0273-2289/98/6903—0191$11.00

Microbial Photodegradation of Aminoarenes Metabolism of 2-Amino-4-Nitrophenol by *Rhodobacter capsulatus*

C.-P. WITTE,[1] R. BLASCO,[2] AND F. CASTILLO*,[2]

[1]*Botanisches Institut, University of Braunschweig, Braunschweig, Germany; and* [2]*Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Universidad de Córdoba, 14071 Cordoba, Spain*

Received June 10, 1997; Accepted October 23, 1997

ABSTRACT

The phototrophic bacterium *Rhodobacter capsulatus* photoreduces 2,4-dinitrophenol to 2-amino-4-nitrophenol, which is further metabolized by an aerobic pathway that is also light-dependent. The catabolism of 2-amino-4-nitrophenol requires O_2 and the presence of alternative carbon (C) and nitrogen (N) sources, preferably acetate and ammonium. *Rhodobacter capsulatus* B10, a bacterium unable to assimilate nitrate, releases negligible amounts of nitrite when growing with 2-amino-4-nitrophenol, thus suggesting that an oxygenase, nitrite-producing activity is not involved in the metabolization of the compound. The diazotrophic growth of *R. capsulatus* increases in the presence of 2-amino-4-nitrophenol, but growth with ammonium is clearly inhibited by the compound. Mutant strains of *R. capsulatus* B10, which are affected in *nifHDK*, *nifR1*, or *nifR4* genes, unable to fix dinitrogen, do not grow with 2-amino-4-nitrophenol as the sole N source. This indicates that the compound cannot be used as a N source. The *nif* mutants degrade 2-amino-4-nitrophenol to the same extent as the wild-type in the presence of ammonium. The compound is not used as a C source by the bacterium, either. Aromatic stable intermediates, such as 2,4-diaminophenol or 4-nitrocatechol, are not detectable in microaerobic cultures of *R. capsulatus* growing with 2,4-dinitrophenol or 2-amino-4-nitrophenol.

Index Entries: Aminoaromatic; photodegradation; *Rhodobacter capsulatus*

*Author to whom all correspondence and reprint requests should be addressed. E-mail: bb1carof@uco.es

Abbreviations: 2,4-DNP, 2,4-dinitrophenol; ANP, 2-amino-4-nitrophenol; CFU, colony-forming units.

INTRODUCTION

Substituted aromatic amines are common, hazardous xenobiotic compounds produced by the chemical industry in manufacture of pesticides, dyes, and drugs *(1,2)*. Some aminoaromatic sulphonates and chloroanilines are also produced by the microbial breakdown of herbicides *(3–5)*. A frequent natural mechanism yielding aromatic amines is the reduction of the nitroaromatic groups catalyzed by bacterial nitroreductases *(6–10)*. These enzymes have been isolated and characterized from several microorganisms, and are NAD(P)H-dependent flavopro-teins *(11–14)* or ironsulfur proteins *(8)*. There are also extracellular nitroreductases produced by bacteria from the human gastrointestinal tract *(9)*. Nitroreductase from *Rhodobacter capsulatus* and *Nocardia* are unstable FMN-linked enzymes that transfer six electrons from NAD(P)H to the nitroaromatic group *(11,15)*; other bacterial nitroreductases produce nitrosamines and hydroxylamines as partially reduced intermediates *(14,16)*.

Because of the mutagenic properties of the aromatic amines, the bacterial reduction of nitroaromatics is important from an ecological point of view. Besides, the production of aromatic hydroxylamines, nitrosamines, and amines by the colon microflora is rising as an important problem in human health. These mutagens are formed from some nitroaromatic compounds that are very often present in drugs, natural or canned foods for humans, or feed for livestock *(17)*. Thus, the nitroreductase activity of enterobacteria considerably increases the toxicity and mutagenicity of some nitroaromatic compounds *(18)*. As a matter of fact, genetically engineered bacteria overproducing nitroreductase activity are specially sensitive to the mutagenicity of nitroarenes *(19,20)*. By contrast, nitroreductase-deficient bacteria are affected to a lesser extent by nitroaromatic compounds *(21)*. There is a recent report that the composition of intestinal flora gives rise to the risk of colorectal cancer *(22)*. Moreover, the hepatic activation of arylamines yields mutagenic metabolites *(23)*, and there are also reports on the mutagenicity of nitrofurans *(24)* and aromatic hydroxylamines *(25)*. The advances in knowledge on the molecular properties of bacterial nitroreductases have allowed their use in cancer treatments, such as the antibody-directed enzyme prodrug therapy, (ADEPT) *(26,27)*.

Taking into account the mutagenic potential of aromatic amines, the study of the biodegradation of these compounds and their lifetime in natural environments is of great interest. The persistence of aminoarenes in natural environments depends on O_2 pressure, since anoxia prevents aromatic amines from polymerization into recalcitrant macromole-

cules *(28)*. Sometimes, aerobic and anaerobic steps of aromatic metabolism are combined in the same microorganism. Thus, a novel pathway for the degradation of 2-aminobenzoate, consisting of the oxidation of the 2-amino-benzoyl coenzyme A to the 5-keto derivative, has been reported in a denitrifying strain of *Pseudomonas (29)*. Bacterial communities are also able to degrade amino-naphtalenesulfonates, which are intermediates in the industrial synthesis of azo dyes *(30)* and other aromatic pollutants *(31)*. The microbial metabolism of chloroanilines has been improved by natural gene exchange within *Pseudomonas* strains *(32)*. To date, there are no other reports on the photodegradation of these compounds by photosynthetic bacteria. This work has studied the light-dependent metabolization of 2-amino-4-nitrophenol (ANP), a compound derived from the anaerobic reduction of 2,4-dinitrophenol (2,4-ANP) by the phototrophic bacterium *R. capsulatus (10)*. The coupling between anaerobic and aerobic pathways for the degradation of nitroaromatic compounds to nonaromatic products has been studied. The role of a second substrate in the biodegradation of ANP has also been investigated.

MATERIALS AND METHODS

Organisms and Growth Conditions

Rhodobacter capsulatus wild-type strain B10 and the mutant strains lacking dinitrogen (N)-fixing capacity were supplied by W. Klipp (Bielefeld, FRG). The cells were cultured, as previously described, with 10 m*M* acetate as a carbon (C) source *(10)*. Where indicated, the following compounds were used as N source: 0.2 m*M* ANP, dissolved dinitrogen (up to 16 mg/L at 30°C under air), 10 m*M* sodium glutamate, or 19 m*M* ammonium chloride. Anaerobic conditions were achieved by filling up screw-capped bottles with culture medium. Strict anaerobiosis was done by bubbling the cultures with high-purity argon. Microaerobic conditions were produced by filling with culture medium only half of the available volume of the same bottles under a gas phase of sterile air. Aerobic conditions were achieved by partially filling Erlenmeyer flasks capped with sterile cotton stoppers and shaking at 140 rpm under air. The cultures were normally maintained at 30°C under light (40 W/m^2) or, where indicated, at the same temperature in the dark. Cell growth was monitored by measuring the turbidity of the cultures at 680 nm, or by CFU counting on rich-agar medium.

For reinoculation of cells in fresh medium, the cultures were centrifuged (12,000 *g* for 10 min) and resuspended in an aliquot of fresh medium under sterile conditions. These aliquots were then used as the inoculum.

Analytical Determinations

2,4-DNP, ANP, and possible catabolic intermediates were measured by HPLC, as previously described *(10)*. The chromatography was carried out using a 125 × 4 mm Spherisorb ODS-2 column (Tracer, Barcelona, Spain). The mobile phase was 60% methanol and 40% of an aqueous phase (100 m*M* acetic acid and 0.8 m*M* triethylamine in Milli Q, Millipore, Bedford, MA water) at a flow rate of 1 mL/min. The effluent was monitored at 260 nm.

Nitrite was measured at 540 nm by the diazotization procedure *(33)*. Ammonium was detected by the Conway microdiffusion technique *(34)*. Protein was determined by the Lowry procedure *(35)*.

RESULTS AND DISCUSSION

The phototrophic bacterium *R. capsulatus* is a versatile microorganism able to grow either in the light or in the dark by using a variety of C and N sources, artificial electron acceptors, and xenobiotic compounds *(10, 36–38)*. Anaerobic photometabolism of 2,4-DNP takes place with the simultaneous and stoichiometric release of ANP, but the production of ANP is not stoichiometric under microaerobic conditions *(10)*. Because aromatic aminoarenes are potent mutagens and hazardous chemicals for every type of cell, further degradation of ANP by the purple bacterium *R. capsulatus* under aerobic conditions has been studied. The authors have used several *R. capsulatus* strains affected in their diazotrophic capacity, or in their ability to assimilate nitrite. Thus, *R. capsulatus* E1F1 and *R. capsulatus* B10 are diazotrophic bacteria, but only the strain E1F1 is able to assimilate nitrate and nitrite. On the other hand, mutants of *R. capsulatus* B10, affected in the structural *nifHDK* genes or in the regulatory *nifR1* and *nifR4* genes, are unable to fix dinitrogen. *R. capsulatus* B10 was unable to grow with ANP as the sole C source, and did not use ANP as a sole N source, since the mutants affected in *nifR1*, *nifR4*, or *nifHDK* genes did not grow with ANP as sole N source under microaerobiosis (Table 1). *R. capsulatus* B10 degraded ANP with acetate as the C source and glutamate as the N source under light-aerobic conditions (Fig. 1A), but not under light-anaerobic conditions (Fig. 1B). The process was significantly enhanced under air, despite cell growth being inhibited in aerobic cells under light (Fig. 1A). The first phase of the ANP degradation was fast, and depended on the presence of cells. The second phase of ANP degradation (from 3 to 20 h), which showed a considerably lower rate (Fig. 1A), was probably caused a photochemical process. It was observed that ANP was slowly degraded under aerobic conditions in the light and in the absence of living cells. A photochemical, Fe-dependent degradation of aminoaromatic compounds has been previously described that facilitates aminophenol

Table 1
Diazotrophic Growth and ANP Degradation by Wild-type Strain and *nif* Mutants of *R. capsulatus* B10

	Absorbance at 680 nm			ANP degraded (%)	
Bacterial strain	- ANP	+ANP	+ANP $+NH_4^+$	$- NH_4^+$	$+ NH_4^+$
R. capsulatus B10 wild-type	0.60	0.9	0.63	60	80
mutant *nifΔHDK*	-	-	0.95	30	70
mutant *nifR1*⁻	-	-	0.65	25	75
mutant *nifR4*⁻	-	-	0.58	28	77

The cells were cultured microaerobically under light with dissolved N_2, in the absence or in the presence of 0.2 m*M* ANP. After 48 h growth, the $A_{680\,nm}$ of the cultures were measured, the cell suspensions were centrifuged at 15,000 *g*, and the ANP concentration was determined in the supernatants.

(−) Negligible growth ($A_{680\,nm} \leq 0.05$).

Results of one experiment are given. Standard deviations were less than 10% of the mean values obtained in four independent experiments.

biodegradation *(39)*. Since auto-oxidation of aromatic amines is pH-dependent, and, taking into account that bacterial growth on acetate increases the pH of the medium, the possibility of an indidect degradation of ANP was checked, because of the pH rise in *R. capsulatus* cultures. The highest pH value observed after the bacteria grew on acetate was 8.5; ANP was stable at pH 9.0 for 24 h under the same conditions. This result suggests a biological degradation of ANP in *R. capsulatus* cultures.

Photodegradation of ANP does not seem to be an inducible process, since it took place rapidly after inoculation, independently of the presence of ANP in the previous culture. Both *R. capsulatus* E1F1 and B10 strains photodegraded ANP at a similar rate and extent.

Similar results concerning cell growth and ANP degradation were obtained when the N source was dissolved N_2, instead of glutamate (Table 1). No ANP catabolism was observed when the cells were cultured under dark-anaerobic or dark-aerobic conditions.

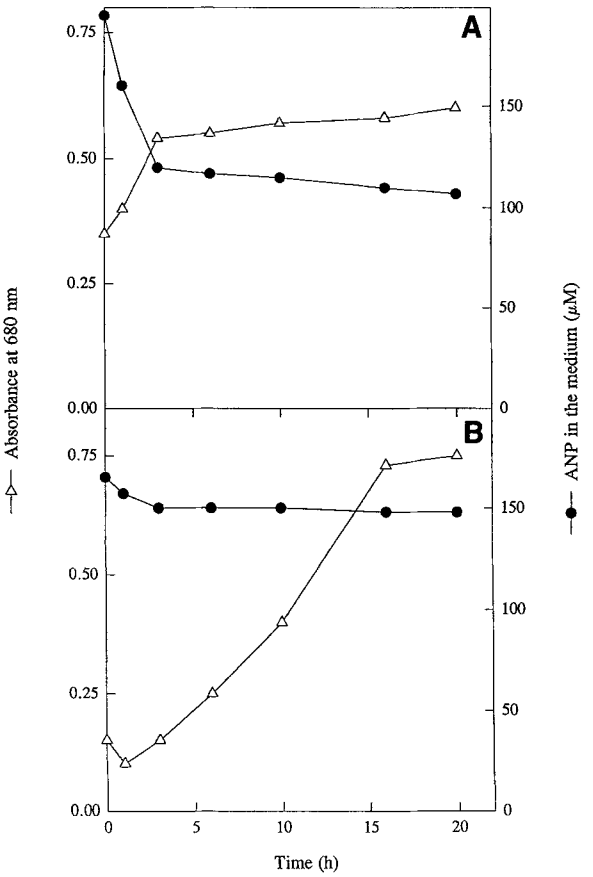

Fig. 1. Effect of O_2 on ANP photodegradation by *R. capsulatus* B10. The cells were cultured with glutamate as N source and acetate as C source under aerobiosis **(A)** or anaerobiosis **(B)**. The absorbance at 680 nm and the ANP concentration in the medium were determined at the indicated times. Results of one experiment are given. Standard deviations were less than 10% of the mean values obtained in four independent experiments.

From this first set of experiments, one can conclude that the degradation of ANP by *R. capsulatus* requires light and O_2. Because O_2 inhibits nitrogenase activity and ANP cannot be used as a N source by the bacterium, the degradation of ANP under diazotrophic conditions became inhibited at pO_2 pressures above 0.2 atm.

To assess possible effects of alternative N sources, ANP degradation was followed in the presence of glutamate or ammonium. Figure 2 shows the time-courses of ANP disappearance with acetate as the C source and glutamate (A) or ammonium chloride (B) as the N source, under light and aerobiosis. The bacterium did not grow well with ammonium and ANP, but in the presence of glutamate and ANP, the cells showed higher growth

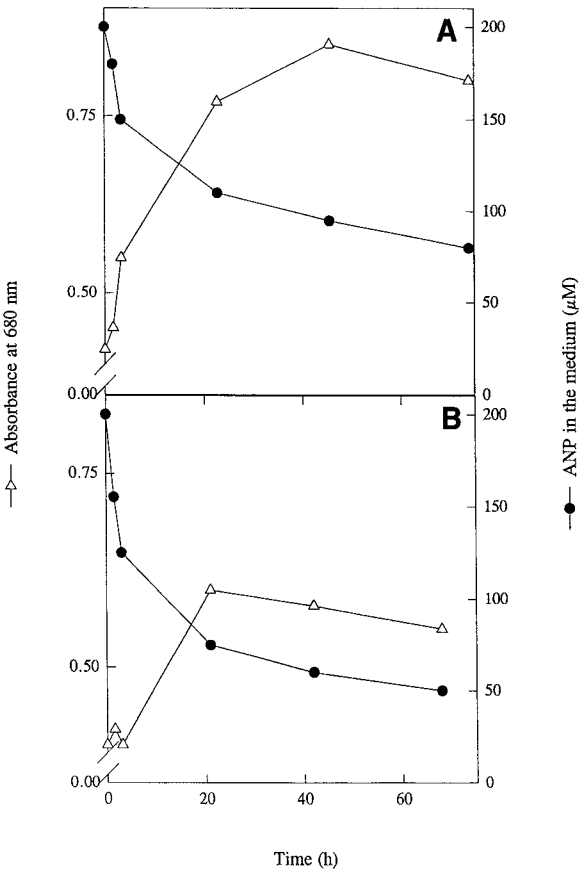

Fig. 2. Effect of ammonium on ANP photodegradation by *R. capsulatus*. The cells were cultured with acetate as C source under light and aerobiosis with glutamate **(A)** or ammonium chloride **(B)** as N source. Absorbance at 680 nm and the ANP concentration in the medium were determined at the indicated times. Results of one experiment are given. Standard deviations were less than 10% of the mean values obtained in four independent experiments.

levels (Fig. 2A). Nevertheless, ANP degradation was higher and faster in ammonium-growing cells (Fig. 2B).

The toxicity of ANP and its putative derivatives for *R. capsulatus* was tested by increasing the ANP concentration in the presence of glutamate and acetate as alternative substrates. Figure 3 shows that ANP inhibited bacterial growth at concentrations above 0.3 m*M*. The degradation of the compound was inhibited above 0.2 m*M* ANP. As previously suggested, the second phase observed in the time-course of ANP degradation (Fig 1A) was a photochemical process. To check if the cessation of biological activity was caused by the accumulation of a toxic derivative from ANP, both the cell survival and ability to degrade ANP were assayed by reinoculation experiments. Figure 4 shows the effect of the age of the culture on the abil-

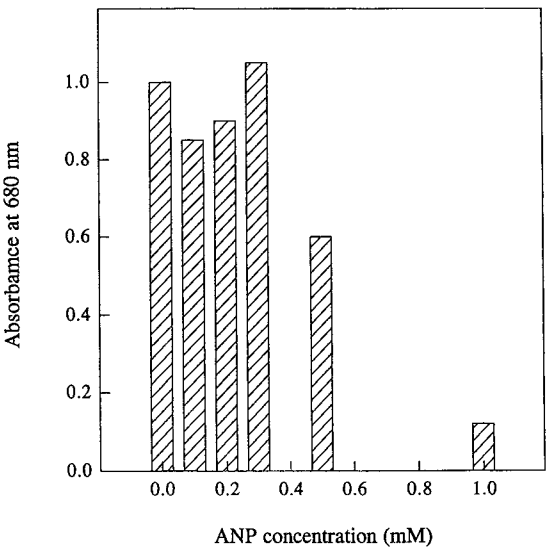

Fig. 3. Effect of ANP concentration on the growth of *R. capsulatus*. The cells were cultured under light-aerobiosis with glutamate as N source and acetate as C source. The absorbance at 680 nm was measured 48 h after inoculation. Results of one experiment are given. Standard deviations were less than 10% of the mean values obtained in four independent experiments.

ity of the cells to grow and the capacity for degrading ANP. When the same cells were successively reinoculated with nontoxic concentrations of ANP, the increase in CFU/mL completely ceased after two reinoculation cycles. CFU in the presence of ANP only decreased by one order of magnitude, compared with an untreated control (Fig. 4A). Therefore, it can be concluded that toxic metabolites from ANP were not accumulated. Neither the rate nor the extent of ANP degradation seemed to be affected by the culture age (Fig. 4B).

We did not detect aromatic intermediates by HPLC from the degradation of ANP by *R. capsulatus*. The nitroreductase activity is low in aerated cultures, and it is repressed by ammonium *(12)*. Therefore, a second reduction of ANP at the level of the 4-nitro group, yielding the diamino derivative, is unlikely. An oxidative reaction could be a more plausible mechanism as a first step in ANP catabolism. Oxygenase reactions involved in aminoaromatic metabolism have been reported in the degradative pathway for 2-amino-bencenesulphonates by *Alcaligenes (40)*. Moreover, the existence of enzymes of the *ortho* pathway have been observed in the metabolism of anilines by *Pseudomonas acidovorans (3,4)*. Actually, this organism is a typical chloroaniline degrader, which has been used in bioremediation assays *(41)*. From the results shown in Fig. 1, we can conclude that ANP was not metabolized by a mono-oxygenase reac-

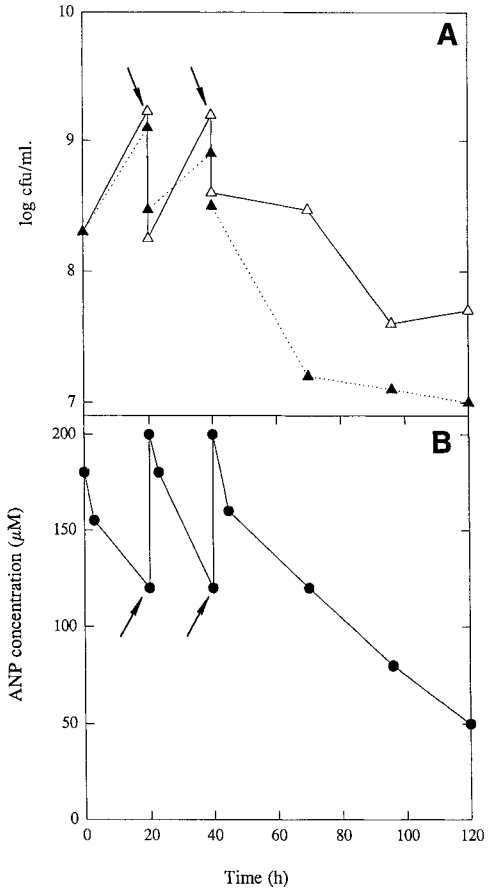

Fig. 4. Effect of the age of the cultures on cell viability and ANP degradation by *R. capsulatus*. At the times indicated by the arrows, the cells were reinoculated in fresh media with ANP (filled symbols) and without ANP (hollow symbols). CFU **(A)** and ANP concentrations **(B)** were determined at the indicated times. Results of one experiment are given. Values were reproducible within one order of magnitude in four separate experiments.

tion via 4-nitrocatechol and ammonium, because if ANP were actually degraded via 4-NC, we would have detected this latter compound by HPLC, and because *R. capsulatus* B10 degrades 4-NC under microaerobiosis, with stoichiometric release of nitrite *(42)*. Under light and microaerobic conditions, we only observed up to 20 μ*M* nitrite from 0.2 m*M* ANP. Since *R. capsulatus* B10 lacks nitrite reductase activity, and these cells do not accumulate nitrite inside *(42)*, the nitrite observed in the medium corresponded to the amount of nitrite produced photochemically. Any other type of oxidative reaction yielding ammonium is unlikely to take place, since the mutant strains *nifΔHDK* (lacking functional genes encoding for dinitrogenase reductase and dinitrogenase proteins), *nifR*1$^-$ and *nifR*4$^-$

(lacking functional genes encoding the transcriptional activator NIFR1 and a σ^{54} factor, respectively *(43)* were unable to use ANP as the sole N source (Table 1). Nevertheless, the mutants degraded ANP to the same extent as the wild-type in the presence of ammonium as a second substrate. These results support the conclusion that an alternative N source is necessary for ANP metabolization. Therefore, the increase in absorbance at 680 nm shown by *R. capsulatus* B10, cultured with ANP as N source, is not caused by the metabolization of ANP. The cell growth in cultures with ANP is probably based on the utilization of acetate and dissolved N_2. Equally, no growth was observed in cells cultured with ANP in the absence of alternative C sources, thus confirming that ANP was actually co-metabolized by *R. capsulatus*. Cometabolism of aromatic amines has been reported in *Rhodococcus rhodochrous*, which increases degradation of 2-methylaniline and its chlorinated isomers in the presence of ethanol *(44)*.

Previous results, and those shown in this work, demonstrate that the metabolization of 2,4-DNP by *R. capsulatus* requires two different pathways: first, 2,4-DNP is transported inside the cells, and it is photoreduced by a nitroreductase, with stoichiometric releasing of ANP into the medium. This process is inhibited in ammonium-growing cells, and takes place either under strict anaerobiosis, or, more slowly, at pO_2 above 0.2 atm *(10,12)*. During a second step, ANP is further photodegraded by a pathway that requires pO_2 of about 0.2 atm, and that is enhanced by ammonium. Aerobic photodegradation of ANP by *R. capsulatus* depends on alternative C and N sources, and eliminates the aromatic properties of the compound. Both phases of nitroaromatic degradation could be catalyzed by phototrophic bacteria thriving in natural microaerobic environments. Therefore, the process could take place without accumulating aromatic amines, which are common products of the anaerobic bacterial metabolism of nitroaromatic pollutants.

ACKNOWLEDGMENTS

The authors wish to thank W. Klipp (University of Bielefeld, FRG) for the supply of deletion mutants. The financial support of DGICYT (Grant no. PB92 0783), Junta de Andalucia (PAI n. 3036) (Spain), and the Alexander von Humboldt Foundation (FRG) is also gratefully acknowledged.

REFERENCES

1. Kearney, P. C. and Kaufman, D. D. (1975), *Herbicides: Chemistry, Degradation and Mode of Action*, 2nd ed., Marcel Dekker, New York.
2. Meyer, U. (1981), in *Microbial Degradation of Xenobiotics and Recalcitrant Compounds*, Leisinger, T., Hütter, R., Cook, A. M. and Nüesch, J., eds., Academic, London, pp. 371–385.

3. Hinterhegger, C., Loidl, M., and Streichbier, F. (1994), *J. Basic. Microbiol.* **34,** 77–85.
4. Zeyer, J., Wasserfallen, A., and Timmis, K. N. (1985), *Appl. Environ. Microbiol.* **50,** 447–453.
5. Häggblom, M. M. (1992), *FEMS Microbiol. Rev.* **103,** 29–72.
6. Hallas, L. E. and Alexander, M. (1983), *Appl. Environ. Microbiol.* **45,** 1234–1241.
7. Kinoushi, T. and Ohnishi, Y. (1983), *Appl. Environ. Microbiol.* **46,** 596–604.
8. McCormick, N. G., Feeherry, F. E., and Levinson, H. S. (1976), *Appl. Environ. Microbiol.* **31,** 949–958.
9. Rafii, F., Franklin, W., Heflich, R. H., and Cerniglia, C. L. (1991), *Appl. Environ. Microbiol.* **57,** 962–968.
10. Blasco, R. and Castillo, F. (1992), *Appl. Environ. Microbiol.* **58,** 690–695.
11. Villanueva, J. R. (1964), *J. Biol. Chem.* **239,** 773–776.
12. Blasco, R. and Castillo, F. (1993), *Appl. Environ. Microbiol.* **59,** 1774–1778.
13. Bryant, C. and DeLuca, M. (1991), *J. Biol. Chem.* **226,** 4119–4125.
14. Sommerville, C. C., Nishino, S. F., and Spain, J. C. (1995), *J. Bacteriol.* **177,** 3837–3842.
15. Blasco, R., Aparicio, P. J., and Castillo, F. (1995), *Arch. Microbiol.* **163,** 248–253.
16. Duque, E., Haidour, A. Godoy, F., and Ramos, J. L. (1993), *J. Bacteriol.* **175,** 2278–2283.
17. McCalla, D. R. (1983), *Environ. Mutagen.* **5,** 745–765.
18. Venitt, S. and Crofton-Sleigh, C. (1987), *Mutagenesis* **2,** 375–381.
19. Watanabe, M., Ishidate, M., Jr., and Nohmi, T. (1989), *Mutat. Res.* **234,** 337–348.
20. Jurado, J., Alejandre-Durán, E., and Pueyo, C. (1994), *Environ. Mol. Mutagen.* **23,** 286–293.
21. Speck, W. T., Blumer, J. L., Rosenkranz, E. J., and Rosenkranz, H. S. (1981), *Cancer Res.* **41,** 2305–2307.
22. Moore, W. E. C. and Moore, L. H. (1995), *Appl. Environ. Microbiol.* **61,** 3202–3207.
23. Rodríguez-Ariza, A., Diaz-Mendez, F. M., Navas, J. I., Pueyo, C., and López-Barea, J. (1995), *Environ. Mol. Mutagen.* **25,** 50–57.
24. McCalla, D. R. and Voutsinos, D. (1974), *Mutat. Res.* **26,** 3–16.
25. Weissburger, J. H. (1988), in *Carcinogenic and Mutagenic Responses to Aromatic Amines and Nitroarenes*, King, C. M., Romano, L. J., and Schuetzle, D., eds., Elsevier, New York, pp. 3–19.
26. Knox, R. J., Friedlos, F., and Boland, M. P. (1993), *Cancer and Metast. Rev.* **12,** 195–212.
27. Mauger, A. B., Burke, P. J., Somani, H. H., Friedlos, F., and Knox, R. J. (1994), *J. Med. Chem.* **37,** 3452–3458.
28. Carpenter, D. F., McCormick, N. G., Cornell, J. H., and Kaplan, A. M. (1978), *Appl. Environ. Microbiol.* **35,** 949–954.
29. Altenschmidt, U., Bokranz, M., and Fuchs, G. (1992), *Eur. J. Biochem.* **207,** 715–722.
30. Nörtemann, B., Baumgarten, J., Rast, H.-G., and Knackmuss, H.-J. (1986), *Appl. Environ. Microbiol.* **52,** 1195–1202.
31. Field J. A., Stams, A. J. M., Kato, M., and Schraa, G. (1995), *Anton. Leeuw. Int. J. G.* **67,** 47–77.
32. Latorre, J., Reineke, W., and Knackmuss, H.-J. (1984), *Arch. Microbiol.* **140,** 159–165.
33. Snell, F. D. and Snell, C. T. (1949), in *Colorimetric Methods of Analysis*, Van Nostrand Reinhold, Princeton, New Jersey, pp. 804–805.
34. Conway, E. J. (1962), in *Microdiffusion Analysis and Volumetric Error*, 5th ed., Crosby Lockwood, London, pp. 105–110.
35. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265–275.
36. Ferguson, S. J., Jackson, J. B., and McEwan, A. G. (1987), *FEMS Microbiol. Rev.* **46,** 117–143.

37. Castillo, F. and Cárdenas, J. (1982), *Photosynth. Res.* **3,** 3–18.
38. Roldán, M. D., Caballero F. J., and Castillo, F. (1994), *Curr. Microbiol.* **29,** 119–122.
39. Pulgarin, C. and Kiwi, J. (1995), *Langmuir.* **11,** 519–526.
40. Junker, F., Field, J. A., Bangerter, F., Ramsteiner, K., Kohler, H.-P., Joannou, C. et al. (1994), *Biochem. J.* **300,** 429–436.
41. Brunsbach, F. R. and Reineke, W. (1993), *Appl. Microbiol. Biotechnol.* **40,** 402–407.
42. Roldán, M. D. (1996) PhD Thesis, University of Córdoba, Spain.
43. Masepohl, B. and Klipp, W. (1996), *Arch. Microbiol.* **165,** 80–90.
44. Fuchs, K., Schreiner, A., and Lingens, F. (1991), *J. Gen. Microbiol.* **137,** 2033–2039.