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Degradation of 4-aminobenzenesulfonate by a two-species bacterial coculture

Physiological interactions between Hydrogenophaga palleronii S1 and Agrobacterium radiobacter S2

Esther Dangmann, Andreas Stolz, Andrea E. Kuhm, Angela Hammer, Burkhard Feigel, Naruemol Noisommit-Rizzi[1], Manfred Rizzi[1], Matthias Reuß[1] & Hans-Joachim Knackmuss*
Institut für Mikrobiologie der Universität Stuttgart, Allmandring 31, 70569 Stuttgart, Germany; [1] *Institut für Bioverfahrenstechnik der Universität Stuttgart, Allmandring 31, 70569 Stuttgart, Germany (* Corresponding author)*

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Abstract

The mutualistic interactions in a 4-aminobenzenesulfonate (sulfanilate) degrading mixed bacterial culture were studied. This coculture consisted of *Hydrogenophaga palleronii* strain S1 and *Agrobacterium radiobacter* strain S2. In this coculture only strain S1 desaminated sulfanilate to catechol-4-sulfonate, which did not accumulate in the medium but served as growth substrate for strain S2. During growth in batch culture with sulfanilate as sole source of carbon, energy, nitrogen and sulfur, the relative cell numbers (colony forming units) of both strains were almost constant. None of the strains reached a cell number which was more than threefold higher than the cell number of the second strain. A mineral medium with sulfanilate was inoculated with different relative cell numbers of both strains (relative number of colony forming units S1:S2 2200:1 to 1:500). In all cases, growth was found and the proportion of both strains moved towards an about equal value of about 3:1 (strain S1:strain S2). In contrast to the coculture, strain S1 did not grow in a mineral medium in axenic culture with 4-aminobenzenesulfonate or any other simple organic compound tested. A sterile culture supernatant from strain S2 enabled strain S1 to grow with 4-aminobenzenesulfonate. The same growth promoting effect was found after the addition of a combination of 4-aminobenzoate, biotin and vitamin B_{12}. Strain S1 grew with 4-aminobenzenesulfonate plus the three vitamins with about the same growth rate as the mixed culture in a mineral medium. When (resting) cells of strain S1 were incubated in a pure mineral medium with sulfanilate, up to 30% of the oxidized sulfanilate accumulated as catechol-4-sulfonate in the culture medium. In contrast, only minor amounts of catechol-4-sulfonate accumulated when strain S1 was grown with 4ABS in the presence of the vitamins.

Abbreviations: 4ABS – 4-aminobenzenesulfonate, CFU-colony forming units, 4CS – catechol-4-sulfonate, 4HB – 4-hydroxybenzoate

Introduction

The degradation of recalcitrant compounds with xenobiotic character requires unusual catabolic activities which may not be found in a single organism. Characteristically, the microbial degradation of sulfonated aromatics is often accomplished by mixed cultures. Thus the mineralization of 4-aminobenzenesulfonate (sulfanilate; 4ABS) and the vinylogous compound 6-aminonaphthalene-2-sulfonate is accomplished by

a mutualistic interaction of two bacterial species (Nörtemann et al. 1986; Diekmann et al. 1988; Feigel & Knackmuss 1988; Thurnheer et al. 1988; Jiménez et al. 1991; Rozgaj & Glancer-Šoljan 1992; Feigel & Knackmuss 1993). A two species microbial community, which grows with 4ABS as sole source of carbon, nitrogen, sulfur, and energy is currently studied in our laboratory (Feigel & Knackmuss 1988, 1993). This mixed culture consists of *Hydrogenophaga palleronii* strain S1 and *Agrobacterium radiobacter* strain S2. The analysis of the degradative pathway for 4ABS demonstrated that only strain S1 was able to oxidize the growth substrate. 4ABS was converted by this strain to catechol-4-sulfonate (4CS) which was utilized by both organisms, strain S1 and strain S2. Although a complete enzyme sequence for the degradation of 4ABS *via* 4CS was identified in strain S1, productive degradation of 4ABS was observed only in coculture with strain S2 (Feigel & Knackmuss 1988, 1993). In the present study, the factors which govern the behaviour of the mixed culture and the interactions between the two strains were analyzed.

Material and methods

Organisms

The 4-aminobenzenesulfonate degrading mixed culture consisting of *Hydrogenophaga palleronii* strain S1 and *Agrobacterium radiobacter* strain S2 has been described previously (Feigel & Knackmuss 1988, 1993).

Culture conditions

The nitrogen- and sulfur-free mineral medium (pH 7.4) contained 8.0 g $K_2HPO_4 \times 3\ H_2O$, 2.0 g $NaH_2PO_4 \times H_2O$, 0.1 g $MgCl_2$, 0.02 g $FeCl_2$ and 2.0 ml of a trace element solution per litre. The stock solutions were prepared as described before (Feigel & Knackmuss 1988). For growth experiments with catechol-4-sulfonate and 4-hydroxybenzoate, $(NH_4)_2SO_4$ (0.5 g/l) was added as nitrogen and sulfur source.

HPG medium was composed of 3.0 g yeast extract, 5.0 g peptone from casein and 1.0 g glucose per litre. The SHPG medium was supplemented additionally with 2.0 g 4ABS per litre.

A vitamin stock solution was used, which contained 2 mg biotin, 2 mg folic acid, 10 mg pyridoxal hydrochloride, 5 mg thiamine hydrochloride, 5 mg riboflavine, 5 mg nicotinic acid, 5 mg calcium pantothenate, 2 mg vitamin B_{12}, 5 mg 4-aminobenzoate, and 5 mg lipoic acid per litre (Wolin et al. 1963).

The organisms were cultivated in batch-culture in 100 ml Erlenmeyer flasks with baffles containing 10 ml of medium on a rotary shaker (100 rpm) at 30° C.

Measurement of growth

Growth of the bacterial cultures was monitored spectrophotometrically by measuring the optical density at 650 nm with a Kontron Uvikon 710 spectrophotometer (Kontron, Eching, Germany). For *Hydrogenophaga palleronii* S1 an optical density $OD_{650nm} = 1.0$ corresponded to a biomass concentration of 0.7 g dry weight per litre. An optical density $OD_{650nm} = 1.0$ corresponded to 2.9×10^9 (*Hydrogenophaga palleronii* S1) or 1.2×10^9 (*Agrobacterium radiobacter* strain S2) cells/ml, respectively.

Analytical methods

4ABS and 4SC were analyzed by reverse phase HPLC (HPLC data and chromatography control station model 840, equipped with a programmable multi-wavelength detector model 490; Waters Associates, Milford, MA, USA). A reverse-phase column (250 mm × 4.6 mm; Grom, Herrenberg, Germany), packed with 5 μm particles of LichroSorb RP8 (Merck, Darmstadt, Germany) was used. The separated compounds were detected photometrically at 210 or 235 nm. The following solvent system was used (v/v): 98% water, 1% methanol and 1% H_3PO_4 (85%). The usual flow rate was 0.7 ml/min.

Differentiation between strain S1 and strain S2

After four days incubation on a complex medium containing 4ABS (SHPG-medium), both strains formed characteristic types of colonies. Those of strain S1 were yellow and small. In contrast, the colonies formed by strain S2 were whitish with a dark center and 2-3-fold larger than those of strain S1. Usually a violet halo was observed around colonies of strain S1 due to the excretion of catechol-4-sulfonate which forms a colored complex with Fe^{3+}-ions. Therefore the composition of the mixed culture during growth with 4ABS could be easily analysed by plating on SHPG-medium and counting the colony forming units.

Chemicals

The sources of all chemicals have been described before (Feigel & Knackmuss 1988).

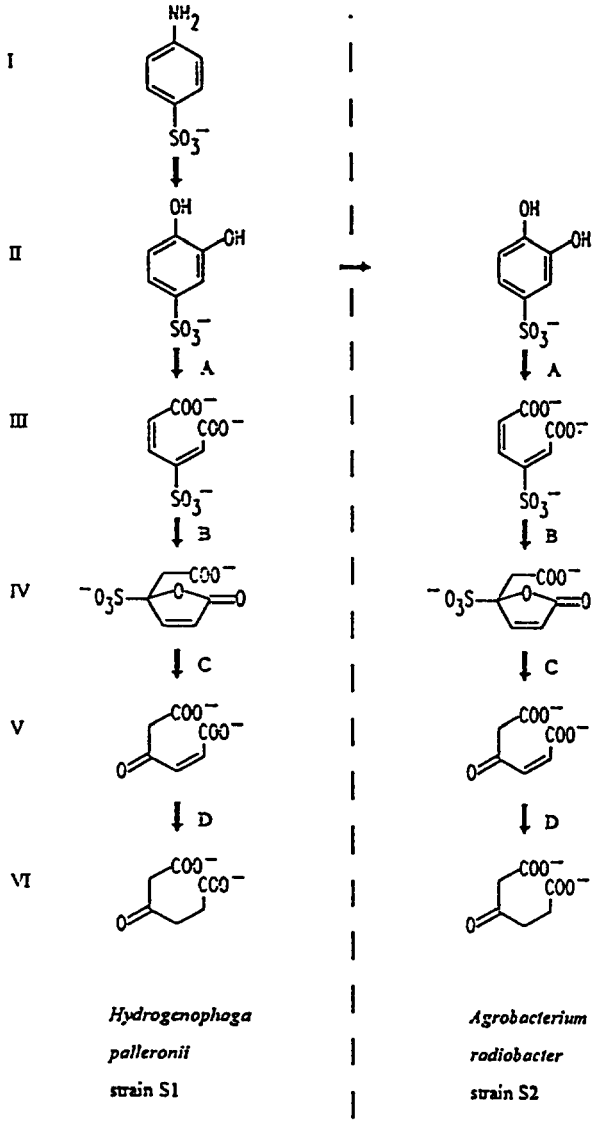

Figure 1. Proposed pathway for the degradation of 4-aminobenzenesulfonate by a mixed culture of *Hydrogenophaga palleronii* S1 and *Agrobacterium radiobacter* S2 (Feigel & Knackmuss 1993). Key to enzymes: A, protocathechuate 3,4-dioxygenase type II; B, 3-carboxymuconate cycloisomerase type II; C, sulfolactone hydrolase; D, maleylacetate reductase. Key to compounds: I, 4-aminobenzenesulfonate (sulfanilate, 4ABS); II, catechol-4-sulfonate (4CS); III, 3-sulfomuconate; IV, 4-sulfomuconolactone (4-carboxymethyl-4-sulfobut-2-en-4-olide); V maleylacetate; VI 3-oxoadipate.

Results

Population analysis during growth with 4ABS

For the degradation of 4ABS by the mixed culture of *Hydrogenophaga palleronii* strain S1 and *Agrobacterium radiobacter* strain S2, the degradative pathway shown in Figure 1 has been proposed (Feigel & Knackmuss 1993). To analyze the composition of the mixed culture, a 4ABS grown inoculum of the consortium was added (1% v/v) to a mineral medium with 4ABS as sole source of carbon, nitrogen, sulfur, and energy. At different time intervals, aliquots were taken, the optical density was determined and the cell numbers of both strains were analyzed by plating. During the entire growth experiment, the ratio of the cell numbers of strain S1 to strain S2 was almost constant. The number of colony forming units (CFU) of strain S1 was about 3-fold higher than the corresponding value for strain S2 (Figure 2).

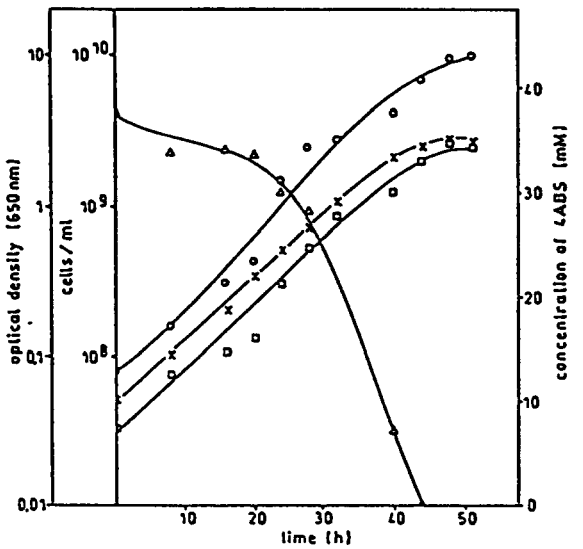

Figure 2. Population analysis of the *Hydrogenophaga palleronii* S1 and *Agrobacterium radiobacter* S2 coculture during growth with 4ABS. The mixed culture was grown in a mineral medium with 4ABS to an optical density of $OD_{650nm} = 1.55$. From this preculture, 0.02 ml was transferred to 20 ml of a mineral medium with 4ABS as sole source of carbon, nitrogen, sulfur, and energy (c_{4ABS} = 28.9 mM = 5 g/l). Growth was determined spectrophotometrically at 650 nm (X), the concentration of 4ABS by HPLC (△) and viable cell counts of strain S1 (○) and strain S2 (□) was carried out by plating on SHPG medium after appropriate dilutions.

In order to analyze the stability of the consortium, pure cultures of strain S1 and strain S2 were mixed in different proportions. These mixtures were used to inoculate mineral media with 4ABS to the same initial optical density. The initial ratio of the cell-numbers (relative number of CFU) of strain S1 and strain S2 were varied between 2200:1 and 1:500 (S1:S2). Finally, growth and consumption of 4ABS was observed

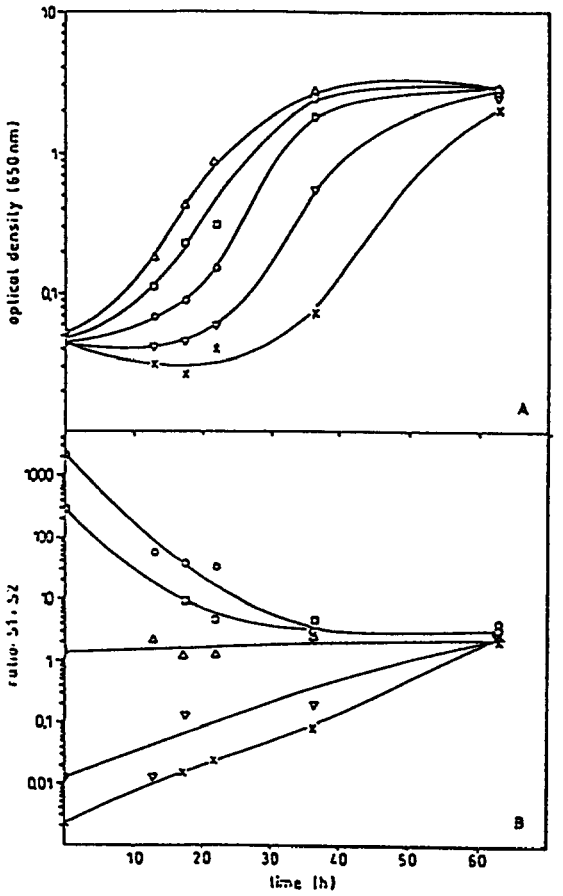

Figure 3. Growth of mixed cultures of *Hydrogenophaga palleronii* S1 and *Agrobacterium radiobacter* S2 with 4ABS inoculated with different initial ratios of both strains. Precultures of strain S1 and S2 were grown in SHPG-medium or HPG-medium with 4SC (5 mM), respectively. These cultures were separately centrifuged, the cells washed with 0.9% (w/v) NaCl and resuspended in Na/K-phosphate buffer (54 mM, pH 7.4) to a final optical density (OD_{650nm}) of 0.25. The following amounts of these two cell suspensions were mixed together: 4 ml S1 and 1 µl S2 ((○), 4 ml S1 and 40 µl S2 (□), 2 ml S1 and 2 ml S2 (△), 40 µl S1 and 4 ml S2 (▽), 1 µl S1 and 4 ml S2 (X). These mixtures were added to 5 Erlenmeyerflasks (20 ml culture volume) with mineral medium and 5 g/l 4ABS. Growth was measured spectrophotometrically (Figure 3A) and the ration of strain S1 to strain S2 determined on SHPG-agar plates (Figure 3B).

with all cultures, although the lag-periods differed considerably (Figure 3A). Pronounced longer lag-phases were observed particularly with those cultures which contained low initial cell numbers of strain S1. During growth with 4ABS, the composition of these mixed cultures moved towards an almost equal relative cell number of the two organisms (Figure 3B).

Growth of the mixed culture with catechol-4-sulfonate

Strain S1 and strain S2 were both able to utilize catechol-4-sulfonate (4CS), whereas only strain S1 oxidized 4ABS (Feigel & Knackmuss 1988, 1993). Therefore it was of interest to study the growth characteristics of the mixed culture growing with 4CS. A 4ABS grown mixed culture (doubling time [t_d] = 7.2 h) was transferred to a mineral medium with 4CS (5 mM) as sole source of carbon and energy. After growth with 4CS, the mixed culture was twice transferred to fresh medium with 4CS (inoculum 1% v/v, each; t_d of the final culture = 8.0 h). Cultures were analyzed by plating on HPG agar. During these experiments a continuous decrease of CFU from strain S1 was found. After two transfers less than 0.5% of the CFU were due to strain S1.

Growth of strain S1 or S2 in axenic cultures

When a culture of strain S1 was transferred (1% v/v) from complex medium (SHPG) into mineral medium with 4ABS (1 g/l, c_{4ABS} = 5.8 mM) as sole source of carbon, energy, nitrogen and sulfur, the maximal optical density reached by the bacterial culture was less than OD_{650nm} = 0.1 (compared to OD_{650nm} = 0.58 for the mixed culture under the same conditions). Nevertheless, after 10 days of incubation about half of the initial amount of 4ABS had been consumed by the cells. The concentration of the substrate decreased linearly with time. About 30% of the 4ABS converted were accumulated in the culture medium as 4CS.

In a parallel experiment $(NH_4)_2SO_4$ (0.5 g/l) was added to the mineral medium as nitrogen- and sulfur-source. Under these conditions (c_{4ABS} = 5.8 mM) the cultures of strain S1 reached an optical density of OD_{650nm} = 0.5 within 2 days. When these cultures were transferred (1% v/v) to fresh medium of the same composition, growth discontinued. It was therefore concluded that during transfer of the cells from complex medium to mineral medium some growth factors had been carried along. Axenic cultures of strain S1 also showed no growth in a mineral medium with 4-hydroxybenzoate (4HB) or 4CS as sole sources of carbon and energy.

Strain S2 did not grow with 4ABS, but could be transferred for an unlimited number of generations in mineral media with 4CS (t_d = 8.0 h) or 4HB (t_d = 2.6 h) (5 mM, each).

Growth promoting effect of strain S2 on strain S1

A promotive effect of strain S2 on the growth of strain S1 was clearly visible on agar plates with 4ABS in a mineral medium. When both strains were inoculated in separate streaks on distant parts of the agar-plate, no growth was found for strain S2 and only weak growth of strain S1 was visible. When both strains were streaked only by a few millimeters apart, growth of strain S2 occurred and growth of strain S1 was clearly stimulated.

In a similar experiment both strains were streaked out in an analogous manner on agar plates which contained both gluconate and 4HB (2 mM each). It was shown earlier that 4HB supported growth of strain S2 (Feigel & Knackmuss 1993), and oxidation of gluconate by strain S1 was found during a taxonomic study using the Biolog identification system (GN MicroPlates, Biolog Inc., Hayward, CA, USA). In this experiment pronounced growth of strain S2 was found irrespective of the presence of strain S1. Again, growth of strain S1 was observed only if strain S2 was present in the neighbourhood (distance about 2–5 mm). This demonstrated that the growth promoting effect of strain S2 on strain S1 was independent of 4ABS as growth substrate. It was therefore concluded that strain S2 must excrete factors which support the growth of strain S1.

This effect was also demonstrated in liquid culture by growing strain S2 in a mineral medium with 4CS and using sterilized culture supernatant for growth experiments with strain S1. In the presence of this culture supernatant, growth of strain S1 with 4ABS was observed. In a control experiment without the S2 culture supernatant and in a further control without added 4ABS no growth of strain S1 was found. Therefore it was concluded that strain S2 supplied strain S1 with growth factors and that these factors did not serve as a source of carbon or energy for strain S1.

Identification of growth factors required by strain S1

A positive effect of 4-aminobenzoate (4AB) for growth of strain S1 has been observed before (Noisommit-Rizzi et al. 1991). Therefore, strain S1 was grown with HPG-medium and transferred (1% v/v) to several flasks, which contained 10 ml of a mineral medium with $(NH_4)_2SO_4$ (0.5 g/l) and 4ABS (5.8 mM) as sole source of carbon and energy. Different compounds were added to the respective flasks: 4AB (1.5 μM or 15 μM) or 10 μl of a vitamin solution (also containing 4AB, see Material and methods) or a mixture of 4AB (1.5 μM) and 10 μl of the vitamin solution. With the vitamin solution, growth was clearly stimulated and final optical densities $OD_{650nm} > 0.5$ was reached. These cultures could be repeatedly subcultivated in the same medium. By testing different mixtures of vitamins as growth factors it was found that axenic cultures of strain S1 could be repeatedly subcultivated with 4ABS if 4AB (5 μg/l), vitamin B_{12} (2 μg/l) and biotin (2 μg/l) were added to the medium.

When strain S1 was grown with 4ABS (5.8 mM) plus the supplines mentioned above, the doubling times ($t_D \cong 6$ h) and final cell densities ($OD_{650nm} = 0.55$) were almost the same as those observed with the mixed culture of strain S1 and S2 if growing with 4ABS in a vitamin-free mineral medium. Surprisingly, only marginal amounts of 4CS (< 0.15 mM) were accumulated in the culture fluid by axenic cultures of strain S1 growing in the presence of the vitamins.

With 5 g/l 4ABS the final optical density of the axenic culture ($OD_{650nm} = 2.1$) was only about 75% of the value found for the mixed culture, although 4ABS was completely utilized. When the amount of supplines added was increased to 20 mg/l 4AB, 2 μg/l vitamin B_{12}, and 2 μg/l biotin the pure culture of strain S1 reached the same final optical density as the mixed culture. This increase was not due to the supplementary amount of 4AB added (= 0.15 mM). During growth of the axenic culture in the mineral medium with 5 g/l 4ABS (= 29 mM), at most 0.64 mM 4SC were accumulated in the culture medium.

It was tested whether strain S2 would be able to survive in the mixed culture if the 4ABS mineral medium was supplemented by the vitamins essential for strain S1. Therefore, the mixed culture was transferred to a medium with 4ABS (5 g/l) plus 20 mg/l 4AB, 2 μg/l vitamin B_{12}, and 2 μg/l biotin. These cultures were transferred eight times (0.5%, v/v) and the relative amounts of the two strains were determined by plating. During the first subcultivation, the relative number of CFU of strain S1 compared those of strain S2 shifted to a ratio of 40:1 which thereafter remained almost constant during subcultivation.

Discussion

Currently two mixed bacterial cultures which totally degrade arylsulfonates are studied in this laboratory: A culture which is able to mineralize different (substituted) naphthalenesulfonates (Nörtemann et al. 1986,

1994) and the 4ABS degrading culture described in the present publication (Nörtemann et al. 1986; Feigel & Knackmuss 1988, 1993; Nörtemann et al. 1994). In the case of the naphthalenesulfonates degrading mixed culture one strain (strain BN6) is able to oxidize sulfonated naphthalenes so (non-sulfonated) substituted salicylates. This strain grows in mineral media with many naphthalenesulfonates if they are supplied in appropriate concentrations. The function of the accompanying strains in this mixed culture can be described by their ability to metabolize the salicylates which are excreted in stoichiometric amounts by strain BN6. They are therefore necessary for the total degradation of the naphthalenesulfonates. Furthermore, these accompanying strains remove those salicylates (especially salicylate and 4-hydroxysalicylate) which are toxic for strain BN6. Therefore, the presence of these strains allows growth of strain BN6 with the corresponding naphthalenesulfonates (Nörtemann et al. 1986, 1994).

In the 4ABS degrading mixed culture described here, the interactions among the organisms are much more complex. It was earlier shown that only strain S1, but not strain S2 is able to oxidize 4ABS. Therefore the presence of strain S1 is crucial for the degradation of 4ABS. The inability of strain S1 to grow in axenic culture in a pure mineral medium with 4ABS demonstrated a pivotal role of strain S2 for growth of strain S1. The mixed culture has by now been cultivated in the laboratory for almost 10 years (Feigel 1985). If strain S2 would be only a commensal during degradation of 4ABS it should have been overgrown by strain S1 during this long time period. The importance of strain S2 for the productive degradation of 4ABS by the mixed cultures was further demonstrated by the growth experiments starting with different ratios of cell numbers of strain S1 and strain S2 (Figure 3). Since strain S2 is not able to convert 4ABS to 4CS but can totally degrade the latter even in axenic culture, its growth with 4ABS within the mixed culture only depends on the excretion of 4CS by strain S1.

The growth experiments with pure cultures of strain S1 and strain S2 with 4CS, 4HB or gluconate as carbon sources indicated that strain S2 can grow in pure culture in mineral media with these compounds, whereas strain S1 could not. The experiments performed with mixed cultures of strain S1 and strain S2 on mixed substrates (4HB plus gluconate) showed that, under these conditions, strain S1 was able to grow only in the presence of strain S2. It was thus demonstrated that the promotive effect of strain S2 on growth of strain S1 was independent from 4ABS as growth substrate and cannot be explained by the elimination of potentially toxic metabolites formed by strain S1 from 4ABS oxidation.

The experiments performed with culture supernatants from strain S2 showed that strain S2 excreted factors which enabled strain S1 to grow, but which were not utilized as carbon sources by strain S1. The growth promoting effect of the culture supernatant from strain S2 on strain S1 could be completely replaced by the addition of 4-aminobenzoate, biotin, and vitamin B_{12}.

Clearly strain S1 supplies strains S2 with carbon, energy, nitrogen, and sulfur sources formed from 4ABS. The results obtained by addition of the vitamins to axenic cultures of strain S1 suggest that in return for this provision of 4CS, strain S2 supplies strain S1 with 4-aminobenzoate, biotin, and vitamin B_{12}. It is well known that interspecies transfer of growth factors is of major importance in natural populations (Alexander 1961). A comparable coculture between a cellulolytic, N_2-fixing *Clostridium* sp. and a non-cellulytic *Klebsiella* sp. has been recently described (Cavedon & Canale-Parola 1992). In this coculture the *Clostridium* sp. hydrolysed cellulose to soluble sugars that served as fermentable substrates for the *Klebsiella* sp. In turn, the *Klebsiella* sp. excreted biotin and 4-aminobenzoate, which were required by the *Clostridium* sp.

For the mixed culture of *Hydrogenophaga palleronii* S1 and *Agrobacterium radiobacter* S2, it seems reasonable to assume that strain S1 supplies strain S2 with 4CS as a source of carbon and energy. The experiments performed with the mixed culture growing on 4ABS demonstrated that in these cultures about one third of the cells belonged to strain S2. Thus it must be assumed that a corresponding amount of the carbon and energy originating from 4ABS is transferred from strain S1 to strain S2. It was previously suggested that 4CS was the metabolite which is excreted by strain S1 and taken up by strain S2 (Feigel & Knackmuss 1993). Because both strains metabolize 4CS presumably by the same degradative pathway, any other intermediate formed from 4ABS could also play this role.

The main argument for the interspecies transfer of 4CS was the observation that resting cells of strain S1 (pregrown with SHPG medium) converted 4ABS to 4CS, which became visible as a Fe^{3+}-complex causing an intensive violet coloration of the medium (Feigel & Knackmuss 1988, 1993). In a typical experiment, where the cells were incubated on a rotary shaker, maximally about 30% of the 4ABS converted were temporarily excreted as 4CS (Feigel & Knackmuss

1993). This can be utilized by strain S2 as sole growth substrate. In the present publication, however, it was shown that strain S1 excretes only minor amounts of 4CS when growing with 4ABS in the presence of the vitamins added. Therefore, if 4CS is the actual metabolite transferred it must be postulated that its excretion is increased under vitamin deficiency. This would lead to the situation that a limiting number of cells from strain S2 will cause strain S1 to excrete larger amounts of 4CS which then promotes growth of strain S2. Therefore growth of the two partner strains may be balanced by supplying 4CS to strain S2 to such an extent that enough vitamins are produced for strain S1. This would explain the constant ratio of cell numbers for both strains during growth with 4ABS.

The other possibility is that another metabolite instead or in addition to 4CS is transferred from strain S1 to S2. Actually, two or three signals of low intensity and unknown identity were observed during the HPLC-analysis of the culture supernatants of strain S1 converting 4ABS.

The amount of 4CS which is accumulated by strain S1 can be influenced by the dissolved oxygen tension in the medium (Noisommit-Rizzi et al. 1991a; Nörtemann et al. 1994). It is currently attempted to combine the effects of these different parameters in a mathematical model to describe the mutualistic interactions in the mixed culture under different process conditions (Noisommit-Rizzi et al. 1991b; Noisommit-Rizzi 1994).

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