pOB as the substrate.

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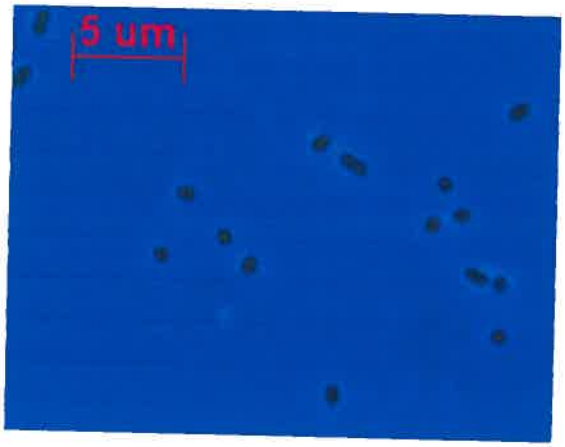

Morphology of isolate 5

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Morphology of isolate 19

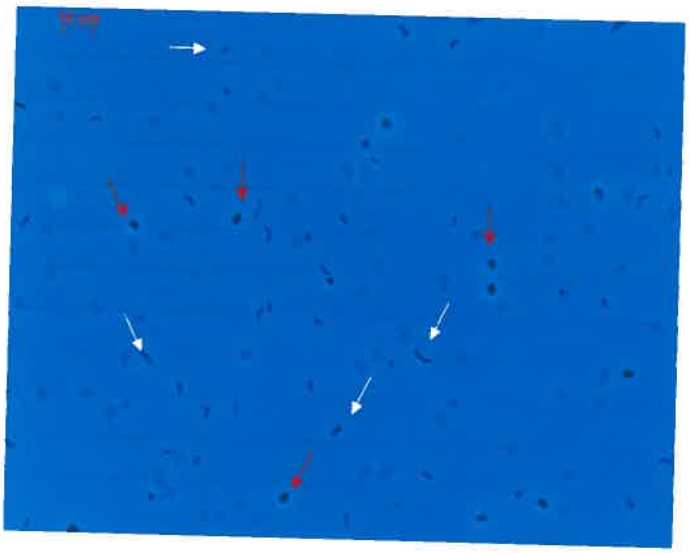

Fig. 2: Pictures above show the different morphologies of isolate 5 and 19. The below picture shows a mixture of isolate 5 and 19, red arrows show isolate 5, white arrows show isolate 19

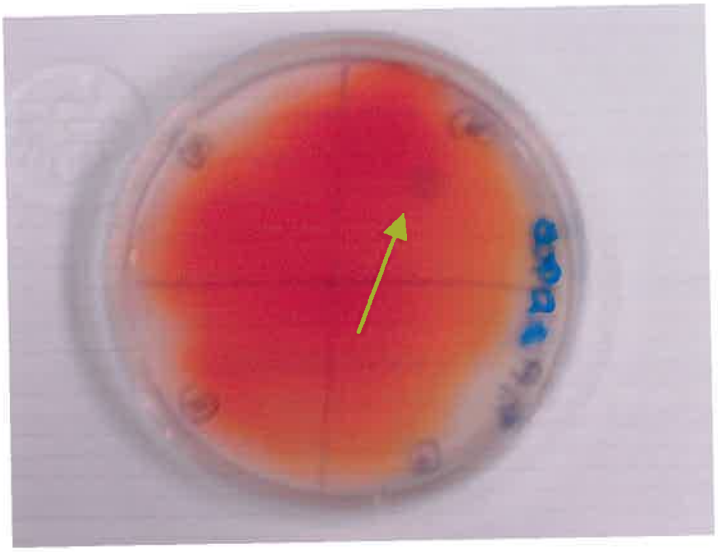

Fig. 11: Development of brown precipitate on plates and in tubes due to protechatechuate or cathecol development. Positives are indicated with green arrows.