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Anaerobic Microbial Associations Degrading Aminoaromatic Acids

I. B. Kotova*, O. V. Savel'eva*, A. T. D'yakonova*, V. I. Sklyar, S. V. Kalyuzhnyi**, A. Stams***, and A. I. Netrusov***

**Moscow State University, Faculty of Biology, Moscow, 119992 Russia*
e-mail: anetrusov@mail.ru
***Moscow State University, Faculty of Chemistry, Moscow, 119992 Russia*
****Wageningen University, Wageningen 6703CT, The Netherlands*

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Abstract—Anaerobic microbial associations have been isolated that degrade aminoaromatic acids to methane and carbon dioxide at high rates. Significant differences between the morphological, cytological, and physiological traits of cultures isolated from samples of adapted and unadapted sludge are shown. The effects of cultivation temperature, illumination, and presence of mineral nitrogen and bicarbonate in the medium upon adaptation of enrichment cultures to substrates and subsequent behavior of the anaerobic associations have been studied. Intermediate and final products of degradation of aminoaromatic compounds and the sequence of their formation in the cultures have been determined. We have also studied the effects of exogenous electron acceptors and additional carbon sources on the degradation of aminoaromatic compounds.

Aminoaromatic compounds studied in this work belong to the category of xenobiotics. Their release into the environment has become a hazard in recent years. Being components of dyes, stabilizers, medicines, and biocides, they are produced by the chemical industry in increasing quantities. The failure of numerous attempts of scientists to degrade such compounds (into inorganic and nontoxic organic substances) using microorganisms is related to the high toxicity and carcinogenicity of these materials [1].

Isolation of microorganisms and microorganism associations capable of degrading the compounds under consideration and determination of appropriate conditions of the process are particularly important. As many such substances are polymerized in the presence of oxygen (giving rise to compounds that are not readily degradable), anaerobic conditions are often more favorable for degradation [2]. Therefore, in recent decades, studies have been performed seeking anaerobic associations capable of degrading various aminoaromatic xenobiotics.

So far, microbial associations and pure bacterial cultures have been obtained which degrade the target compounds via sulfate [3] and nitrate [4, 5] reduction. Their physiological traits and biochemical features of cleavage of the aromatic ring have been studied [6–9]. Much less is known about the methanogenic pathway of degradation of aminoaromatic compounds.

The goal of this work was to isolate and study the properties of microbial associations degrading isomers of aminobenzoic (ABA) and aminosalicylic (ASA) acids under anaerobic methanogenic conditions.

MATERIALS AND METHODS

Biomass source. Experiments were performed with anaerobic sludge of two kinds: (1) mesophilic floccular sludge of works processing hog-farm sewage adapted to various isomers of aminoaromatic compounds (Russia) and (2) unadapted mesophilic floccular sludge of works processing domestic wastes (Bennekom, The Netherlands).

Main reagents: 2-ABA, 3-ABA, 4-ASA, and bromoethanesulfonic acid (BESA) from Sigma (USA); 2-hydroxybenzyl alcohol and 3-hydroxybenzyl alcohol from Aldrich (Germany); 4-ABA, 5-ASA and salicylic acid from Merck (Germany); resazurin from Fluka (Germany); and yeast extract from BDH Chemicals (UK).

Cultivation. Sludge was inoculated in a mineral medium containing 100 mg/l yeast extract, microelements, and 0.2 mg/l resazurin (an indicator of anaerobic conditions); starting pH was in the range 7.0–7.5 [10–12]. Anaerobic conditions were provided by replacement of the gaseous phase in vials sealed with rubber corks with nitrogen and addition of a reducing agent, 0.278 g/l $Na_2S \cdot 9H_2O$. Isomers of aminoaromatic acids 2-, 3-, 4-ABA and 4-, 5-ASA were used as main aminoaromatic substrates. The substrates and other essential additives were stored under anaerobic conditions as sterile stock solutions in the mineral medium and added to vials at proper quantities with a syringe. For nitrate reduction, $NaNO_3$ was added to the medium to final concentrations of 10 or 20 mM. Nitrite, sulfate, sulfite, and thiosulfate were added to concentrations of 10 mM each. For the development of anaerobic

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Table 1. Degradation of isomers of aminobenzoic and aminosalicylic acids by active methanogenic associations

Substrate	Culture source	Lag phase, days	Substrate consumption rate, mM/day	Culture features	Predominant morphotypes
2-ABA	Mesophilic sludge adapted to 2-ABA	87	0.6–0.8	Dark flakes in turbid culture liquid, yellowish-brown precipitate	Rods of various lengths, some of them fragmented, with spores; separate cocci
5-ACA	Mesophilic sludge adapted to 5-ACA	150–240	0.5	Grayish mucous aggregates in turbid culture liquid	Aggregates of small rods, separate cocci, long thin heteromorphous rods
4-ABA	Mesophilic sludge adapted to 4-ABA	300	0.15	Dark flakes in turbid culture liquid, yellowish-brown precipitate	Rods of various lengths, some of them fragmented, with spores; separate cocci

associations, a 5–10% (v/v) homogenized sludge sample was added to the liquid medium containing an aminoaromatic substrate. Enrichment cultures were incubated at 30°C. All platings were performed with sterile syringes under anaerobic conditions.

Assays. Aromatic compounds were assayed by UV spectrometry at characteristic wavelengths (5-ASA, 330 nm; 2-ABA, 310 nm; 3-ABA, 300 nm; 4-ASA, 295 nm; and 4-ABA, at 275 nm) using a Shimadzu UV-1202 spectrometer (Japan) and by RP-HPLC using Chromspher C8 2 × 10 cm and Inertsil ODS3 2 × 10 cm columns (Chrompack, the Netherlands). Detection was performed at 255 nm. Hydrogen and methane were assayed with a 14B gas chromatograph (Shimadzu, Japan). Carbon dioxide and alcohols with few carbon atoms were assayed with Chrompack CP9001 and Chrompack CP9000 chromatographs, respectively (Chrompack). Ammonium was assayed by the Nessler method [13], and sulfide, by the method of Truper and Schlegel [14].

RESULTS AND DISCUSSION

Enrichment cultures from sludges preadapted to ABA and ASA isomers. Anaerobic enrichment cultures degrading ABA and ASA isomers were obtained directly from active methanogenic sludges that had been adapted for degradation of corresponding aminoaromatic acids more than two years previously [15]. To obtain stable anaerobic associations, we made endpoint dilutions in such a way that (1) the resulting associations were still capable of degrading aminoaromatic acids and (2) microorganisms not related to biodegradation were eliminated. The anaerobic sludge suspension (5–10%) was inoculated into vials with 5 ml of mineral medium. Then, those cultures that remained active were inoculated into 500-ml serum bottles with 250 ml of the same medium.

At the first stage of isolation of methanogenic associations from sludges degrading corresponding ABA and ASA isomers, we found that all associations were characterized by certain lag periods (probably required for spatial arrangement of the association, modification of its trophic webs and activation of enzyme sets necessary for degradation of target compounds to final products). Table 1 shows that the stable active enrichment cultures obtained by us varied in the duration of the lag and the rate of substrate consumption.

The only carbon and energy sources in the media were ABA and ASA isomers. The final degradation products of the aminoaromatic acids studied were methane and carbon dioxide. Independent of the aminoaromatic substrate, the stable active methanogenic associations always produced CO_2, H_2, and NH_4^+; then, benzoate and acetate (Fig. 1); and, sometimes, traces of ethanol, propanol, 2-propanol, propionate, and butyrate. Initial slow consumption of an aminoaromatic substrate was accompanied by changes in exterior features and microscopic images of the cultures. Dark

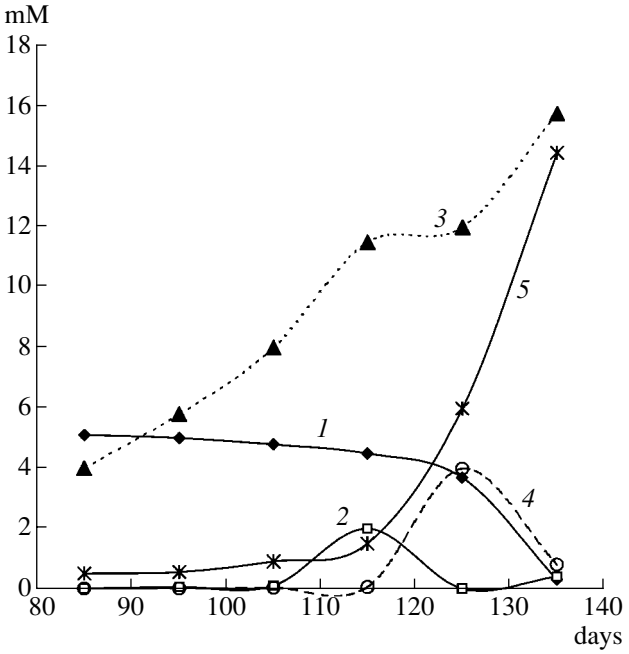

Fig. 1. Degradation of 2-ABA by a methanogenic association: (*1*) 2-ABA, (*2*) benzoate, (*3*) ammonia, (*4*) acetate, (*5*) methane.

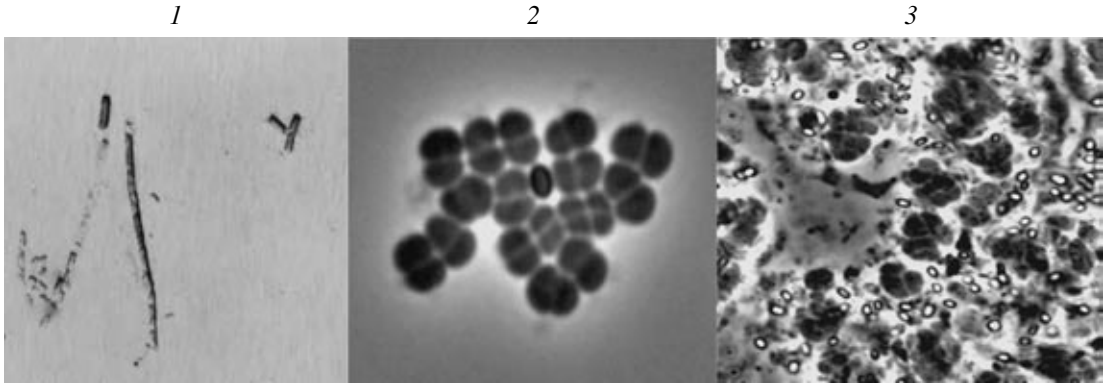

Fig. 2. Cell morphotype at various biodegradation stages: (*1*) chains of heteromorphous rods packed in a common mucous envelope; (*2*) Methanosarcina-like cells; (*3*) methanogenic grains.

grains consisting of thin rods varying in length, spore-forming rods, and cocci incorporated into an amorphous matrix gave way to dusty-white films containing chains and clusters of small rods and cocci. At the end of the lag period, the first aromatic intermediate, a benzoate derivative, was detected spectrophotometrically at 225 nm. Simultaneously, the culture liquid became turbid; dark-brown films formed at the bottom; and white cottonlike flakes formed in the column of the liquid. Then the association began to consume benzoate and rapidly performed the final degradation of the aminoaromatic substrate. After addition of a fresh portion of the aromatic amino acid, an increase in the contents of benzoate, ammonium, and methane was observed, but the content of acetate remained constantly low. In stable active enrichment cultures, the consumption rate did not vary on addition of fresh substrate portions. After passage to fresh mineral medium (10% inoculate), the association started the consumption of the aminoaromatic substrate at a high rate without any lag phase.

The microscopic patterns of active anaerobic associations did not depend on the substrate. The onset of intense consumption always matched (1) the appearance of heteromorphous rods, sometimes fragmented and tending to form very long chains packed in a common mucous envelope (Fig. 2, Panel *1*), and (2) the increase in the number of cells similar to those of *Methanosarcina* species (Fig. 2, Panel *2*). With a shortage of the aminoaromatic substrate, associations formed grains, numerous spores appeared, and the chained heteromorphous rods disappeared (Fig. 2, Panel *3*).

According to microscopic studies, a stable methanogenic association intensely degrading a substrate under study contained no more than six cell morphotypes: (1) short straight or bent rods without spores, sometimes forming long filaments, (2) large cocci in tetrads and 8-membered clusters, (3) vibrios, (4) single small cocci, (5) long thin spore-forming rods, and (6) large spore-forming rods.

Associations degrading 5-ASA were also obtained from mesophilic methanogenic sludge preadapted to salicylic acid (SA). First, active enrichment cultures degrading SA to methane and carbon dioxide were isolated as described above. Then, after complete consumption of the substrate, 5-ASA was added. Degradation of the aminoaromatic substrate required no lag. These cultures consistently consumed 5-ASA, but they were less active than cultures obtained directly from the sludge adapted to 5-ASA (Table 1). The rate of substrate degradation was as low as 0.05 mM/day.

We failed to obtain stable enrichment cultures with a high rate of 3-ABA degradation from the mesophilic sludge, because the starting floccular sludge of hog-farm purification works was virtually unable to consume this substrate [15]. After a long lag, enrichment cultures from thermophilic (55°C) methanogenic sludge adapted to 3-ABA showed a low initial rate of 3-ABA degradation (0.05 mM/day) for 10 days. Then, the rate of substrate consumption decreased, and the duration of the cycle increased. Thirty days later, 3-ABA underwent polymerization (this could be an effect of oxygen, trace amounts of which entered the incubation mixture during sampling, that manifested at high temperatures).

At the beginning of the work, we had no anaerobic sludge adapted to 4-ASA [15] at our disposal. Because of this, we could not obtain anaerobic enrichment cultures degrading 4-ASA by the common approach.

The balance of 2-ABA degradation to the final products (methane, carbon dioxide, and ammonium) was calculated for the enrichment culture showing the most stable and intense degradation from five independent replications. It was in agreement with the theoretical balance of aminobenzoic acid decomposition:

Predicted balance:

$$2C_7H_6NO_2^- + 10H_2O + 4H^+ = 7CO_2 + 7CH_4 + 2\,NH_4^+.$$

Experimental balance:

$$2C_7H_6NO_2^- + 10H_2O + 8\,H^+ = 7.2CO_2 + 6.9CH_4 + 3NH_4^+.$$

The elevated concentration of ammonium ions in the culture liquid (3.0 M instead of the expected 2.0) is apparently related to the error of the Nessler method. This method is sensitive to a wide range of ions and therefore not suitable for quantitative assay of ammonium in media with complex mineral composition. It is also conceivable that some members of the association (e.g., spore-forming rods of clostridium type) could fix nitrogen from the gaseous phase of the anaerobic vials, thereby adding to the total amount of ammonium in the liquid medium.

Properties of highly active stable enrichment cultures. Stable, highly active methanogenic associations quite readily degraded not only the main substrate on which they had been enriched but also its isomers, i.e., without any lag phase. For example, the culture adapted to 2-ABA degraded other substrates as follows (in order of decreasing rates): 5-ASA > 4-ABA > 3-ABA > 4-ASA. The last substrate was consumed for no more than two successive cycles. The culture adapted to 5-ASA also intensely degraded 2-ABA. However, the association adapted to 4-ABA consumed 2-ABA at a higher rate than the main substrate (Table 2). A different situation was observed with active methanogenic sludges from which these stable associations had been isolated. Sludges adapted to 5-ASA and 2-ABA did not degrade other isomers of these aminoaromatic acids [15].

To mimic the purification of industrial sewage (usually containing more than one pollutant), we studied the consumption of aminoaromatic substrates by active enrichment cultures from binary mixtures of their aqueous solutions. It was shown that, in the mixture 5-ASA + 2-ABA, both substrates were consumed simultaneously at equal rates. In experiments with 2-ABA + 4-ABA, the former was consumed first, and then 4-ABA was consumed at a lower rate. Of a mixture of two ASA isomers, the association first rapidly consumed 5-ASA and then, at its low residual content, slow degradation of 4-ASA began. The degradation of 4-ASA in a binary mixture is of special interest, because methanogenic associations did not consume 4-ASA as the sole substrate. It is worth noting that the combination of substrates caused a general decrease in the biodegradation rate.

Effects of some factors on the duration of adaptation and biodegradation efficacy. We varied cultivation conditions in order to shorten the duration of the lag and to increase the efficacy of biodegradation. Enrichment cultures were incubated at various temperatures (55, 30, and 20°C), in the light and in the dark, with addition of nitrate or sulfate (as electron acceptors) and other organic substrates. We also used media without nitrogen sources or bicarbonate. The rate of the process did not depend significantly on light or absence of mineral nitrogen or bicarbonate.

Table 2. Consumption of other aminoaromatic substrates by methanogenic associations adapted to a certain isomer

Enrichment culture	Substrate consumption rate, mM/day	
	main	others
2-ABA	0.6–0.8	5-ACA, to 0.6
		4-ABA, to 0.3–0.4
		3-ABA, to 0.1
		4-ACA, to 0.06–0.15
5-ACA	to 0.5	2-ABA, to 0.5
4-ABA	0.15	2-ABA, to 0.3

While developing associations degrading aminoaromatic compounds from active methanogenic sludges, we found that increasing the incubation temperature to 55°C or decreasing it to 20°C did not affect the duration of the lag. However, the incubation temperature affected the rate of degradation of aminoaromatic compounds by these associations. Degradation of 2-ABA at 20°C was even faster than at 30°C. The thermophilic cultivation conditions (55°C) completely arrested the degradation. Changes to the mesophilic conditions recovered the ability of the association to degrade the aminoaromatic substrate after a short adaptation period (about 20 days). The next cycle of substrate consumption under the mesophilic conditions (30°C) started without adaptation. It was shown that short-term temperature changes do not result in irreversible loss of the ability of the associations to degrade aminoaromatic acids.

We studied the effect of various electron acceptors on the degradation of ABA and ASA isomers by anaerobic enrichment cultures obtained from the floccular sludge of purification works treating hog-farm sewage. For this purpose, nitrate and sulfate were added (to final concentrations of 10 and 20 mM) to methanogenic associations not having high degradation rates. Isomers of aminobenzoic (2-ABA, 3-ABA, and 4-ABA) and aminosalicylic (5-ASA) acids were the only carbon and energy sources in the associations under study. None of the anaerobic associations obtained from adapted methanogenic sludge showed nitrate- or sulfate-dependent degradation of the target substances.

Another anaerobic sludge type, mesophilic floccular sludge of purification works treating domestic sewage, was sampled directly from the works without preadaptation. The initial sludge showed both methanogenic activity and the capacity for sulfate reduction and denitrification. Enrichment cultures of this sludge most readily degraded 2-ABA, 3-ABA, and 4-ABA under methanogenic or nitrate-reducing conditions, whereas the rates of 4-ASA and 5-ASA degradation were much lower. Degradation of 2-ABA, 3-ABA, and 5-ASA under sulfate-reducing conditions also produced methane. Judging

from the ratio between the consumed aromatic amino acid and sulfide produced, we suggest that, in enrichment cultures degrading 4-ABA and 5-ASA, the process is related to sulfate-reducing microorganisms.

We added a variety of readily consumable carbon sources to intensify biodegradation in anaerobic associations during their enrichment from sludges and after they lost the ability to degrade aminoaromatic substances as a result of long-term incubation without these substrates ("starvation"). It is known from the literature that *Desulfovibrio vulgaris* PY1 can reduce aromatic carboxylic acids to the corresponding alcohols when fermenting pyruvate in the absence of sulfate [16]. We added substrates most commonly used for sulfate reduction: lactate, a mixture of volatile fatty acids (formate, acetate, propionate, and butyrate, 10 mM each), pyruvate, or acetate. Only pyruvate accelerated mineralization of 5-ASA to methane and carbon dioxide (a threefold increase was detected in comparison with the growth on 5-ASA as the sole carbon and energy source). Microscopic images showed that the methanogenic association grown with an additional source of carbon, pyruvate, was similar to the association grown on the aminoaromatic substrate alone. However, vibrio-like cells were more abundant, and the association was dominated by short straight or bent rods without spores. This anaerobic association fermented pyruvate and simultaneously degraded aminosalicylic acid. The following intermediates were detected: hydrogen, acetate, formate, and propionate. Thus, addition of pyruvate intensified degradation of aminoaromatic acids by the methanogenic association.

Suppression of methanogenesis by adding BESA to the enrichment culture growing on a medium with pyruvate did not affect 5-ASA consumption, but, in addition to hydrogen, carbon dioxide, acetate, and traces of volatile acids (formate, propionate, and lactate), the culture liquid contained a product of 5-ASA conversion, later identified by HPLC as 2-hydroxybenzyl alcohol. The microscopic pattern of the association changed: *Methanosarcina*-like and vibrio-like cells disappeared, as well as long, thin spore-forming rods. The detection of the aromatic intermediate under the conditions of suppressed methanogenesis is in agreement with data from the literature that the benzene ring is not cleaved at initial stages of degradation [17].

Major differences between enrichment cultures and adapted sludges. A comparison of features of highly active enrichment cultures and the sludges from which they were obtained shows that the enrichment cultures are microbial associations with few members directly involved in degradation of the substrates. Unlike sludge, which is highly specific to certain substrates [15], the enrichment cultures can degrade any of the target compounds without a lag, although at variable rates. The different rates of biodegradation of ABA and ASA isomers provide additional proof of the significance of the position of substituents in aromatic compounds for their mineralization by active microbial associations [10, 12]. The rate of degradation by the associations remained high from cycle to cycle, not changing after passages. It is shown that active sludge of purification works contain microorganisms capable of degrading these compounds to produce methane, as well as to perform sulfate- and nitrate-dependent degradation.

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