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# Acquisition of the ability for *Rhodopseudomonas palustris* to degrade chlorinated benzoic acids as the sole carbon source

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## Abstract

Three strains of *Rhodopseudomonas palustris* were isolated from phototrophic enrichment cultures containing 3-chlorobenzoate (3-CBA) and benzoate (BA). These new strains as well as several previously described strains of *R. palustris* were tested in this study and shown to degrade 3-CBA if grown in media that contained BA as a co-substrate. All of the pure cultures that originally required BA for the degradation of 3-CBA acquired the ability to degrade 3-CBA as the sole carbon source after long periods of incubation that ranged from 1 to 3 months. After this adaptation period, the 3-CBA-degrading capabilities of all variants were stable, and the rates of 3-CBA degradation were significantly enhanced as compared to the parental strains. Furthermore, the variants had also acquired the ability to metabolize 2- and 4-CBA as sole carbon sources indicating that the enhanced ability to metabolize 3-CBA was accompanied by an expanded ability to metabolize chlorinated benzoates. These data indicate that acquisition of the ability to degrade 3-CBA may be rather common among strains of *R. palustris* and mutations that confer the ability to metabolize 3-CBA may provide a selective advantage to *R. palustris* under specific environmental conditions. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

*Keywords:* Phototrophic enrichment; 3-Chlorobenzoate; Adaptation; *Rhodopseudomonas palustris*

## 1. Introduction

Anoxic mineralization of halogenated compounds by photoheterotrophic bacteria has attracted relatively little attention [1–5] although anoxygenic phototrophs are known to abound in various aquatic environments that are known to accumulate organic pollutants, including surface sediments of shallow ponds, rivers, and lakes [6]. *Rhodopseudomonas palustris*, a purple nonsulfur bacterium, is known to be one of the most physiologically versatile bacteria with respect to the anaerobic degradation of aromatic compounds [7–9]. However, only three strains have been reported to utilize 3-chlorobenzoate (3-CBA) as a source of carbon and reducing equivalents. Strain WS17 is able to degrade 3-CBA if grown with benzoate (BA) as a co-substrate [2], whereas strains DCP3 [4] and RCB100 [1] can grow on 3-CBA as the sole carbon source. Strains WS17 and DCP3 were isolated using prolonged enrichment procedures under phototrophic conditions in liquid cultures [2,4]. The fact that strains of *R. palustris* able to use 3-CBA as the sole carbon source have infrequently been isolated suggests they are rare in natural environments. Alternatively, it is possible that genetic and physiological adaptations occur during certain enrichment regimes that result in the isolation of *R. palustris* able to degrade 3-CBA.

It has been well established that microbial communities may adapt to metabolize persistent compounds as energy and/or carbon sources [10,11]. Such adaptations may simply involve the induction of catabolic enzymes after exposure of the community to the new substrate. However, genetic changes and selection of specific bacterial populations able to metabolize the substrate are also known to be involved in these adaptation processes [12] and the frequency of adaptive mutations that result in acquisition of degradative abilities may be influenced by the phase of growth of organisms or specific environmental signals [13–16]. In the course of our current study on the ecological role of *R. palustris* in degradation of xenobiotic com-

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pounds in natural environments, we sought to achieve a better understanding of the adaptive responses and subsequent selection of degradative abilities of *R. palustris*. In this paper, we investigated the transition from the ability to degrade 3-CBA only in the presence of BA to the newly-acquired ability to degrade 3-CBA as the sole carbon source, using strains of *R. palustris* as model organisms.

## 2. Materials and methods

### 2.1. Media and growth conditions

Batch cultures were routinely incubated under saturating light conditions at 30°C in screw-cap tubes (16 ml) containing anoxically prepared low-chloride minimal (LCM) medium (with 0.01% of yeast extract) [4] and an $N_2$ gas phase. After autoclaving of the basal LCM medium, 25 ml of 1 M autoclaved $K(NH_4)PO_4$ solution (pH 7.0) was added per liter to a final concentration of 25 mM and 2 ml of filter-sterilized vitamin solution [4] was added per liter to the medium. Carbon sources were added from separately autoclaved stock solutions at the concentrations indicated in the text. Unless mentioned otherwise, inocula were added to 2% of the final volume of fresh media.

Continuous cultivation was performed in a chemostat vessel (working volume 500 ml) on LCM medium containing 0.5 mM of each 3-CBA and BA as carbon sources and the dilution rate was set to 0.02 $h^{-1}$. The temperature was set to 30°C and the chemostat was illuminated by three 40 W light bulbs at a distance of 20 cm. The culture vessel was kept anoxic by flushing the headspace with sterile $N_2$ gas (300 ml $h^{-1}$). After a steady state was reached (at least five volume changes), samples were taken and optical densities (ODs) (660 nm), 3-CBA, and BA concentration were measured. At each sampling time point, aliquots of the culture were transferred into fresh media containing only 3-CBA (1 mM) and these batch cultures were incubated under anoxic conditions in the light.

Duplicate sets of LCMPY plates (LCM medium supplemented with 0.3% peptone, 0.3% yeast extract, and 1.5% agar) were inoculated with culture dilutions and incubated in the presence and absence of oxygen and light using a BBL GasPak anaerobic jar system (Becton Dickinson and Company, Cockeysville, MD, USA) to confirm culture purity.

### 2.2. Enrichment cultures and isolation of 3-CBA-degrading photoheterotrophic bacteria

Sediment samples used as inocula for enrichment cultures were collected from the top layer (about 0.5 cm) of three different sites in The Netherlands. Two were from uncontaminated ditches (Appèlbergen and Haren) and one from a fresh water marsh (De Biesbosch) and all were used without prior storage. The sediment samples (10 g wet material) were suspended in 40 ml of anoxically prepared LCM medium (without yeast extract). The suspensions were sonicated briefly three times for 10 s (Branson B3 sonicator, Germany) and subsequently shaken for 2 h at room temperature. After sedimentation, aliquots of the supernatant were transferred into serum bottles and a mixture of 3-CBA plus BA or 3-CBA alone was added as substrates from stock solutions to 1 mM final concentrations. These enrichment cultures were incubated at 30°C and illuminated by two 40 W light bulbs at a distance of 20 cm. Algal growth was suppressed by the addition of 5 μM 3(3,4-dichlorophenyl)1,1-dimethylurea (DCMU) from an ethanolic 1 mM solution to all enrichment cultures [17]. After 2 months of incubation, cultures in which 3-CBA was metabolized were plated on LCM media containing 1 mM 3-CBA, 1 mM BA, and 1.5% agar, and incubated anoxically ($N_2$ gas atmosphere) in the light.

### 2.3. Microorganisms

Photoheterotrophic bacterial strains were obtained in pure cultures from various enrichment cultures by transferring on agar plates several times. Three strains named AP1, KD1, and BIS10 were isolated from enrichment cultures containing a mixture of 3-CBA plus BA and were capable of degrading 3-CBA. A previously described 3-CBA-degrading *R. palustris* strain WS17 [2] was also included. *R. palustris* strain NCIB8288 was a kind gift from Dr. W. Crielaard, University of Amsterdam and type strain *R. palustris* NCIMB8252 was purchased from the National Collections of Industrial, Food and Marine Bacteria (NCIMB; Aberdeen, UK).

To determine the closest relatives of the new isolates, nearly complete 16S rRNA genes were amplified from genomic DNA using fD1 and rD1 as primers [18]. Genomic DNA was isolated from liquid cultures grown on 15 mM succinate under anoxic conditions in the light. Cells (3 ml) were harvested by centrifugation and resuspended in 750 μl of TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). Lysozyme (2.5 mg $ml^{-1}$ final concentration) and RNAse (20 μg $ml^{-1}$ final concentration) were added and the suspension was incubated with gentle agitation for 1 h at 37°C. After the addition of 1% (w/v) sodium dodecyl sulfate (SDS) and 1 h further incubation at 50°C, proteinase K was added to the suspension at a final concentration of 100 μg $ml^{-1}$ and incubated with gentle agitation for 2 h at 50°C. The suspension was extracted twice with phenol: chloroform (1:1) and once with chloroform, and DNA was precipitated from the aqueous phase by addition of two volumes of absolute ethanol. The DNA was collected by centrifugation and washed in 70% ethanol, and resuspended in 100–200 μl of TE buffer.

PCR products were purified with the QIAquick PCR Purification kit (Qiagen GmbH, Germany) and used for direct sequencing. Sequencing was performed using an

ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). The sequencing primers used for 16S rRNA fragments were 27F (*Escherichia coli* positions 8–27), 536F (519–536), 926F (907–926), 1392R (1406–1392), 907R (926–907), and 519R (536–519). The 16S rRNA sequences were compared to sequences in the GenBank database.

### 2.4. Selection of R. palustris strains to growth on 3-CBA as the sole carbon source

Cultures (approximately $5\times10^8$ cells per ml) grown on a mixture of 3-CBA and BA (1 mM each) were transferred into fresh media containing 1 mM 3-CBA as the sole carbon source. These subcultures were cultivated under anoxic conditions in the light.

To examine the frequency and reproducibility of adaptation to growth on 3-CBA as the sole carbon source, a 25% glycerol stock culture (−80°C) of strain NCIB8288 was plated onto LCMPY plates and incubated in a BBL GasPak anaerobic jar system (Becton Dickinson and Company) for approximately 10 days. From these agar plates 35 single colonies were picked and cultivated in liquid cultures containing 15 mM succinate as the sole carbon source. These 35 independent succinate-grown cultures (approximately $10^9$ cells per ml) were transferred into liquid cultures containing a mixture of 3-CBA and BA (1 mM each) and incubated under anoxic conditions in the light. After 3 weeks incubation, cultures were transferred into fresh media containing only 1 mM 3-CBA and incubated under anoxic conditions in the light for up to 6 months. Growth was monitored by measuring the optical densities at 660 nm in 10 out of a total of 35 cultures.

To confirm that 3-CBA-degrading variants were derived from their parental strains, repetitive extragenic palindromic (rep)-PCR fingerprints were compared. Purified genomic DNA (see Section 2.3) was used to generate rep-PCR fingerprints using a BOX A1R primer as previously described [19] and PCR products were separated by electrophoresis on 1.5% agarose gel.

### 2.5. Analytical procedures

Growth was monitored by measuring the optical densities at 660 nm using a STARRCOL SC-60-S (R&R Mechatronics, The Netherlands) or a Shimadzu UV-1601 UV-visible spectrophotometer. The concentrations of benzoate and chlorinated benzoates were determined by reverse-phase HPLC using a MicroSpher C18 column (CHROMPACK, The Netherlands) with methanol–water–acetic acid (50:49.5:0.5 (vol/vol)) as an eluent, and the absorbance of the effluent at 254 nm was monitored using a Jasco UV-975 Intelligent UV/VIS detector (Tokyo, Japan). Chloride concentrations were measured colorimetrically with NaCl as a standard [20].

### 2.6. Chemicals

All chemicals were of analytical grade. 2-CBA, 3-CBA, 4-CBA, 2,4-CBA, and 2,6-CBA were purchased from Merck (Darmstadt, Germany); 2,3-CBA and 2,5-CBA were from Janssen (Geel, Belgium); 3,4-CBA was from Aldrich (Gillingham, UK); 3,5-CBA was from Fluka (Buchs, Switzerland); BA was from Sigma (St. Louis, MO, USA).

### 2.7. Nucleotide sequence accession numbers

The 16S rRNA gene sequences of strains AP1, KD1, and BIS10 have been submitted to GenBank database under accession numbers AF314062, AF314063, and AF314064, respectively.

## 3. Results and discussion

### 3.1. Isolation and characterization of 3-CBA-degrading photoheterotrophic bacteria

To isolate 3-CBA-degrading photoheterotrophic bacteria, anoxic phototrophic enrichment cultures were set up using a mixture of 3-CBA plus BA or 3-CBA alone. These enrichments were inoculated with aquatic sediment samples from three different sites (Appèlbergen, Haren, and Biesbosch). In all enrichment cultures that contained a mixture of 3-CBA plus BA, BA was completely degraded, and 3-CBA was degraded to about 30% of the initial concentration (1 mM) after 2 months of incubation under anoxic conditions in the light. However, when 3-CBA was provided as the sole carbon source, no degradation of 3-CBA was observed in any of the enrichments (data not shown).

Several photoheterotrophic bacteria that were able to degrade 3-CBA in the presence of BA were isolated from these enrichment cultures and obtained in pure culture. Three of these strains, designated AP1 (Appèlbergen), KD1 (Haren), and BIS10 (Biesbosch) were selected for further analysis. Based on their partial 16S rRNA gene sequences (1256 bp), the three isolates could all be identified as belonging to the species *R. palustris* ($>99\%$ sequence similarity to *R. palustris* strain ATCC17001, GenBank accession number D25312). These three strains as well as *R. palustris* strain WS17 [2], strain NCIB8288, and the type strain NCIMB8252 were tested for the ability to degrade 3-CBA. None of these strains was able to degrade 3-CBA (1 mM) as the sole carbon source. However, in the presence of BA (1 mM), all strains were able to degrade 3-CBA (Fig. 1A; growth curve for *R. palustris* strain NCIB8288 only), and the final cell densities of the cultures increased proportionally when both substrates were completely degraded. This observation is consistent with the previously reported *R. palustris* strains WS17 [2]

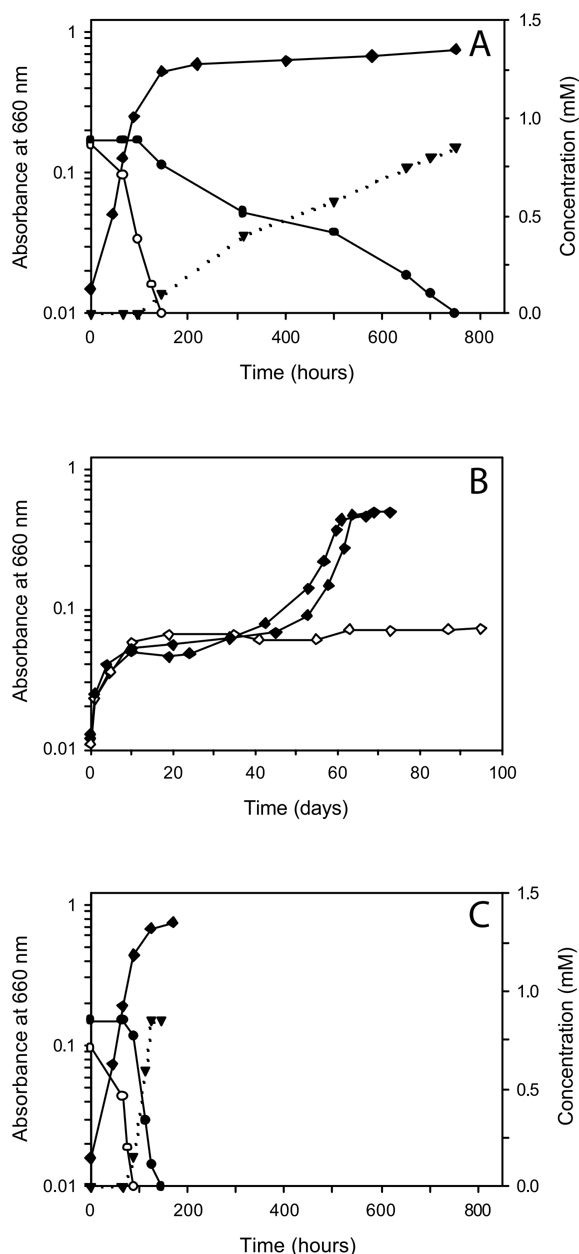


Fig. 1. A: Growth of the parental strain of *R. palustris* NCIB8288 on a mixture of 3-CBA and BA under anoxic conditions in the light. B: Growth of two independent cultures of the parental strain of *R. palustris* NCIB8288 on 1 mM 3-CBA as the sole carbon source (♦) after long-term incubation plus one culture without 3-CBA (◇) under the same conditions. C: Growth of a 3-CBA-degrading variant of *R. palustris* NCIB8288 on a mixture of 3-CBA and BA under anoxic conditions in the light. 0.01% of yeast extract was present in all cultures. Symbols: $OD_{660\ nm}$ (♦); 3-CBA (●); BA (○); $Cl^-$ (▼).

and CGA009 [1] that could degrade 3-CBA if BA was present as a co-substrate.

The degradation of 3-CBA by strains tested in this study depended on the nature of the primary growth substrate. Cultures grown on a mixture of 3-CBA and succinate did not degrade 3-CBA indicating that the presence of BA may be required as a structurally related compound to activate the synthesis of one or more enzymes involved in the stable degradation of 3-CBA. The requirement for a structurally related compound as a primary growth substrate has also been observed for degradation of 3,4-dichloroaniline and of substituted aromatic compounds by *Pseudomonas* strains [21,22].

### 3.2. Acquisition of 3-CBA metabolizing ability in *R. palustris* strain NCIB8288

Kamal and Wyndham [2] reported that the enrichment culture containing a mixture of 3-CBA and BA from which *R. palustris* strain WS17 was isolated degraded 3-CBA only after adaptation for a period of several weeks. Similarly, the phototrophic 3-CBA-degrading enrichment culture from which *R. palustris* strain DCP3 was isolated was no longer dependent on BA as a co-substrate for metabolism of 3-CBA after several successive transfers in medium containing 3-CBA plus BA [4]. These studies suggest that acquisition of the ability to degrade 3-CBA after long-term exposure to this compound can occur in phototrophic enrichment cultures from which *R. palustris* was the dominant bacterium. Therefore, we tested whether axenic cultures of *R. palustris* that initially required BA for the degradation of 3-CBA also acquired the ability to degrade 3-CBA as the sole carbon source after long-term exposure to this compound. Furthermore, the frequency, reproducibility, and time required to acquire the ability to degrade 3-CBA were investigated.

All three new isolates (AP1, KD1, and BIS10) as well as strains WS17, NCIB8288, and NCIMB8252 were unable to degrade 3-CBA as the sole carbon source during 1 month of incubation. However, after two more months, some of these axenic cultures appeared to grow on 3-CBA (data not shown). To investigate this in more detail, additional experiments were done using *R. palustris* strain NCIB8288. The strain was grown on a mixture of 3-CBA and BA, then transferred into fresh media containing 3-CBA alone and incubated under anoxic conditions in the light. Growth of strain NCIB8288 ceased following depletion of the small amount of yeast extract present in the medium (Fig. 1B). After a lag time of about 40 days in which no significant growth could be observed ($OD_{660\ nm} < 0.1$), the strain started to grow with a doubling time of approximately 6 days, whereas in this same time period no growth ($OD_{660\ nm} < 0.1$) was observed in the control culture without 3-CBA (Fig. 1B). During growth of strain NCIB8288 on 3-CBA, complete disappearance of 3-CBA and accumulation of chloride in the culture medium was observed. After plating one of these 3-CBA-grown cultures on LCM agar containing 1 mM 3-CBA under anoxic conditions in the light, a 3-CBA-degrading variant was isolated and obtained in pure culture. Rep-PCR genomic DNA fingerprints obtained using a BOX A1R primer [19] showed the 3-CBA-degrading variant and the parental strain NCIB8288 had identical fingerprint patterns (Fig. 2) indicating that the 3-CBA-de-

grading variant originated from the parental strain. This variant retained the ability to grow on 3-CBA as the sole carbon source without a lag phase even after being propagated several times in media that contained succinate as the sole carbon source. The stability of the variant phenotype and the elimination of a prolonged lag phase indicate that this character results from genetic changes rather than physiological adaptation (e.g. induction of enzymes involved in 3-CBA degradation) resulting in the ability of *R. palustris* to grow on 3-CBA as the sole carbon source. The rate of 3-CBA degradation by the variant had increased more than 10-fold as compared to the parental strain NCIB8288 when it was grown on a mixture of 3-CBA and BA (Fig. 1C), although its maximum specific growth rate on BA alone remained identical ($\mu_{max} = 0.073$ h$^{-1}$). These results indicate that adaptation to the use of 3-CBA as the sole carbon source was probably not achieved by simple overexpression of enzymes involved in the formation of benzoyl-CoA required for benzoate degradation [23–25].

To examine the frequency and reproducibility of the acquisition of the ability to degrade 3-CBA, 35 independent cultures of strain NCIB8288 were transferred into fresh media containing 3-CBA as the sole carbon source and incubated for up to 6 months. Five of the 35 cultures grew on 3-CBA after 2 months of incubation, and 25 cultures grew after 4 months of incubation (Fig. 3A). In those cultures showing growth, the cell doubling times ranged from 5 to 20 days (Fig. 3B). These results indicate that spontaneous 3-CBA-degrading variants developed in the course of long-term incubations, and that the time of their appearance following inoculation varied considerably. Interestingly, no growth ($OD_{660\,nm} < 0.1$) was observed in 10 out of the 35 cultures even after 6 months of incubation (Fig. 3A). The failure to observe growth in all cultures is consistent with the notion that mutations rather than physiological adaptation confer the ability for *R. palustris* to degrade 3-CBA.

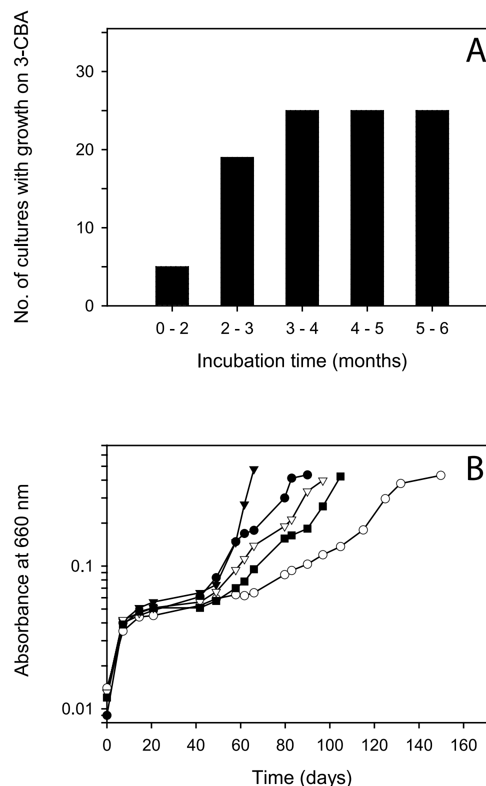


Fig. 3. Number of *R. palustris* strain NCIB8288 cultures of a total of 35 cultures showing growth ($OD_{660\,nm} > 0.4$) on 1 mM 3-CBA as the sole carbon source (A) and growth of five independent cultures (B) after 40–70 days of incubation. The different symbols represent five independent cultures.

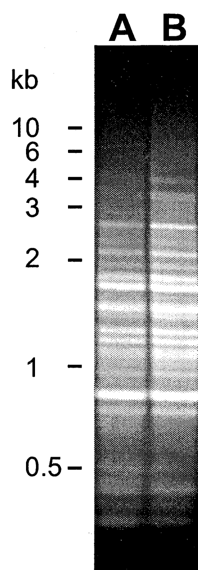


Fig. 2. Comparison of BOX-PCR genomic DNA fingerprints of the parental strain (A) and a 3-CBA-degrading variant (B) of *R. palustris* NCIB8288.

### 3.3. Acquisition of 3-CBA metabolizing ability in other *R. palustris* strains

To investigate whether the observed acquisition of the ability to metabolize 3-CBA as the sole carbon source was a property shared with other strains of *R. palustris*, strains AP1, KD1, BIS10, WS17, and type strain NCIMB8252 were tested. These strains were cultivated on a mixture of 3-CBA and BA and subsequently transferred into fresh media containing 3-CBA as the sole carbon source. All strains grew on 3-CBA after 1–3 months of incubation. Subsequent experiments showed that maximum specific growth rates of all variants on 3-CBA ranged from 0.006 to 0.029 h$^{-1}$ (Table 1) and that the rates of 3-CBA degradation by these variants had increased 10- to 15-fold as compared to their parental strains when grown on a mixture of 3-CBA and BA (data not shown). Thus, the acquisition of the ability to degrade 3-CBA may be a rather common phenomenon among strains of *R. palustris*.

Table 1
Growth rates of *R. palustris* strains on BA, 3-, 2-, and 4-CBA as the sole carbon source under anoxic conditions in the light

| Strain[a] | Maximum specific growth rate ($h^{-1}$) on[b]: | | | |
|---|---|---|---|---|
| | Parental strain | Variant | | |
| | BA | 3-CBA | 2-CBA | 4-CBA |
| AP1 | 0.060 | 0.029 | 0.005 | 0.003 |
| KD1 | 0.074 | 0.019 | (0.002)[c] | NG[d] |
| BIS10 | 0.039 | 0.020 | 0.005 | 0.002 |
| WS17 | 0.062 | 0.020 | 0.008 | 0.002 |
| NCIB8288 | 0.073 | 0.018 | 0.011 | 0.003 |
| NCIMB8252 | 0.032 | 0.006 | 0.005 | NG |

[a]All parental strains were only able to degrade 3- and 2-CBA when BA (1 mM) was present as a co-substrate.
[b]BA was used at 2 mM final concentration and 3-,2-, and 4-CBA were used at 1 mM final concentration. The data shown are mean values of duplicate measurements.
[c]Growth started only after 5 weeks of incubation.
[d]NG, no growth ($OD_{660\ nm} < 0.1$).

In batch cultures of all six strains tested, acquisition of 3-CBA metabolizing ability was only observed after at least 1 month of incubation. However, when a mixed culture of three strains, AP1, KD1, and WS17 was cultivated under BA-limiting conditions in a chemostat culture in the continuous presence of excess of 3-CBA, the culture density did not change significantly for a period of 5 months during which BA was continuously degraded to undetectable levels and 3-CBA was degraded to about 10–20% of its initial concentration. At regular intervals, samples from the chemostat were subcultured in batches with 3-CBA as the sole carbon source. No growth was observed after 1 month of incubation in any of these batch cultures, but after 3–4 months of incubation all batch cultures could grow on 3-CBA. This adaptation period was similar to that observed for all six strains in separate batch cultures. Apparently the ability to use 3-CBA as the sole carbon source and to degrade it at increased rates does not provide sufficient selective advantage under conditions of continuous growth in a BA-limited chemostat to yield stable 3-CBA-degrading variants as obtained in long-term batch incubations.

### 3.4. Utilization of other chlorinated benzoates by the 3-CBA-degrading variants

Representative 3-CBA-degrading variants derived from parental strains NCIB8288, AP1, KD1, BIS10, WS17, and type strain NCIMB8252 were tested for the utilization of mono- (2-CBA and 4-CBA) or dichlorinated benzoates (2,3-CBA, 2,4-CBA, 2,5-CBA, 2,6-CBA 3,4-CBA, and 3,5-CBA) both in the presence and in the absence of BA. None of the dichlorinated benzoates was used as carbon source. However, with the exception of the variant of KD1, all variants appeared to concurrently acquire the ability to degrade 2-CBA as the sole carbon source along with their acquired ability to grow on 3-CBA (Table 1). Although the variant of KD1 was not able to grow on 2-CBA alone during 3 weeks of incubation, growth started after adaptation for another 2 weeks. Most interestingly, the variants of AP1, BIS10, WS17, and NCIB8288 had also acquired the ability to degrade 4-CBA completely (based on 4-CBA disappearance, stoichiometric chloride formation, and growth). Their parental strains could not degrade 4-CBA at all, not even in the presence of BA before the adaptation period. While the rates of growth on chlorinated benzoates decreased in the order 3-, 2-, and 4-CBA in all cases, the ratio of the growth rates comparing these three compounds varied substantially (Table 1).

### 3.5. Conclusions

The data presented in this study showed that acquisition of the ability to degrade 3-CBA as the sole carbon source frequently and reproducibly occurred among strains of *R. palustris*. The following observations strongly suggest the events leading to acquisition of the ability to degrade 3-CBA were stochastic in nature and that different kinds of mutations conferred the ability to degrade 3-CBA: (i) of the 35 independent cultures of strain NCIB8288 tested, not all acquired the ability to grow on 3-CBA (Fig. 3A); (ii) of the cultures of strain NCIB8288 that showed growth on 3-CBA, the trait developed after various periods of incubation and the cultures had different growth rates (Fig. 3B); (iii) among the *R. palustris* strains tested, not all 3-CBA-degrading variants acquired the ability to degrade 2- and 4-CBA even after long-term exposure to 3-CBA (Table 1); and (iv) the ratio of the growth rates on 3-, 2-, and 4-CBA differed among variants derived from the parental strains. Further research is underway to elucidate the mechanisms responsible for the adaptive changes.

An important outcome of this study was that all of the axenic cultures of *R. palustris* tested acquired new metabolic properties in fairly short periods of time. This is consistent with the findings of Sasikala et al. [26] who reported that after long-term exposure to heterocyclic aromatic compounds belonging to the pyridine and pyrazine groups, a pure culture of *R. palustris* adapted to photometabolize these compounds. Thus, adaptive mutations that expand the ability to degrade xenobiotic compounds may commonly occur in *R. palustris*.

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## References


[1] Egland, P.G., Gibson, J. and Harwood, C.S. (2001) Reductive, coenzyme A-mediated pathway for 3-chlorobenzoate degradation in the phototrophic bacterium *Rhodopseudomonas palustris*. Appl. Environ. Microbiol. 67, 1396–1399.

[2] Kamal, V.S. and Wyndham, R.C. (1990) Anaerobic phototrophic metabolism of 3-chlorobenzoate by *Rhodopseudomonas palustris* WS17. Appl. Environ. Microbiol. 56, 3871–3873.

[3] Montgomery, L. and Vogel, T.M. (1992) Dechlorination of 2,3,5,6-tetrachlorobiphenyl by a phototrophic enrichment culture. FEMS Microbiol. Lett. 94, 247–250.

[4] van der Woude, B.J., de Boer, M., van der Put, N.M.J., van der Geld, F.M., Prins, R.A. and Gottschal, J.C. (1994) Anaerobic degradation of halogenated benzoic acids by photoheterotrophic bacteria. FEMS Microbiol. Lett. 119, 199–208.

[5] McGrath, J.E. and Harfoot, C.G. (1997) Reductive dehalogenation of halocarboxylic acids by the phototrophic genera *Rhodospirillum* and *Rhodopseudomonas*. Appl. Environ. Microbiol. 63, 3333–3335.

[6] Imhoff, J.F. and Trüper, H.G. (1992) The genus *Rhodospirillum* and related genera. In: The Prokaryotes Vol. III (Balows, A., Trüper, H.G., Dworkin, M., Harder, W. and Schleifer, K.-H., Eds.), pp. 2141–2155. Springer Verlag, New York.

[7] Harwood, C.S. and Gibson, J. (1988) Anaerobic and aerobic metabolism of diverse aromatic compounds by the photosynthetic bacterium *Rhodopseudomonas palustris*. Appl. Environ. Microbiol. 54, 712–717.

[8] Gibson, J. and Harwood, C.S. (1995) Degradation of aromatic compounds by nonsulfur purple bacteria. In: Anoxygenic Photosynthetic Bacteria (Blankenship, R.E., Madigan, M.T. and Bauer, C.E., Eds.), pp. 991–1003. Kluwer Academic Publishers, Dordrecht.

[9] Sasikala, C. and Ramana, C.V. (1998) Biodegradation and metabolism of unusual carbon compounds by anoxygenic phototrophic bacteria. Adv. Microb. Physiol. 39, 339–377.

[10] Spain, J.C. and van Veld, P.A. (1983) Adaptation of natural microbial communities to degradation of xenobiotic compounds: effects of concentration, exposure time, inoculum, and chemical structure. Appl. Environ. Microbiol. 45, 428–435.

[11] Aelion, C.M., Swindoll, C.M. and Pfaender, F.K. (1987) Adaptation to and biodegradation of xenobiotic compounds by microbial communities from a pristine aquifer. Appl. Environ. Microbiol. 53, 2212–2217.

[12] van der Meer, J.R., de Vos, W.M., Harayama, S. and Zehnder, A.J.B. (1992) Molecular mechanisms of genetic adaptation to xenobiotic compounds. Microbiol. Rev. 56, 677–694.

[13] Thomas, A.W., Lewington, J., Hope, S., Topping, A.W., Weightman, A.J. and Slater, J.H. (1992) Environmentally directed mutations in the dehalogenase system of *Pseudomonas putida* strain PP3. Arch. Microbiol. 158, 176–182.

[14] Foster, P.L. (1993) Adaptive mutation: the uses of adversity. Annu. Rev. Microbiol. 47, 467–504.

[15] van der Meer, J.R. (1997) Evolution of novel metabolic pathways for the degradation of chloroaromatic compounds. Antonie Van Leeuwenhoek 71, 159–178.

[16] Massey, R.C., Rainey, P.B., Sheehan, B.J., Keane, O.M. and Dorman, C.J. (1999) Environmentally constrained mutation and adaptive evolution in *Salmonella*. Curr. Biol. 9, 1477–1480.

[17] de Wit, R., van Boekel, W.H.M. and van Gemerden, H. (1988) Growth of the cyanobacterium *Microcoleus chtonoplastes* on sulfide. FEMS Microbiol. Ecol. 53, 203–209.

[18] Weisburg, W.G., Barns, S.M., Pelletier, D.A. and Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703.

[19] Rademaker, J.L.W., Louws, F.J. and de Bruijn, F.J. (1997) Characterization of the diversity of ecologically important microbes by rep-PCR genomic fingerprinting. In: Molecular Microbial Ecology Manual, Suppl. 3 (Akkermans, A.D.L., van Elsas, J.D. and de Bruijn, F.J., Eds.), Ch. 3.4.3, pp. 1–26. Kluwer Academic Publishers, Dordrecht.

[20] Bergman, J.G. and Sanic, J. (1957) Determination of trace amounts of chlorine in naphtha. Anal. Chem. 29, 241–243.

[21] You, I.-S. and Bartha, R. (1982) Stimulation of 3,4-dichloroaniline mineralization by aniline. Appl. Environ. Microbiol. 44, 678–681.

[22] Haigler, B.E., Pettigrew, C.A. and Spain, J.C. (1992) Biodegradation of mixtures of substituted benzenes by *Pseudomonas* sp. strain JS150. Appl. Environ. Microbiol. 58, 2237–2244.

[23] Egland, P.G., Gibson, J. and Harwood, C.S. (1995) Benzoate-coenzyme A ligase, encoded by *badA*, is one of three ligases able to catalyze benzoyl-coenzyme A formation during anaerobic growth of *Rhodopseudomonas palustris* on benzoate. J. Bacteriol. 177, 6545–6551.

[24] Harwood, C.S., Burchhardt, G., Herrmann, H. and Fuchs, G. (1999) Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. FEMS Microbiol. Rev. 22, 439–458.

[25] Villemur, R. (1995) Coenzyme A ligases involved in anaerobic biodegradation of aromatic compounds. Can. J. Microbiol. 41, 855–861.

[26] Sasikala, C., Ramana, C.V. and Raghuveer Rao, P. (1994) Photometabolism of heterocyclic aromatic compounds by *Rhodopseudomonas palustris* OU 11. Appl. Environ. Microbiol. 60, 2187–2190.