Degradation of dichloroaniline isomers by a newly isolated strain, *Bacillus megaterium* IMT21

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An efficient 3,4-dichloroaniline (3,4-DCA)-mineralizing bacterium has been isolated from enrichment cultures originating from a soil sample with a history of repeated exposure to diuron, a major metabolite of which is 3,4-DCA. This bacterium, *Bacillus megaterium* IMT21, also mineralized 2,3-, 2,4-, 2,5- and 3,5-DCA as sole sources of carbon and energy. These five DCA isomers were degraded via two different routes. 2,3-, 2,4- and 2,5-DCA were degraded via previously unknown dichloroaminophenol metabolites, whereas 3,4- and 3,5-DCA were degraded via dichloroacetanilide.

INTRODUCTION

Dichloroanilines (DCAs) are widely used intermediates in commercial syntheses of various azo dyes, herbicides, paints, cosmetics and other industrial chemicals. However, their stability and toxicity also render them hazardous when released into the environment (NTP Comparative Toxicity Studies, 1998; Argese *et al.*, 2001; Lo *et al.*, 1990; Padmanabhan *et al.*, 2006). 3,4- and 3,5-DCA are particularly noxious, although 2,3-, 2,4- and 2,5-DCA are also highly toxic (Claver *et al.*, 2006; Götz *et al.*, 1998; Peng *et al.*, 2005; Valentovic *et al.*, 1995). 3,4-DCA is the most commonly used DCA isomer in the chemical industry (Giacomazzi & Cochet, 2004; Valentovic *et al.*, 1995) and it is also the major breakdown product of the widespread phenylamide herbicides diuron, linuron and propanil. In fact it is detected more frequently in environmental samples than the parent herbicides (Claver *et al.*, 2006; Giacomazzi & Cochet, 2004).

Several bacterial strains have been isolated that can degrade 3,4-DCA, and at least one of these has also been reported to degrade 2,3- 2,4- and 2,5-DCA (Table 1). A complete degradation pathway has not been elucidated for 3,4-DCA, but 4-chloroaniline, 3-chloro-4-hydroxyaniline, 4-chloro-3-hydroxyaniline, 4,5-dichlorocatechol and 1,2-dichlorobenzene have been suggested as intermediates, ultimately leading to mineralization via different catabolic routes (Table 1). Furthermore, 3,4-dichloroacetanilide, 3,3′,4,4′-tetrachloroazobenzene and 3,4-dichloro-*N*-(3,4-dichlorophenyl)benzamide have also been reported as transformation products or minor intermediates, suggesting that different degradation pathways could be operating in some of these strains (Table 1).

In this communication we report the isolation of a new bacterial strain, *Bacillus megaterium* IMT21, which is able to mineralize five DCA isomers, 2,3-, 2,4-, 2,5-, 3,4- and 3,5-DCA, as sole sources of carbon and energy. We find that the catabolic pathway used depends on the isomer. 3,4- and 3,5-DCA are degraded via dichloroacetanilide, i.e. one of the previously reported pathways for 3,4-DCA. However, the other three isomers are degraded via previously undescribed dichloroaminophenol intermediates. The mechanistic basis for this isomer-specific degradation is discussed.

METHODS

Chemicals. All DCA isomers, 3,4-dichloroacetanilide and formic acid were obtained from Sigma-Aldrich. Solvents were obtained from Merck.

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Abbreviation: DCA, dichloroaniline.

The GenBank accession number for the 16S rRNA gene sequence reported in this paper is GU479395.

Table 1. Known DCA-degrading bacterial strains

Bacterial strain	Activity on	Comments on metabolic route	Reference
Acidovorax sp.	3,4-DCA	3,4-DCA mineralization but no metabolites observed	Dejonghe *et al.* (2002)
Alcaligenes faecalis H1	3,4-DCA	3,4-DCA mineralization via. 4,5-dichloropyrocatechol	Surovtseva *et al.* (1993)
Comamonas testosteroni WDL7	3,4-DCA	Plasmid-mediated mineralization of 3,4-DCA but no metabolites observed	Dejonghe *et al.* (2002); Surovtseva *et al.* (1993)
Delftia acidovorans WDL34	3,4-DCA	3,4-DCA mineralization but no metabolites observed	Dejonghe *et al.* (2003)
Paracoccus denitrificans 3XA	3,4-DCA	3,4-DCA mineralization but no metabolites observed	Surovtseva *et al.* (1981)
Pseudomonas diminuta INMI KS-7	3,4-DCA	Different dehalogenated products identified during 3, 4-DCA mineralization	Surovtseva *et al.* (1985, 1986)
Pseudomonas fluorescens 26-K	3,4-DCA	3,4-DCA mineralization via 2-amino-4-chlorophenol; trace amounts of 3,4-dichloroacetanilide and 3,3′,4,4′-tetrachloroazoxybenzene also identified	Surovtseva *et al.* (1981)
Pseudomonas putida strain	3,4-DCA	Cometabolic mineralization of 3,4-DCA (along with aniline) via 4,5-dichlorocatechol, 3,4-dichloro-muconate, 3-chlorobutenolide, 3-chloromaleyl acetate, and 3-chloro-4-ketoadipate	You & Bartha (1982)
Pseudomonas sp. Bk8	3,4-DCA	Diuron mineralization via 3,4-DCA; no further metabolites observed	El-Deeb *et al.* (2000)
Pseudomonas sp. KB35B	3,4-DCA	3,4-DCA mineralization and chlorocatechol-2,3-dioxygenase activity observed	Kim *et al.* (2007)
Rhodococcus sp. strain 2	3,4-DCA	Co-metabolic transformation of 3,4-DCA under anaerobic conditions; 3,4-dichloroacetanilide, 3,4-dichloro-*N*-(3,4-dichlorophenyl)benzamide and 1,2-dichlorobenzene identified as intermediates	Travkin *et al.* (2002)
Rhodococcus sp. T1-1	5 DCA isomers	Vinclozolin mineralization via 3,5-DCA; 2,3-, 2,4-, 2,5- and 3,4-DCA also mineralized but no metabolites observed during growth on any of the DCA isomers	Lee *et al.* (2008)
Variovorax sp. SRS16	3,4-DCA	Diuron and linuron mineralization via 3,4-DCA; no further metabolites observed	Sørensen *et al.* (2008)
Variovorax sp. WDL1	3,4-DCA	Linuron mineralization via 3,4-DCA; no further metabolites observed	Dejonghe *et al.* (2003)

Media and growth conditions. Minimal medium (MM) consisted of 1.36 g KH_2PO_4, 1.78 g $Na_2PO_4.2H_2O$, 0.50 g $MgSO_4.7H_2O$ and 0.50 g NH_4Cl per litre. The pH was adjusted to 7.2 with NaOH, and 1 ml of trace elements solution [0.10 g $Al(OH)_3$, 0.05 g $SnCl_2.2H_2O$, 0.05 g KI, 0.05 g LiCl, 0.08 g $MnSO_4$, 0.05 g H_3BO_3, 0.10 g $ZnSO_4.7H_2O$, 0.01 g $CoCl_2$, 0.01 g $NiSO_4.6H_2O$, 0.05 g $BaCl_2$ and 0.05 g $(NH_4)_6Mo_7O_{24}.4H_2O$] per litre was also added. Individual DCA isomers were added to the MM from stock solutions prepared in dichloromethane and used at final concentrations of 0.30 mM (50 p.p.m.). The dichloromethane was evaporated by shaking the medium well before inoculation. Nutrient agar (NA) and nutrient broth (NB) were used as rich media for bacterial growth.

Soil enrichment and isolation of the 3,4-DCA-mineralizing micro-organism. Soil samples were collected from a local agricultural field in Chandigarh, India, with a history of repeated exposure to the herbicide diuron. MM (100 ml) containing 0.3 mM 3,4-DCA was inoculated with 1 g of pooled soil samples and incubated at 28 °C in an Erlenmeyer flask on a rotary shaker at 150 r.p.m., with the addition of a further 0.3 mM 3,4-DCA after 15 days. After a further 15 days this culture (1 %, v/v) was transferred to 100 ml fresh medium supplemented with 0.3 mM 3,4-DCA as above and then incubated at 28 °C for another month, again with the addition of a further 0.3 mM 3,4-DCA at day 15. After five rounds of such enrichment cycles the growth medium was plated onto MM agar plates containing 0.3 mM 3,4-DCA as sole carbon source and incubated at 28 °C for 48 h. Individual bacterial colonies were then tested for 3,4-DCA mineralization in MM by HPLC as described below.

Identification of 3,4-DCA-degrading bacteria. The IMT21 strain which was found to be kinetically most efficient at degrading 3,4-DCA was identified by the Microbial Type Culture Collection (MTCC), Chandigarh, India, using polyphasic taxonomic methods (Vandamme *et al.*, 1996). The nucleotide sequence of the 16S rRNA gene (1448 bp) was determined by the direct PCR sequencing method using the universal 16S primers 8F and 1492R (Baker *et al.*, 2003). The nucleotide sequence was compared with sequences in the non-redundant database using a BLAST search (Zhang *et al.*, 2000).

HPLC and LC-MS analysis. An Agilent series LC system controlled by Agilent TOF software (version A.01.00) (Agilent Technologies) was used for quantitative analysis of DCA isomers and metabolites. The compounds were separated at 25 °C on an Aqua C18 (5 μm particle size, 250 × 4.60 mm) column (Phenomenex) using acetonitrile/water (50 : 50, v/v; both containing 0.1 %, v/v, formic acid) as mobile phase at a flow rate of 0.7 ml min^{-1}. Substrate and products were monitored at 250 nm.

Qualitative analysis of DCA isomers was performed using an LC/MS TOF mass spectrometer (Agilent Technologies) with an electrospray ionization (ESI) source. The mass spectrometer was connected to the HPLC stream after the DAD detector. Nitrogen was used at a flow rate of 12 l min^{-1} as drying gas. The capillary temperature was 350 °C and the spray voltage was 3 kV. The scanning was done in positive-

ion mode and the fragmenter and skimmer were set at 120 V and 60 V, respectively for scans in the range 50–450 *m/z*.

Growth and resting-cell studies. For studies of growth kinetics, 0.5 ml aerobically grown seed culture of strain IMT21 in nutrient broth was inoculated in 50 ml minimal medium containing 0.3 mM of one of the five DCA isomers tested and incubated with shaking (200 r.p.m.) at 28 °C. Samples (0.5 ml) were collected from the incubated cultures at different time points, centrifuged at 12 000 ***g*** and filtered with 0.22 μm filters (Millipore). The filtrate was used for the HPLC and LC-MS analysis.

For resting-cell studies, NB-grown cells of strain IMT21 (300 ml) were harvested in the mid-exponential phase (OD_{600} ~0.5), washed with 300 ml ice-cold MM and finally resuspended in 30 ml ice-cold MM. A DCA isomer was added as a filter-sterilized solution at a final concentration of 0.30 mM and 0.5 ml aliquots were removed at different time points, centrifuged and filtered prior to analysis by HPLC and LC-MS. Quantitative analysis was done with HPLC using a standard curve prepared with authentic standards. The experiments were performed in triplicate with appropriate controls.

RESULTS AND DISCUSSION

Approximately 50 % degradation of 0.3 mM 3,4-DCA was observed within 15 days after five rounds of enrichment culture (data not shown). Different dilutions of this enrichment were plated onto MM plates containing 3,4-DCA as a sole carbon source, and 12 morphologically distinct bacterial colonies appeared after 30 days of incubation at 30 °C. These were picked and checked individually for 3,4-DCA degradative activity in liquid cultures as described above. The fastest-degrading strain, IMT21, degraded ~60 % of 0.3 mM 3,4-DCA within 20 days.

Strain IMT21 was a Gram-positive, motile, sporulating, catalase- and oxidase-positive, long rod-shaped bacterium which formed tiny creamish-white colonies after overnight incubation on NB plates at 30 °C. The strain hydrolysed aesculin, Tween 20, starch and gelatin, and showed acid production from D-fructose, glycerol, maltose, galactose, D-mannose and D-ribose. Tetradecanoic acid (16 : 0), 13-methylpentadecanoic acid (15 : 0 *iso*) and 12-methyltetradecanoic acid (15 : 0 *anteiso*) were its major fatty acids. After PCR amplification and sequencing, its 16S rRNA gene (GenBank accession no. GU479395) showed highest sequence similarity with the genus *Bacillus*, with 100 % identity to several *Bacillus megaterium* strains (GenBank accession numbers GU191918, GQ406847, EU880506 and EU723818). Based on its physiological characteristics, morphological and biochemical tests and 16S rDNA gene sequence, strain IMT21 was therefore named *Bacillus megaterium* IMT21.

Strain IMT21 mineralized 2,3-, 2,4-, 2,5-, 3,4- and 3,5-DCA as sole sources of carbon and energy in MM (Fig. 1). 2,3-, 2,4- and 2,5-DCA were degraded at comparable rates, and complete degradation was observed within 20 days. However, 30 days were required for complete degradation of 3,4- and 3,5-DCA (data not shown). No metabolites were detected by LC-MS during growth of IMT21 on any of the five tested DCA isomers. The relative degradation rates for the different DCA isomers by this strain are somewhat different from those reported by Lee *et al.* (2008) for *Rhodococcus* sp. T1-1 (2,5-DCA>2,4-DCA>3,5-DCA>2,3-DCA>3,4-DCA), which is the only other bacterium so far reported that can also mineralize these five DCA isomers. *Rhodococcus* sp. T1-1 was isolated by enrichment culture from a pesticide-contaminated soil sample and was reported to mineralize the fungicidal carbamate vinclozolin (Lee *et al.*, 2008). 3,5-DCA is a toxic metabolite produced in the (bio)degradation of vinclozolin. Interestingly, the isomer degraded most slowly by both

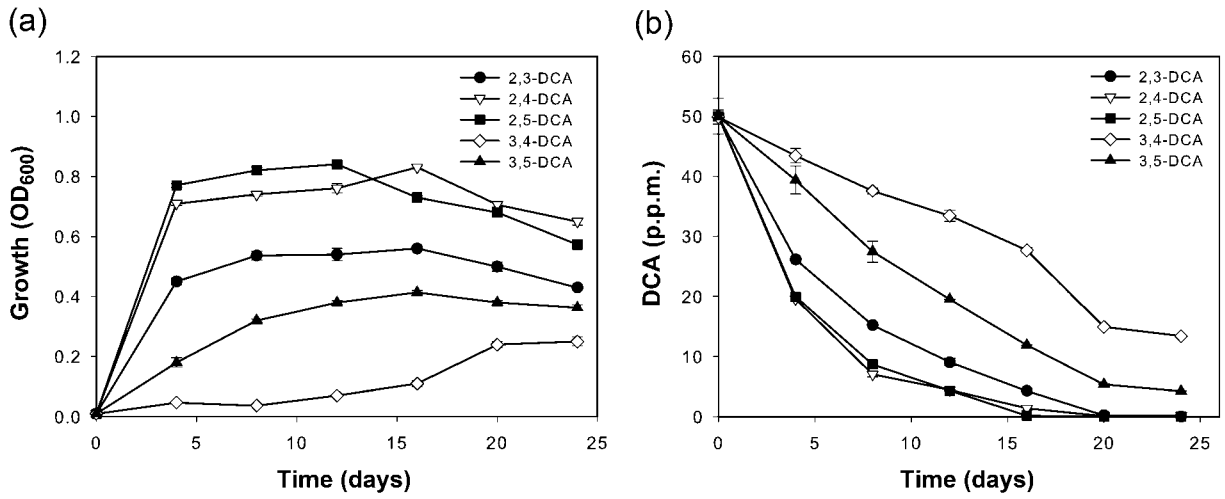

Fig. 1. Growth (a) and degradation kinetics (b) of *B. megaterium* IMT21 on different DCA isomers as sole sources of carbon and energy.

the strains was 3,4-DCA, which is also the most toxic of the five isomers (NTP Comparative Toxicity Studies, 1998; Padmanabhan *et al.*, 2006; Valentovic *et al.*, 1995). Another similarity with our findings is that Lee *et al.* (2008) were also unable to detect any metabolites during the growth of strain T1-1 on any of the five DCA isomers tested by HPLC.

Indeed no metabolites have yet been reported nor catabolic pathway proposed for most of the 3,4-DCA-degrading bacterial strains so far characterized (Table 1). However, those few reports where metabolites have been detected implicate three different 3,4-DCA catabolic pathways (Table 1). The first involves direct oxidative deamination of the aromatic nucleus, resulting in dichlorocatecholic intermediates which are assimilated via ring cleavage. This pathway has been exemplified in a *Pseudomonas putida* strain in which 3,4-DCA is co-metabolically mineralized in the presence of aniline via 4,5-dichlorocatechol, which is assimilated via 3,4-dichloromuconate, 3-chlorobutinolide, 3-chloromaleylacetate and 3-chloro-4-ketoadipate (You & Bartha, 1982). A second pathway, in this case initiated by reductive deamination, has been described for *Rhodococcus* sp. strain 2 (Travkin *et al.*, 2002). In this case 1,2-dichlorobenzene (1,2-DCB) was identified as a catabolic intermediate under nitrate-reducing anaerobic conditions (Travkin *et al.*, 2002). Although no further intermediate was observed in this strain, degradation of 1,2-DCB or other dichlorobenzene isomers via catecholic intermediates has been described for other bacterial strains (Haigler *et al.*, 1988; Spain & Nishino, 1987; Spiess *et al.*, 1995; Vandamme *et al.*, 1996). The third pathway is proposed to involve an initial oxidative removal of a chlorine atom from the 3,4-DCA molecule. Evidence for this pathway is the transient appearance of 2-chloro-4-aminophenol during 3,4-DCA mineralization by *Pseudomonas fluorescens* 26-K (Travkin *et al.*, 2003). Enhanced catechol-2,3-dioxygenase activity observed in cultures of strain 26-K and *Pseudomonas* sp. KB35B when grown in the presence of 3,4-DCA also suggests that this pathway proceeds further via catecholic intermediates (Kim *et al.*, 2007; Travkin *et al.*, 2003).

Resting-cell studies with NB-grown cultures were used to elucidate the catabolic pathways of the different DCA isomers by strain IMT21. Aqueous samples collected at different time points were analysed by LC-MS (ES+). Culture supernatants incubated with 2,4- and 2,5-DCA each resulted in one transient metabolite peak at 24 h and 48 h with the elemental composition of $C_6H_5NCl_2O+H^+$ (*m/z* 177.98), at retention times 8.46 and 5.01 min, respectively. Two metabolite peaks at 4.69 and 7.54 min with exactly the same elemental composition appeared transiently in the case of 2,3-DCA at 24 h. This empirical elemental composition is interpreted as the monohydroxylated metabolite of the DCA isomers, dichloroaminophenol. The observed mass spectra of the metabolite matched precisely with the *in silico*-generated mass spectra of the elemental composition above. Dichloroaminophenol would probably result from direct hydroxylation of the aromatic ring without dechlorination or deamination. This metabolite has not been reported previously for DCA degradation, but another phenolic metabolite, 2-amino-4-chlorophenol, has been reported as an intermediate in the degradation of 3,4-DCA by *P. fluorescens* 26-K and it is argued that this is then further

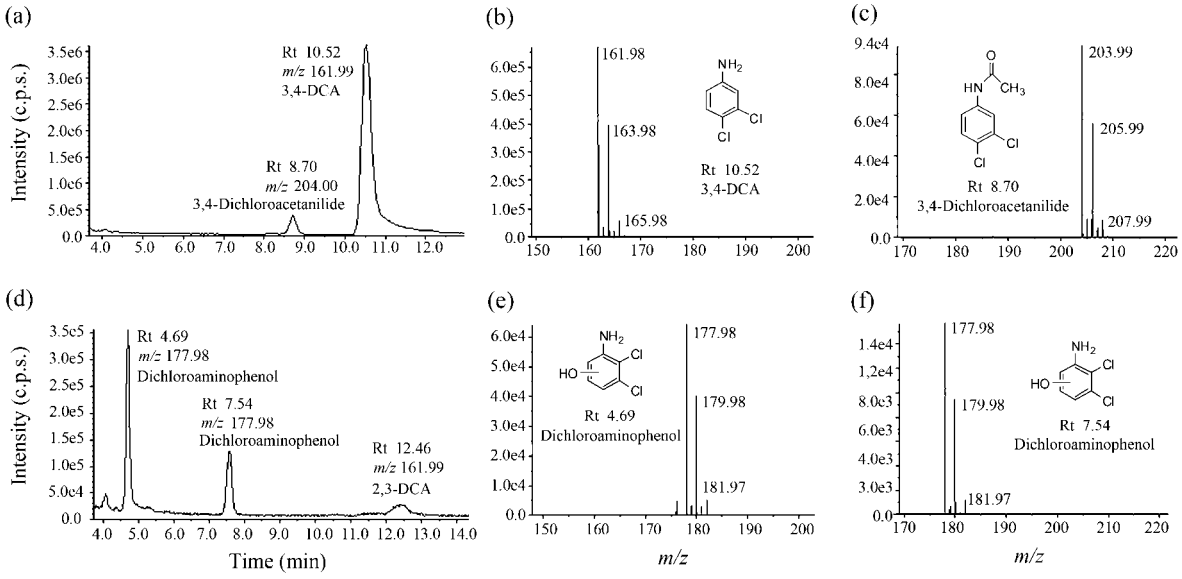

Fig. 2. LC-MS extracted ion chromatograms showing metabolite peaks of (a) 3,4-DCA and (d) 2,3-DCA, and LC-MS TOF spectra of the pseudomolecular ions ($m/z+H^+$) of 3,4-DCA (b), the 3,4-dichloroacetanilide (c) from panel (a), and the dichloroaminophenol metabolites (e and f) from panel (d). Rt, retention time.

degraded via catechol (see above and Kim *et al.*, 2007). Simultaneous monohydroxylation and dechlorination have also been reported previously for highly chlorinated benzenes, such as hexachlorobenzene (HCB). This bacterial degradation of HCB via a monooxygenation reaction to form pentachlorophenol has been shown in a pentachlorophenol degrader, *Sphingobium chlorophenolicum* ATCC 39723, after a cytochrome P-450$_{cam}$ variant was inserted into it (Yan *et al.*, 2006) A similar reaction has recently been shown in the HCB-degrading *Nocardioides* sp. strain PD653 (Takagi *et al.*, 2009). Interestingly, pentachlorophenol is further degraded via a hydroquinone pathway in both these latter strains, which raises the possibility that dichloroaminophenol produced by the monohydroxylation of 2,3-, 2,4- and 2,5-DCA in strain IMT21 may also be further degraded via a hydroquinone pathway.

A single metabolite peak with the elemental composition $C_8H_7Cl_2NO+H^+$ (*m/z* 203.99) transiently appeared in culture supernatant incubated with 3,4- or 3,5-DCA (retention times 10.52 and 10.49 min, respectively) at 24 h. This empirical formula is interpreted as dichloroacetanilide. The observed mass spectra of the metabolite we have detected match precisely with the *in silico*-generated mass spectra of its elemental composition. Furthermore, the retention time and mass spectra matched those of an authentic 3,4-dichloroacetanilide standard (data not shown). Representative LC-MS profiles of 2,3- and 3,4-DCA along with pseudomolecular ions ($m/z+H^+$) of dichloroaminophenol and 3,4-dichloroacetanilide are shown in Fig. 2.

3,4-Dichloroacetanilide has previously been observed as a minor metabolite in the degradation of 3,4-DCA in *Rhodococcus* sp. strain 2 under anaerobic conditions, although a complete pathway involving it as a catabolic intermediate was not established (Travkin *et al.*, 2002) Twenty-two different *Pseudomonas* strains were tested by Vol'nova *et al.* (1980) for the ability to acetylate 3,4-DCA under co-metabolic conditions, as none of them could mineralize 3,4-DCA as a sole source of carbon and energy. Eleven of the tested strains were found to have the acetylation activity. A recent study involving the model fungus *Podospora anserina* has also conclusively demonstrated acetylation as a detoxification strategy for various toxic aromatic amines, including 3,4-DCA (Martins *et al.*, 2009). Targeted gene-disruption experiments showed that an arylamine *N*-acetyltransferase enzyme was essential for growth and survival of the fungus in the presence of 3,4-DCA and other toxic aromatic amines. This acetyltransferase, named PaNAT2, was further demonstrated to bioremediate 3,4-DCA in experimentally contaminated soils in a proof-of-concept bioremediation experiment (Martins *et al.*, 2009).

To the best of our knowledge, no predicted catabolic pathway nor any intermediate has previously been reported for the aerobic degradation of 2,3-, 2,4- and 2,5-DCA by a single bacterial isolate, although reductive dehalogenation of 2,4-DCA via 3-chloroaniline and 4-chlorocatechol has been reported for an anaerobic consortium of bacteria (Struijs & Rogers, 1989).

In conclusion, surprisingly little work has been done on the microbial degradation of DCA isomers despite their notoriety as environmental pollutants. To the best of our knowledge, *Bacillus* sp. IMT21 and the previously described *Rhodococcus* sp. T1-1 are the only known bacterial strains that can mineralize the five different DCA isomers in question without a requirement for any other carbon source. No metabolic intermediate or pathway has been proposed for strain T1-1 and, although several metabolites belonging to three catabolic routes have been proposed for 3,4-DCA degradation in various bacteria (see above), no degradation intermediates or catabolic routes have so far been proposed for the other four DCA isomers. Strain IMT21 degrades 2,3-, 2,4- and 2,5-DCA via the respective dichloroaminophenols and it degrades 3,4- and 3,5-DCA via acetanilide intermediates.

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