Aerobic versus Anaerobic Metabolism of Halogenated Anilines by a *Paracoccus* sp.

JEAN-MARC BOLLAG AND STEFAN RUSSEL

Laboratory of Soil Microbiology
Department of Agronomy
The Pennsylvania State University 16802

Abstract. A *Paracoccus* sp. which transforms aniline and different halogen-substituted derivatives under aerobic and anaerobic conditions was isolated from the soil. In experiments with ^{14}C-ring-labeled 4-chloroaniline, approximately 60% of the radioactive material disappeared from the growth medium after incubation under anaerobiosis within 48 hr, but under aerobic conditions no decrease of radioactivity in the growth medium was observed, although 4-chloroaniline was completely metabolized. Acetylation appears to constitute, especially under aerobic conditions, a major transformation mechanism by the bacterium, since almost 50% of the acetylated compound could be detected and identified if aniline, 2-, 3-, and 4-chloroaniline served as substrate. The formation of different metabolites under aerobic and anaerobic conditions clearly indicates the existence of two separate pathways in the metabolism of aniline compounds depending on the oxygen status of the environment.

Aromatic amines are established intermediary products during the decomposition of numerous pesticides. In particular, phenylurea, phenylcarbamates, and acylanilide herbicides are degraded during microbial metabolism to anilines, but the fate of these intermediates in nature is only partially clarified. Several different transformation products of the halogenated anilines have been found, but a pathway of complete degradation, i.e., decomposition into mineral products, has not yet been elaborated.

So far, three major biological transformation reactions of halogenated anilines have been described: (a) dimerization to an azo-derivative [2] or to a diphenylamine [6]; (b) acylation resulting in a formylated [8] or acetylated [9] aniline; and (c) oxidation of the amine to a nitro group [7].

In general it can be stated that all results which were obtained so far relate to the transformation of anilines under aerobic conditions, but there is little knowledge of the extent to which anaerobic metabolism occurs.

In this paper the activity of a bacterium, a *Paracoccus* sp. isolated from soil, capable of transforming various anilines under aerobic and anaerobic conditions is described, and the resulting metabolic products under the various conditions are compared.

MICROBIAL ECOLOGY 3:65–73 (1976)

Materials and Methods

Approximately 20 bacterial strains were isolated from Hagerstown Silt Loam soil which had been treated with the herbicide Maloran [chlorbromuron, 3-(3-chloro-4-bromophenyl)-1-methoxy-1-methylurea]. The bacterium which was most active in the metabolism of 4-chloroaniline was a *Paracoccus* sp., and this strain was subsequently selected for further study. It was determined that this microbe was also capable of growing under anaerobic conditions whereby nitrate was reduced to nitrite.

The *Paracoccus* sp. was grown in a Czapek-Dox broth at 28°C. Aerobic conditions were provided by shaking the cultures in Erlenmeyer flasks on a rotary shaker (150 oscillations/min), and anaerobic conditions were attained by flushing the bottles with helium until all air was removed as indicated by gas chromatographic analysis [4]. The various anilines were dissolved in absolute ethanol, sterilized by membrane filtration (0.22-μm pore size Millipore filter), and added to the growth medium. The final concentration of the various anilines in the growth medium was 20 ppm, and the amount of ethanol was 0.1%. At least three replicates were included in each test, and every experiment was repeated two or three times. In all experiments, sterile controls were simultaneously incubated and analyzed in order to establish that no chemical change or volatilization of the applied aniline took place.

The following chemicals were used: 4-chloroaniline-(ring-UL-^{14}C) sulfate (specific activity 4.5 μCi/m*M*; California Bionuclear Corp., Sun Valley, Calif.); aniline (J. T. Baker Chemical Co., Phillipsburg, N. Y.); 2-chloroaniline, 3-chloroaniline, 4-chloroaniline, 2,3-dichloroaniline, 2,4-dichloroaniline, 2,5-dichloroaniline, 3,4-dichloroaniline (Aldrich Chemical Co., Inc., Milwaukee, Wis.). 4-Chloroaniline was recrystallized several times from hexane and its purity established by thin-layer chromatography (TLC), melting point determination, and mass spectral analysis. The purity of ^{14}C-4-chloroaniline was verified by cochromatography on TLC with different solvent systems.

For indication of metabolic transformation of the various anilines in the culture medium, a colorimetric method was applied using a *p*-dimethylaminobenzaldehyde–reagent (1 gm of the chemical was dissolved in 30 ml ethanol, 3 ml HCl, and 180 ml *n*-butanol); 3 ml acetic acid was mixed with 1 ml of the sample and 0.5 ml of the reagent was added. The reaction mixture was allowed to stand for 30 min and was then measured spectrophotometrically at 450 nm. A standard curve was prepared for each of the anilines.

For TLC analysis, the culture medium was centrifuged to remove suspended bacterial cells. Five milliliters of the supernatant was extracted with an equal volume of diethyl ether. Subsequently, 3 ml of the ether was concentrated to a volume of 0.5 ml which was applied on the thin-layer plate. TLC plates with a thickness of 0.25 mm of silica gel F-254 (Brinkmann Instruments, Inc., Westbury, N. Y.) were used for routine and preparative thin-layer analyses. Two solvent systems were employed for all thin-layer data: ether–hexane (4:1, v/v) and benzene–dioxane–acetic acid (90:25:4, v/v). Spots on the plates were made visible with ultraviolet light at 254 nm. Aromatic amines were detected by spraying the plates with the previously described *p*-dimethylaminobenzaldehyde–reagent. Indication for the formation of 4-chlorophenylhydroxylamine was obtained by using a 0.2% (w/v) aqueous solution of trisodium pentacyanoammine [4] which produced a magenta color after spraying on the thin-layer plates.

Radioactive samples were measured in a modified Bray solution composed of 8 gm of Omnifluor (New England Nuclear Corp., Boston, Mass.), 60 gm of naphthalene, and 100 ml of methanol in 1 liter of dioxane. Radioactivity was determined with a Nuclear-Chicago Isocap-300 liquid scintillation counter. Radioactive zones on thin-layer plates were detected by autoradiography using an X-ray film (Kodak RD Royal X-Omat, Rochester, N. Y.). Radioactive spots from TLC were counted after scraping the entire spot into the scintillation solution and adding Cab-O-Sil (Thixotropic suspension powder, Cabot Corp., Boston, Mass.) at a concentration of 6%.

Radioactive materials released from the growth medium were trapped by passing the air (aerobiosis) or nitrogen (anaerobiosis) under slight pressure through a 5% solution of NaOH or 1-butanol. To measure the trapped radioactive material, aliquots of the NaOH solution or butanol were analyzed in Bray solution supplied with Cab-O-Sil.

Mass spectra were determined using an AEI MS-902 mass spectrometer at an ionization potential of 70 eV using the direct insertion probe.

Results

The *Paracoccus* sp., isolated from soil, which showed the strongest activity in the transformation of 4-chloroaniline of all the isolated bacteria, metabolized the substrate both under aerobic and anaerobic conditions (Fig. 1). After 2 days all the substrate or reacting transformation products were decomposed under anaerobic conditions as determined by the applied colorimetric method, but in the presence of oxygen only 75% disappearance was indicated. The decomposition of the aniline is related to the growth of the bacterium, but despite better growth aerobically, more rapid transformation was indicated under anaerobic conditions.

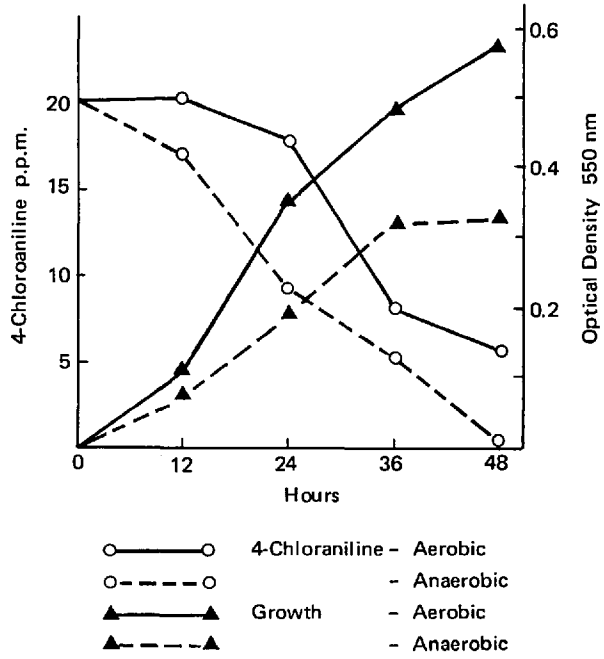

Fig. 1. Disappearance of 4-chloroaniline and products reacting with the applied colorimetric method in relation to the growth of a *Paracoccus* sp. under aerobic and anaerobic conditions.

When ^{14}C-ring-labeled 4-chloroaniline was used as substrate in the growth medium of *Paracoccus* sp., no apparent decrease of radioactivity could be detected after 48 hr under aerobic conditions (Fig. 2). However, incubation of the bacterial culture during the same time period under anaerobic conditions resulted in the loss of approximately 65% of the radiolabeled material. If nitrogen was continuously flushed through the culture solution and subsequently passed through a solution of 5% NaOH, it was possible to trap approximately 40% of the radiolabeled material, but flushing through 1-butanol resulted in a full recovery of the volatilized radioactive compounds. No significant amount of radiolabeled material could be found in the trapping reagents if air was flushed through the aerobic cultures.

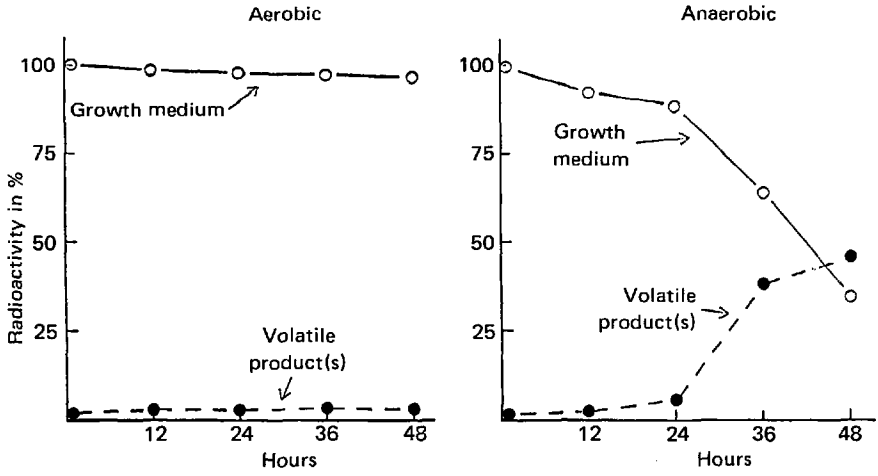

Fig. 2. Transformation of ^{14}C-ring-labeled 4-chloroaniline by a *Paracoccus* sp. under aerobic and anaerobic conditions, as indicated by disappearance of radioactivity from the culture medium and trapping of volatile metabolites in an alkaline solution.

The metabolism of ^{14}C-labeled 4-chloroaniline during growth of a *Paracoccus* sp. was also followed by TLC analysis of the ether-extract from the culture medium, whereas the spots on the TL-plate were detected by autoradiography. Subsequently, the radioactive spots were removed for counting of the radioactivity and for further characterization or identification. Figure 3 shows that only 24 hr of incubation were needed for all 4-chloroaniline to be metabolized under aerobic conditions, and concurrently the formation of ^{14}C-containing spots was observed. Especially, the accumulation of a product with an R_f-value of 0.84 (ether–hexane; 4:1, v/v) was noted after 24 hr, and this product disappeared with further incubation. This compound was very unstable, but from the R_f-value and the reaction of the spot after spraying with an aqueous solution of sodium pentacyanoammine ferroate, it was assumed that this metabolite was 4-chlorophenylhydroxylamine which was previously detected as an intermediate in studies with *Fusarium oxysporum* by Kaufman *et*

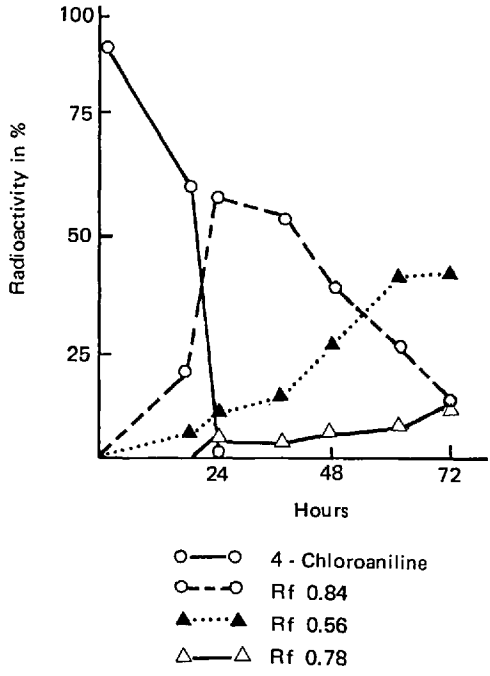

Fig. 3. Metabolism of 4-chloroaniline as indicated by TLC of the ether extract from the culture medium during the growth of a *Paracoccus* sp. under aerobic conditions.

al. [6]. Another metabolite with an R_f-value of 0.56 gradually accumulated in the medium and after 72 hr it amounted to approximately 50% of the original substrate. This chemical was identified by melting point determination, ultraviolet- and mass-spectrometric analysis as 4-chloroacetanilide (Table 3). Some additional metabolites were observed, but they accumulated only in small amounts and they were not further characterized.

If the transformation of 4-chloroaniline under anaerobic conditions was studied by TLC, its complete disappearance was determined after 36 hr, but the formation of a prominent metabolite could not be observed, although several radioactive compounds accumulated (Table 1).

If metabolites formed under aerobic and anaerobic conditions were compared, it appeared that only one with an R_f-value of 0.56 was common, and this

was also isolated and identified from an anaerobic culture as 4-chloroacetanilide. However, the amount of 4-chloroacetanilide which accumulated in the medium varied considerably in the presence or absence of oxygen.

Although most of the experiments were performed using 4-chloroaniline as substrate, the *Paracoccus* sp. also metabolized all other tested chlorinated anilines (Table 2). The transformation of essentially all halogenated anilines or compounds reacting in the colorimetric determination was completed under anaerobic conditions within 48 hr, but lesser amounts of the compounds reacting colorimetrically disappeared aerobically despite the fact that the bacteria showed better growth. It also appeared that under aerobiosis the transformation of the anilines was dependent on the position of the halogen substitution, and the resistance to bacterial attack was different. There was not much difference in the amount of transformation of the various dichlorinated anilines.

Since the major metabolite which accumulated under aerobic conditions in the medium from 4-chloroaniline was the acetylated derivative, attempts were made to isolate the corresponding compound from other anilines. This was indeed the case as can be seen from Table 3. Aniline, as well as 2-, 3-, and 4-chloroaniline, were acetylated as could be shown after TLC-analysis of the 2-day-old growth medium and subsequent identification by melting point determination and mass spectrometry. Preliminary results indicated that acetylation also occurred with dihalogenated anilines, but conclusive identification was not yet obtained.

Table 1

Metabolism of ^{14}C-Labeled 4-Chloroaniline during the Growth of a Paracoccus *sp. under Anaerobic Conditions as Indicated by Thin-Layer Chromatography of the Ether Extract from a Culture Medium*

		Radioactivity on thin-layer plate (dpm)				
R_f[a]	Compound	0 hr	18 hr	24 hr	36 hr	48 hr
0.00	Start point	0	433	661	651	733
0.56	Metabolite-1 (4-Chloroacetanilide)	0	135	369	523	501
0.60	Metabolite-2	0	173	373	341	265
0.71	Metabolite-3	0	135	285	365	263
0.79	4-Chloroaniline	6378	2375	583	0	0
0.93	Metabolite-4	0	178	284	215	123
0.97	Metabolite-5	0	181	334	475	438
Amount of dpm spotted on plate		6510	3810	2970	2610	2370
Amount of dpm discovered on plate		6278	3610	2889	2570	2323

[a]Solvent system: benzene–dioxane–acetic acid (90:25:4, v/v).

Table 2

Metabolism of Chlorinated Anilines under Aerobic and Anaerobic Conditions in a Culture Medium of a Paracoccus *sp. (Concentration of Anilines, 20 ppm; Incubation Time, 48 hr)*

	Aerobic Conditions			Anaerobic Conditions		
	Growth, optical density at 550 nm	Substrate transformed in %[a]	Metabolites formed[b]	Growth, optical density at 550 nm	Substrate transformed in %[a]	Metabolites formed[b]
2-Chloroaniline	0.63	51	3	0.35	100	4
3-Chloroaniline	0.65	63	4	0.36	100	3
4-Chloroaniline	0.62	84	4	0.38	100	5
2,3-Dichloroaniline	0.49	48	2	0.32	91	3
2,4-Dichloroaniline	0.31	53	2	0.34	100	2
2,5-Dichloroaniline	0.35	58	2	0.35	100	2
3,4-Dichloroaniline	0.23	49	3	0.28	100	2

[a]As indicated by colorimetric determination.
[b]As indicated by thin-layer chromatographic analysis.

Table 3

Characteristics of the Acetylated Products from Various Anilines which Accumulated in a 2-Day-Old Culture Medium of a Paracoccus *sp. under Aerobic Conditions*

				Mass spectrometry	
Substrate	Acetylated compound	R_f[a]	Melting point (°C)	Mol. ion	Fragments
Aniline	Acetylanilide	0.23	114	135	119, 93, 77, 43
2-Chloroaniline	2-Chloroacetylanilide	0.20	87	169	153, 127, 75, 43
3-Chloroaniline	3-Chloroacetylanilide	0.27	78	169	153, 127, 75, 43
4-Chloroaniline	4-Chloroacetylanilide	0.22	177	169	153, 127, 75, 43

[a]TLC; ether - hexane (4 = 1, v/v).

Discussion

The known microbial transformation reactions of anilines like acylation, polymerization, or oxidation of the amino group [3] present only part of a possible degradation or detoxication of these compounds. The results described in this paper provide a new aspect for aniline transformation insofar as different metabolic pathways appear to be active under aerobic or anaerobic conditions. The most remarkable observation was that disappearance of radioactive material from the bacterial growth medium supplied with ^{14}C-labeled 4-chloroaniline took place only under anaerobic conditions, whereas no loss of radioactivity was evident if incubation occurred aerobically.

The volatile product formed anaerobically could be trapped in an alkaline solution (5% NaOH solution) and in an organic solvent (1-butanol), indicating that the trapped compound was not carbon dioxide. On the contrary, Bartha [1] found in his studies with partially aerated soil samples that the trapping of radioactivity from ring-labeled 4-chloroaniline in a 1.0 *M* KOH solution indicated the formation of CO_2, but only 2% from the applied material was released within 13 days. Since initial attempts in our experiments to isolate and to identify the volatile product(s) failed, it is not possible to speculate on their specific characteristics. However, there appears little doubt that the bacterium is involved, at least indirectly, in the transformation of 4-chloroaniline to the volatile material.

The major product in an aerobically kept growth medium of the *Paracoccus* sp. was 4-chloroacetanilide which was identified by melting point determination and ultraviolet- and mass-spectroscopy. This observation confirms the reaction of aniline acetylation which was first reported by Tweedy *et al.* [9] using *p*-bromoaniline as substrate. A small amount of 4-chloroacetanilide was detected by Kaufman *et al.* [7] in the culture medium of *Fusarium oxysporum* amended with 4-chloroaniline.

Van Alfen and Kosuge [10] reported the microbial conversion of 2,6-dichloro-4-nitroaniline to 4-amino-3,5-dichloroacetanilide, whereas 2,6-dichloro-*p*-phenylenediamine was detected as an intermediate. They concluded from their experiments that low aeration favored the formation of the acetylated product, but they did not clarify if the increased formation of this compound was only due to a faster reduction of the original substrate to the reduced intermediate. In our study with the *Paracoccus* sp. the accumulation of the acetylated aniline was much greater under aerobic than under anaerobic conditions.

Acknowledgment

This research was authorized for publication as Paper No. 4903 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

References

1. Bartha, R. 1971. Fate of herbicide-derived chloroanilines in soil. *J. Agr. Food Chem.* **19:** 385.
2. Bartha, R., and Pramer, D. 1967. Pesticide transformation to aniline and azo compounds in soil. *Science* **156:** 1617.
3. Bollag, J.-M. 1974. Microbial transformation of pesticides. *Advan. Appl. Microbiol.* **18:** 75.
4. Bollag, J.-M., and Nash, C.L. 1974. Effect of chemical structure of phenylureas and anilines on the denitrification process. *Bull. Environ. Contam. Toxicol.* **12:** 241.
5. Boyland, E., and Nery, R. 1964. Arylhydroxylamines. Part IV. Their colorimetric determination. *Analyst* **89:** 95.
6. Briggs, G. G., and Ogilvie, S. Y. 1971. Metabolism of 3-chloro-4-methoxyaniline and some N-acyl derivatives in soil. *Pest. Sci.* **2:** 165.
7. Kaufman, D. D., Plimmer, J. R., and Klingebiel, U. J. 1973. Microbial oxidation of 4-chloroaniline. *J. Agr. Food Chem.* **21:** 127.
8. Kearney, P. C., and Plimmer, J. R. 1972. Metabolism of 3,4-dichloroaniline in soils. *J. Agr. Food Chem.* **20:** 584.
9. Tweedy, B. G., Loeppky, C., and Ross, J. A. 1970. Metabolism of 3-(*p*-bromophenyl)-1-methoxy-1-methylurea (Metabromuron) by selected soil microorganisms. *J. Agr. Food Chem.* **18:** 851.
10. Van Alfen, N. K., and Kosuge, T. 1974. Microbial metabolism of the fungicide 2,6-dichloro-4-nitroaniline. *J. Agr. Food Chem.* **22:** 221.