Metabolism of 3,4-Dichloroaniline by *Pseudomonas putida*

In-Soon You and Richard Bartha*

3,4-Dichloroaniline (DCA), a biodegradation intermediate of several herbicides, is mineralized in soil only very slowly. In enrichment cultures, DCA failed to serve as the sole substrate, but analogue enrichment yielded a *Pseudomonas putida* strain that, in presence of unchlorinated analogue substrates, mineralized DCA with the release of $^{14}CO_2$ and Cl^-. Mass spectrometric identification of the key biodegradation intermediates 3,4-dichloromuconate, 3-chlorobutenolide, and 3-chlorolevulinic acid revealed that DCA biodegradation occurred through 4,5-dichlorocatechol, 3,4-dichloromuconate, 3-chlorobutenolide, 3-chloromaleylacetate, and 3-chloro-4-ketadipate to succinate plus acetate. Through the above pathway, DCA was converted ultimately to inorganic end products. Comparison of our findings with other reports suggests that the slow mineralization of DCA in soil is not entirely explainable by the inherent recalcitrance of this compound but is also explainable by the competing polymerization and binding reactions that decrease its availability.

3,4-Dichloroaniline (DCA) is released during the biodegradation of several economically important acylanilide, phenylcarbamate, and phenylurea herbicides (Cripps and Roberts, 1978). In soil, some of the liberated DCA is converted by microbial peroxidases to 3,3′,4,4′-tetrachloroazobenzene and other azo products (Bartha and Pramer, 1970), but the bulk of the DCA becomes covalently bound to the soil organic matter by apparently spontaneous chemical reactions (Bartha, 1971; Hsu and Bartha, 1976). As a consequence, mineralization of DCA in soil proceeds slowly with projected half-lives of the order of several years and by metabolic pathways that are as yet unexplored.

Our present knowledge of the in vitro microbial metabolism of DCA and other anilines is rather incomplete. For DCA, to date only dimerization and polymerization reactions (Bartha et al., 1968; Bordeleau et al., 1972), formylation (Kearney and Plimmer, 1972), and N-oxidation (Russel, 1980) were reported. Information on the microbial degradation of other anilines is also scarce. Tweedy et al. (1970) and Russel and Bollag (1977) reported the acylation of 4-bromoaniline and 4-chloroaniline, respectively, but biodegradation was not detected. Kaufman et al. (1973) reported the oxidation of the amino group of 4-chloroaniline over intermediates to a nitro group. Acylation and oxidative condensation were also observed. Briggs and Walker (1973) reported the isolation of an unidentified soil bacterium that could grow on 4-chloroaniline as an only source of carbon and energy. Tentative evidence for hydroxylation ortho to the amino group was obtained through the isolation of the condensation product 7-chloro-2-amino-3*H*-phenoxazin-3-one, but no biodegradation pathway was established. *Bacillus firmus* formed the same chlorophenoxazinone and its reduced phenoxazine analogue from 4-chloroaniline (Engelhardt et al., 1977). Acylation products were also observed. Fletcher and Kaufman (1979) reported the formation of 2-amino-4-chlorophenol and 2-amino-5-chlorophenol from 3- and 4-chloroaniline, respectively, by *Fusarium oxysporum*, but this soil fungus was unable to grow on these monochloroanilines or to degrade them extensively. Recently, Reber et al. (1979) reported that, while unable to serve as growth substrates, monochloroanilines were oxygenated to chlorocatechols by aniline-grown *Pseudomonas multivorans*.

Department of Biochemistry and Microbiology, Rutgers University, Cook College, New Brunswick, New Jersey 08903.

Microbial growth on unhalogenated anilines was reported by Plotho (1948) and by Walker and Harris (1969), but no biodegradation pathway was established. Bachofer et al. (1975) demonstrated that in pyrocatechol formed from aniline by a *Nocardia* mutant both oxygen atoms were derived from molecular oxygen, a finding consistent with a dioxygenase attack on the aniline molecule.

The slow mineralization of DCA in soil and the absence of information on DCA biodegradation in vitro raises the quesion whether DCA is an inherently recalcitrant xenobiotic molecule or whether it becomes resistant due to polymerization and binding reactions that are faster than the biodegradative ones. To answer this question and to establish a degradation pathway, we investigated the biodegradation of DCA in vitro. The use of a pure culture and an in vitro approach was necessary here in order to exclude the polymerization and binding reactions that appear to take precedence in the natural soil environment.

EXPERIMENTAL SECTION

Isolation and Cultivation of the Microorganism. For isolation a DCA degrader, the analogue enrichment technique (Focht and Alexander, 1970) was applied. To a mineral salts medium containing 1.0 g of K_2HPO_4, 1.0 g of KH_2PO_4, 0.41 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $CaCO_3$, and 0.05 g of $FeSO_4 \cdot 7H_2O$ in 1000 mL of H_2O (pH 6.8), 400 ppm of propionanilide was added as the only source of carbon, nitrogen, and energy. Activated sewage sludge obtained from the Raritan Valley Sewage Authority treatment plant at Bridgewater, NJ, was used as inoculum, and incubations were at 28 °C with rotary shaking (200 rpm). For isolation, purification, and maintenance of stock cultures, the above-described medium was solidified by using 2% of agar. Diagnostic tests were performed by conventional techniques (Hugh and Gilardi, 1980) and by the API kit (Otto and Blachman, 1979, Oberhofer, 1979).

In experiments for release of $^{14}CO_2$ from radiolabeled DCA, the mineral medium contained 500 ppm of propionanilide plus 10–60 ppb of radiolabeled DCA. The incubation was in airtight shake flasks of the type described by Kanner and Bartha (1979). During incubation, the flasks were periodically flushed with air through a trapping system (Marinucci and Bartha, 1979) containing, in sequence, the scintillation counting solutions Aquasol and Oxifluor (New England Nuclear, Boston, MS). After each flushing, the trapping vials were detached and counted.

Preparation of resting cell suspensions, the mineral salts medium contained, instead of propionanilide, 1000 ppm

0021-8561/82/1430-0274$01.25/0

of aniline and 50 ppm of DCA. After less than 24 h of incubation, cells were harvested by centrifugation and were resuspended in $^1/_{20}$-volume of fresh mineral medium containing DCA as the only organic substrate. After an appropriate incubation period at 28 °C, cells were removed and the supernatant was analyzed for chloride ions or metabolic intermediates.

Analytical Procedures. Chloride release was determined according to Bergman and Sanik (1957). Radioactivity was determined by using a Beckman LS 230 liquid scintillation counter. Counts were corrected for background and for quenching by using the external standard ratio method. $^{14}CO_2$ was counted in the Oxifluor trapping solution; all other radioactive samples were counted in Aquasol. All measurements were either made on replicate samples or were repeated several times.

For extraction of metabolic intermediates from culture solutions, cells were removed by centrifugation. The supernatant was acidified with HCl and was extracted 3 times with equal volumes of diethyl ether. The combined solvent extracts were dried over andhydrous Na_2SO_4 and concentrated to 2 mL under dry N_2. The extracts were analyzed directly or were methylated prior to chromatographic separations and mass spectrometric (MS) analyses. Methylation was performed with ethereal diazomethane, generated from *N*-methyl-*N*-nitro-*N*-nitrosoquanidine by using an Aldrich MNMG–diazomethane kit according to instructions.

Thin-layer chromatography (TLC) was performed on precoated 250-μm silica gel GF plates (Fisher, Pittsburgh, PA). Solvent systems were (a) chloroform–acetone, 9:1 (v/v), or (b) isooctane–ethyl acetate–acetic acid–water, 110:50:20:100 (v/v). In some cases a combination of solvent systems a and b in a ratio of 4:1 was used. Spots were visualized by UV light (254 nm). For recovery of metabolites or quantitation of radioactivity, the silica gel was scraped from the plates and was eluted by solvent or Aquasol, respectively. Gas chromatographic (GC) analysis was performed on a Model 5700 A Hewlett-Packard instrument equipped with flame ionization detectors and dual 180 cm long 2.5-mm i.d. stainless steel columns packed with 2% OV-17 on 80–100-mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, PA). Operating conditions were as follows: N_2 carrier, 30 mL/min; inlet temperature, 250 °C; detector temperature, 300 °C. Following injection (3 μL) the oven was maintained at 120 °C for 2 min and then programmed at 8 °C/min to 250 °C. A Hewlett-Packard Model 5985 GC–MS instrument interfaced with a computer was used for obtaining mass spectra at an electron impact of 70 eV.

Chemicals. Uniformly ^{14}C-labeled DCA with specific activity of 61 μCi/mg was obtained from Amersham/Searle (Des Plaines, IL). The radiochemical purity, as determined by TLC, was 97%. Unlabeled DCA was obtained from Aldrich Chemical Co. (Milwaukee, WI) and was recrystallized several times from petroleum ether. The melting point of the purified product was 71 °C. Propionanilide, the unchlorinated analogue of the herbicide propanil (3′,4′-dichloropropionanilide), was prepared from aniline and propionyl chloride according to the procedure of Huffman and Allen (1960). Aniline (Aldrich) was redistilled in glass prior to use. All other chemicals were of analytical grade, and the solvents were either pesticide or reagent grade.

RESULTS

Isolation and Characterization of the DCA-Degrading Bacterium. Analogue enrichment from activated sludge followed by isolation and purification on propionanilide–mineral salts agar yielded a short, motile, Gram-negative, oxidase positive rod that produced fluorescent pigment but no pyocyanine. Diagnostic tests identified the isolate as a strain of *Pseudomonas putida*. The isolate was capable of rapid growth on either propionanilide or aniline serving as the only sources of carbon, nitrogen, and energy. The isolate was unable to grow on monochloroanilines or DCA and exhibited only erratic and marginal growth on 3′,4′-dichloropropionanilide. Direct enrichments on DCA as the sole substrate failed to yield any DCA-degrading isolates.

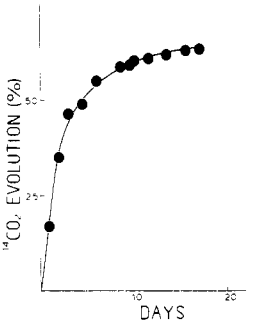

Figure 1. Conversion of radiolabeled DCA (10 ppb) to $^{14}CO_2$ by a *P. putida* suspension growing on propionanilide (500 ppm).

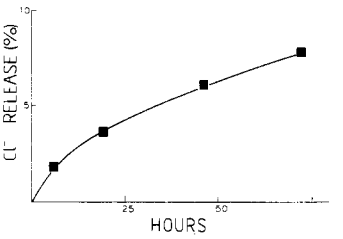

Figure 2. Release of chloride from DCA by an aniline-grown resting cell suspension of *P. putida*.

Production of $^{14}CO_2$ from Labeled DCA and Release of Chloride. In order to prove the ability of *P. putida* to accomplish the degradation of DCA to its inorganic end products (mineralization), we pregrew cell suspensions on mineral salts medium with propionanilide serving as the substrate in the presence of trace amounts of DCA. After addition of 500 ppm of fresh propionanilide along with 10–60-ppb concentrations of radiolabeled DCA, the evolved $^{14}CO_2$ was measured. In several such mineralization experiments, 40–60% of the added radiocarbon was converted to $^{14}CO_2$ within a 2-week incubation period. One of these mineralization experiments is illustrated in Figure 1.

The very slow and low-level chloride release from DCA by growing cells of *P. putida* was difficult to measure accurately, and, therefore, these experiments were subsequently performed with aniline-grown concentrated resting cell suspensions containing 200 ppm of DCA. In this experiment, 7.9% of the available chlorine was released as chloride within 72 h (Figure 2). A comparison of chloride release from DCA and monochloroanilines in similar resting cell incubations showed decreasing dechlorination rates in the order of 4-chloroaniline < 3-chloroaniline < DCA < 2-chloroaniline.

Simultaneous mineralization and dechlorination of 100 ppm of radiolabeled DCA by the same aniline-grown resting cell suspension were compared as illustrated in Figure 3. Evolution of $^{14}CO_2$ under these conditions was limited to 4% in 260 h. Dechlorination during the same time period was 11.3%. The above discrepancy indicated the accumulation of partially or completely dechlorinated biodegradation intermediates. As direct evidence for the

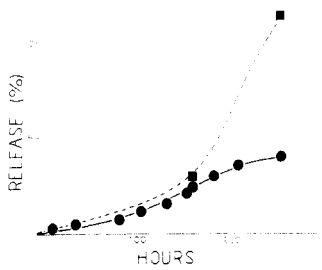

Figure 3. Release of chloride (squares) and $^{14}CO_2$ (circles) from radiolabeled DCA (100 ppm) by an aniline-grown resting cell suspension of *P. putida*.

latter, several radiolabeled spots, not identical with those of DCA, were produced by TLC of solvent extracts of the spent medium.

Identification of DCA Biodegradation Intermediates. Initial efforts to isolate DCA biodegradation intermediates from growing cultures of *P. putida* had only limited success. Under growth conditions, metabolic intermediates were rapidly processed further and did not accumulate. Furthermore, the large excess of metabolites arising from the unchlorinated aniline growth substrate such as catechol (*m/e* 110; λ_{max} 275 nm) made the separation and identification of DCA breakdown products extremely difficult. Better results were obtained through the use of aniline-grown concentrated resting cell suspensions incubated with 100 ppm of DCA. MS evidence was obtained for key metabolic intermediates as shown in Figure 4.

Figure 4A: 3,4-Dichloromuconic Acid Methyl Ester. The molecular ion *m/e* 238 was not observed. This was not unexpected, since $-OCH_3$ or $-COOCH_3$ is easily lost from this type of compound (Rosenberg and Alexander, 1980), but the fragmentation pattern gave a clear indication of the probable parent compound. The ion at *m/e* 203 could be eliminated as being the molecular ion because of the presence of the ion at *m/e* 179. So that *m/e* 179 could be derived from *m/e* 203, the loss of a C_2 fragment (mass 24) would have to be postulated, a clearly unlikely event. In addition, the 3:1 relative intensity ratio of ions 203 to 205 suggested a monochloro compound, while the 9:6:1 intensity ratio of the ions 179 to 181 to 183 indicated a dichloro compound.

Figure 4B: 3-Chloro-4-(carboxymethylene)but-2-enolide Methyl Ester (3-Chlorobutenolide). The molecular ion *m/e* 188 was observed. The 3:1 intensity ratio of the ions 188 to 190 indicated a monochloro compound. The same ratio was observed for *m/e* 157 to 159 and *m/e* 88 to 90, identifying both of these as monochloro fragments. The other observed fragments did not show the presence of Cl.

Figure 4C: 3-Chlorolevulinic Acid Methyl Ester. The molecular ion was observed at *m/e* 164. The 3:1 ratio of *m/e* 164 to 166, 133 to 135, 132 to 134, and 122 to 124 identified these as each containing one chlorine atom; the other fragments were unchlorinated. The fragments *m/e* 122/124 and *m/e* 87 were derived from the molecular ion by the loss of $CH_2{=}C{=}O$ and $CH_2{=}C{=}O$ plus Cl, respectively, via McLafferty rearrangement that is common to carbonyl compounds with a γ hydrogen (Lambert et al., 1976).

DISCUSSION

Recalcitrance of DCA in Vitro and in Soil. The presented data represent the first evidence for the extensive degradation and mineralization of a dichloroaniline by a microbial culture. Although DCA failed to serve as

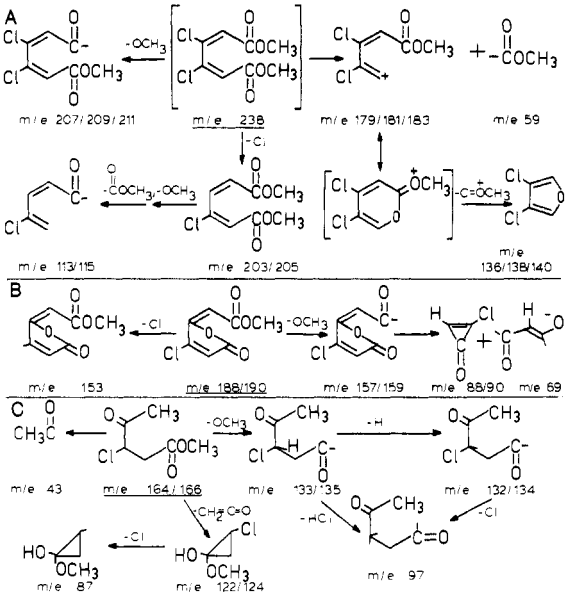

Figure 4. Mass spectrometric evidence for methylated key intermediates of DCA biodegradation by *P. putida*. The ions in brackets were only postulated but not actually observed. In each case, the molecular ion is indicated by underlining; arrows indicate the derivation of fragments. (A) 3,4-Dichloromuconic acid methyl ester; (B) 3-chloro-4-(carboxymethylene)but-2-enolide methyl ester; (C) 3-chlorolevulinic acid methyl ester. For detailed interpretation of the spectra, see the text.

a sole source of carbon, nitrogen, and energy in enrichment cultures, *P. putida* growing on substrate analogues such as aniline or propionanilide mineralized low concentrations of DCA nearly completely and within relatively short time periods. Since microorganisms convert 30–50% of the carbon of a substrate to cell biomass, the 40–60% conversion of DCA radiocarbon to $^{14}CO_2$, as observed in our 2-week experiments, indicated a virtually complete microbial mineralization of this compound. Therefore, we conclude that the high resistance of DCA against mineralization in soil is not entirely an inherent structural characteristic of the DCA molecule but is in part a consequence of the polymerization and binding reactions (Bartha and Pramer, 1970; Chisaka and Kearney, 1970; Bartha, 1971) that compete for DCA in soil and convert it to even more recalcitrant molecules.

Pathway of DCA Biodegradation. The identification of three key biodegradation intermediates allows us to outline in a definitive manner the mineralization pathway of DCA as it occurs in cultures of *P. putida* (Figure 5).

The identified metabolites are consistent with an initial dioxygenase attack on the DCA molecule. An analogous attack was established by Bachofer et al. (1975) on unchlorinated aniline by a *Nocardia* sp. In case of DCA, this attack leads to the formation of a dichlorocatechol and the elimination of the amino group as ammonia. The suggestion that *P. putida* attacks in this manner is supported also by our identificaton of catechol as the primary metabolic product formed from aniline (I.-S. You and R. Bartha, unpublished data). The aniline nitrogen is probably released as ammonia but, being the sole source of nitrogen in the culture medium, is rapidly incorporated and does not accumulate.

We did not succeed in the direct isolation and identification of the dichlorocatechol, but from the identities of the subsequent metabolic products, in particular from the identification of 3-chlorobutenolide, it is evident that the dichlorocatechol in question was the symmetrical 4,5-dichlorocatechol. A chlorine in the γ position to a carboxy

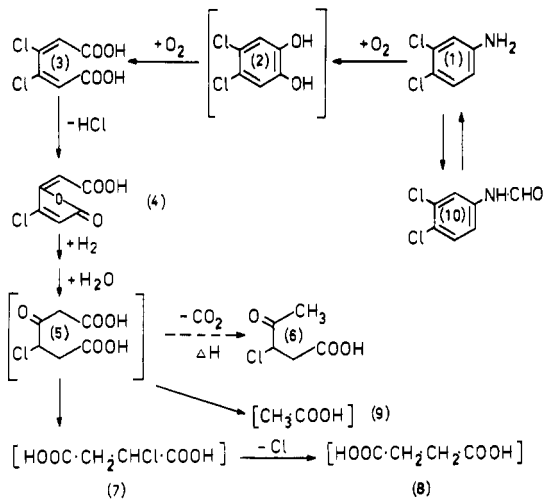

Figure 5. Suggested pathway of DCA biodegradation by *P. putida*. The intermediates in brackets were postulated but not actually identified. Names of the illustrated intermediates are as follows: (1) 3,4-dichloroaniline (DCA); (2) 4,5-dichlorocatechol; (3) 3,4-dichloromuconate; (4) 3-chloro-4-(carboxymethylene)-but-2-enolide (3-chlorobutenolide); (5) 3-chloro-4-ketoadipic acid; (6) 3-chlorolevulinic acid; (7) 2-chlorosuccinic acid; (8) succinic acid; (9) acetic acid; (10) 3′,4′-dichloroformylanilide. For detailed discussion of the pathway, see the text.

group is eliminated with relative ease (Evans et al., 1971), resulting in 3-chlorobutenolide. Should the chlorine in the above compound be situated in a position that is different from the postulated one, the fragments *m/e* 88/90 and 69 in the spectrum of 3-chlorobutenolide (see Figure 4B) would be difficult to explain.

The 4,5-dichlorocatechol ring is opened by ortho cleavage (pyrochatechase attack), resulting in 3,4-dichloromuconate. In the next step a lactonizing enzyme (Sharpee et al., 1973) produces 3-chlorobutenolide with the simultaneous release of one chlorine. This step clearly proves that the first degradation intermediate of DCA was indeed 4,5-dichlorocatechol. Had it been, instead, the asymmetric 3,4-dichlorocatechol, the next steps would have led to 2,3-dichloromuconate and 5-chloro-4-(carboxymethylene)but-2-enolide, a metabolite inconsistent with the previously mentioned fragments *m/e* 88/90 and 69. The only remaining alternative, 2-chloro-4-(carboxymethylene)but-2-enolide, cannot be derived from DCA by any known mechanism.

3-Chlorobutenolide is probably delactonized to form 3-chloromaleylacetic acid (Tiedje et al., 1969; Evans et al., 1971). We did not identify this metabolite directly nor the 3-chloro-4-ketoadipic acid derived by its reduction, but indirect evidence for the latter product was obtained by identification of 3-chlorolevulinic acid that represents a nonbiological decarboxylation product of 3-chloro-4-ketoadipic acid (Kilby, 1948; Tiedje et al., 1969).

3-Chloro-4-ketoadipic acid is subsequently split by an established mechanism (Duxbury et al., 1970) into 2-chlorosuccinic and acetic acids. 2-Chlorosuccinic acid can be dehalogenated with ease (Duxbury et al., 1970; Rosenberg and Alexander, 1980). The remaining unchlorinated acids are common tricarboxylic acid cycle intermediates, and their ultimate mineralization to CO_2 and H_2O follows in course.

TLC analysis of spent media from incubations with radiolabeled DCA indicated the presence of metabolites in addition to those described previously. One of these is identical with 3′,4′-dichloroformylanilide, a DCA acylation product reported earlier by Kearney and Plimmer (1972). Additional work will be required to explore whether this and other yet unidentified metabolites merely represent a temporary and reversible DCA detoxification mechanism or whether these acylated products can be degraded directly by an alternate pathway. The previously described DCA biodegradation pathway will provide a helpful point of departure for such studies and also for work on the environmental fate of other halogenated aniline pollutants.

LITERATURE CITED

Bachofer, R.; Ligens, F.; Schäfer, W. *FEBS Lett.* **1975**, *50*, 288.
Bartha, R. *J. Agric. Food Chem.* **1971**, *19*, 385.
Bartha, R.; Linke, H. A. B.; Pramer, D. *Science (Washington, D.C.)* **1968**, *161*, 582.
Bartha, R.; Pramer, D. *Adv. Appl. Microbiol.* **1970**, *13*, 317.
Bergman, J. G.; Sanik, J. *Anal. Chem.* **1957**, *29*, 241.
Bordeleau, L. M.; Rosen, J. D.; Bartha, R. *J. Agric. Food Chem.* **1972**, *20*, 573.
Briggs, G. G.; Walker, N. *Soil Biol. Biochem.* **1973**, *5*, 695.
Chisaka, H.; Kearney, P. C. *J. Agric. Food Chem.* **1970**, *18*, 854.
Cripps, R. E.; Roberts, T. R. In "Pesticide Microbiology"; Hill, I. R.; Wright, S. J. L., Eds.; Academic Press: London, New York, and San Francisco, 1978; Chapter 11, pp 669–730.
Duxbury, J. M.; Tiedje, J. M.; Alexander, M.; Dawson, J. E. *J. Agric. Food Chem.* **1970**, *18*, 199.
Engelhardt, G.; Wallnöfer, P.; Fuchsbichler, G.; Baumeister, W. *Chemosphere* **1977**, *6*, 85.
Evans, W. C.; Smith, B. S. W.; Fernley, H. N.; Davies, J. I. *Biochem. J.* **1971**, *122*, 543.
Fletcher, C. L.; Kaufman, D. D. *J. Agric. Food Chem.* **1979**, *27*, 1127.
Focht, D. D.; Alexander, M. *Appl. Microbiol.* **1970**, *20*, 608.
Hsu, T.-S.; Bartha, R. *J. Agric. Food Chem.* **1976**, *24*, 118.
Huffman, C. W.; Allen, S. E. *J. Agric. Food Chem.* **1960**, *8*, 298.
Hugh, R.; Gilardi, J. L. In "Manual of Clinical Microbiology", 3rd ed.; Lenette, E. H.; Spaulding, E. H.; Truant, J. P., Eds.; American Society for Microbiology: Washington, DC, 1980; Chapter 22, p 288.
Kanner, D.; Bartha, R. *J. Bacteriol.* **1979**, *139*, 225.
Kaufman, D. D.; Plimmer, J. R.; Klingebiel, U. I. *J. Agric. Food Chem.* **1973**, *21*, 127.
Kearney, P. C.; Plimmer, J. R. *J. Agric. Food Chem.* **1972**, *20*, 584.
Kilby, B. A. *Biochem. J.* **1948**, *43*, V.
Lambert, B. L.; Shurvell, H. F.; Verbit, L.; Cooks, R. G.; Stout, G. H. "Organic Structural Analysis"; Macmillan: New York, 1976: Part 4, Chapter 4, pp 457–460.
Marinucci, A. M.; Bartha, R. *Appl. Environ. Microbiol.* **1979**, *38*, 1020.
Oberhofer, T. R. *J. Clin. Microbiol.* **1979**, *9*, 220.
Otto, A. L.; Blachman, U. *J. Clin. Microbiol.* **1979**, *10*, 147.
Reber, H.; Helm, V.; Karanth, N. G. K. *Eur. J. Appl. Microbiol. Biotechnol.* **1979**, *7*, 181.
Rosenberg, A.; Alexander, M. *J. Agric. Food Chem.* **1980**, *28*, 297.
Russel, S. *Chem. Abstr.* **1980**, *92*, 1524.
Russel, S.; Bollag, J.-M. *Acta Microbiol. Pol.* **1977**, *26*, 59.
Sharpee, K. W.; Duxbury, J. M.; Alexander, M. *Appl. Microbiol.* **1973**, *26*, 445.
Tiedje, J. M.; Duxbury, J. M.; Alexander, M.; Dawson, J. E. *J. Agric. Food Chem.* **1969**, *17*, 1021.
Tweedy, B. G.; Loeppky, C.; Ross, J. A. *J. Agric. Food Chem.* **1970**, *18*, 851.
Von Plotho, O. *Arch. Mikrobiol.* **1948**, *14*, 12.
Walker, N.; Harris, D. *J. Appl. Bacteriol.* **1969**, *32*, 457.

Received for review June 3, 1981. Accepted November 23, 1981. This work was performed under New Jersey Agricultural Experiment Station Project 01200 and was supported by the New Jersey Agricultural Experiment Station and American Cyanamid, Inc.