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Diversity of 3-chloroaniline and 3,4-dichloroaniline degrading bacteria isolated from three different soils and involvement of their plasmids in chloroaniline degradation

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Abstract

Attempts were made to isolate 3-chloroaniline (3-CA) and 3,4-dichloroaniline (3,4-DCA) degrading bacteria from the A- and B-horizon of three different soils. A variety of 3-CA degrading bacteria was obtained from all soils, whereas 3,4-DCA degrading strains were only isolated from one soil. Amongst the 3-CA and 3,4-DCA degraders, two belong to the γ-*Proteobacteria* and seven to the β-*Proteobacteria*. Of the latter group, five are members of the family of the *Comamonadaceae*. Interestingly, all isolates contained an IncP-1β plasmid. These plasmids could be divided into four major groups based on restriction digest patterns. While all plasmids that were detected in the isolates, except one, encode total degradation of 3-CA, no indigenous plasmid that codes for total degradation of 3,4-DCA was found. This is the first study that reports the presence of diverse transferable plasmids that encode mineralisation of 3-CA in different 3-CA degrading species.

Keywords: Chloroaniline; Degradation; Plasmid; Soil enrichment culture; Xenobiotic

1. Introduction

Aniline and its derivatives have been used for a long time in the production of paints, pesticides, plastics, pharmaceuticals and other materials [1] and they mainly accumulate in the environment as a result of the microbial transformation of herbicides such as derivatives of phenylurea, acylanilides, and phenylcarbamates [2]. Since they are known to be toxic and carcinogenic to living organisms [3], many researchers have paid attention to their fate in the environment. Anilines and chloroanilines tend to adsorb to soil particles [4], undergo various spontaneous chemical transformations [5] and turn into non-extractable humic acid-like compounds [6] or persistent xenobiotics such as azobenzenes and triazenes [7]. In spite of these physical and chemical processes, which render (chloro)anilines unavailable for microbial degradation, microorganisms have been shown to metabolise these molecules under monoxenic culture conditions (e.g. [8–14]). Resistance to biodegradation and the extent of the toxicity of the (chloro)anilines for microorganisms are largely dependent on the number and position of the chlorine atoms on the aromatic ring. This becomes evident upon examination of studies of microorganisms that can utilise anilines. There are many more reports of bacteria that can degrade aniline (e.g. [8,14–20]) than studies describing chloroaniline (2-CA, 3-CA, 4-CA, 3,4-DCA) metabolising strains. For one of the most stable and toxic chloroanilines, i.e. 3,4-dichloroaniline [12], only four bacteria have been isolated that can use this compound as the sole source of nitrogen and carbon [12–14]. In contrast to the situation

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for the aniline degradation pathway [20], no specific genes for the transformation of chloroanilines have been described. The presence of plasmids in chloroaniline degrading bacteria has only been investigated in five different strains belonging to the family *Comamonadaceae* in the recent study of Boon et al. [8]. Although all strains contained a plasmid, only one plasmid encoded the oxidative deamination of both aniline and 3-CA, but none of the plasmids conferred total degradation of 3-CA.

Results in the current study indicate that 3-CA and 3,4-DCA, spiked to the A- (0–30 cm depth) and B-horizon (30–60 cm depth) of various soils, rapidly became undetectable after methanol extraction followed by high performance liquid chromatography (HPLC) analysis. Although this may in part be due to the physical and chemical processes mentioned above, microbial degradation could also be one of the causes. To test this hypothesis we tried to isolate 3-CA or 3,4-DCA degrading bacterial strains from these contaminated soils. Since little is known so far about the phylogenetic and metabolic diversity of chloroaniline degrading bacteria, their catabolic genes, and the potential role of mobile genetic elements (MGEs) in the horizontal exchange of these genes, comparison of the isolates obtained in this study with those previously described will extend our knowledge in this area. In parallel, we also examined if in situ transfer of known and previously described 3-CA and 3,4-DCA degradative plasmids from an introduced *Ralstonia eutropha* donor strain to the indigenous soil bacteria could be used to extend the catabolic potential of these soils.

2. Materials and methods

2.1. Chemicals

3,4-Dichloroaniline (3,4-DCA) (Sigma-Aldrich Chemie, Steinheim, Germany) was used as brown crystals in agar medium while it was added as a 25 g l^{-1} stock solution in methanol to liquid media and soils. 3-Chloroaniline (3-CA; Fluka AG Chemische Fabrik, Buchs, Switzerland) was always added as a viscous colourless fluid.

2.2. Media

The mineral medium MMN (mineral medium without any source of nitrogen and carbon) [21,22] contained 1419.6 mg Na_2HPO_4, 1360.9 mg KH_2PO_4, 98.5 mg $MgSO_4$, 5.88 mg $CaCl_2{\cdot}2H_2O$, 1.16 mg H_3BO_4, 2.78 mg $FeSO_4{\cdot}7H_2O$, 1.15 mg $ZnSO_4{\cdot}7H_2O$, 1.69 mg $MnSO_4{\cdot}H_2O$, 0.38 mg $CuSO_4{\cdot}5H_2O$, 0.24 mg $CoCl_2{\cdot}6H_2O$, 0.10 mg MoO_3, and 3.2 mg EDTA in 1 l of distilled water. Luria Bertani (LB) medium [23] containing 10 g of Bacto Peptone (Difco, Detroit, MI, USA), 5 g of Bacto yeast extract (Difco), and 5 g of NaCl in 1 l demineralised water was used as a rich medium. For 1/10 LB medium all concentrations of the LB medium were divided by 10, except the concentration of NaCl was maintained at 5 g l^{-1}. These media were solidified with 15 g l^{-1} of high purity agar (Agar-Agar hochrein, Merck, Germany) for plate growth.

2.3. Bacterial strains

Bacterial strains and their relevant characteristics are presented in Table 1. All strains were cultivated at 28°C on LB agar, except the *R. eutropha* strains JMP228 containing the 3-CA degradative plasmid pC1-3 or the 3,4-DCA degradative plasmid pWDL7, which were maintained on MMN mineral medium with 200 mg l^{-1} 3-CA or 50 mg l^{-1} 3,4-DCA, and 2 g l^{-1} sodium pyruvate. The plasmid pC1-3 encodes partial degradation of 3-CA and was transferred in plate matings from *Delftia acidovorans* CA28 to *R. eutropha* JMP228*gfp* [8]. Plasmid pWDL7 encodes total degradation of 3-CA and 3,4-DCA and was transferred from *Comamonas testosteroni* WDL7 to *R. eutropha* JMP228n (W. Dejonghe, W. Verstraete and E.M. Top, unpublished results). When appropriate (see Table 1), antibiotics were added to the media in the following concentrations: 100 mg l^{-1} nalidixic acid, 100 mg l^{-1} rifampicin, and 50 mg l^{-1} kanamycin.

2.4. Soils

Soil samples were collected from three Belgian sites,

Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
R. eutropha JMP228n	Nx^r, Rif^r	[32]
R. eutropha JMP228n(pWDL7)	Nx^r, Rif^r, 3,4-DCA^{NC}, 3-CA^{NC}	W. Dejonghe, W. Verstraete, and E.M. Top, unpublished results
R. eutropha JMP228*gfp*	Rif^r, Km^r, GFP	[22]
R. eutropha JMP228*gfp* (pC1-3)	Rif^r, Km^r, GFP, 3-CA^N	[8]
E. coli CM404	Km^r, pRK2013	[46]
pWDL7	3,4-DCA^{NC}, 3-CA^{NC}	W. Dejonghe, W. Verstraete, and E.M. Top, unpublished results
pC1-3	3-CA^N	[8]

[a]Rif^r, Nx^r, and Km^r, resistance to rifampicin, nalidixic acid, and kanamycin, respectively; GFP, green fluorescent protein; 3-CA, 3,4-DCA, 3-chloroaniline and 3,4-dichloroaniline, respectively. ^{C}Use of the compound as sole carbon source. ^{N}Use of the compound as sole nitrogen source in the presence of the C source pyruvate.

Table 2
Characteristics of the soils used in this study

Origin	Soil type	Organic matter (%)	Water holding capacity (%)	pH (KCl)	Heterotrophic plate counts[a] (CFU (g fresh soil)$^{-1}$)
Ternat A-horizon	heavy sand loam (22.8% sand, 65.1% loam, 12.1% clay)	3.3	24	6.6	1.7×10^{11}
Ternat B-horizon	heavy sand loam (34.3% sand, 53.8% loam, 11.9% clay)	3.3	24	6.3	5.0×10^{9}
Pittem A-horizon	sandy loam (75.9% sand, 16.8% loam, 7.3% clay)	1.4	16	5.6	3.3×10^{8}
Pittem B-horizon	sandy (83.2% sand, 9.2% loam, 7.6% clay)	0.4	12	5.3	2.0×10^{7}
Melle A-horizon	sandy loam (40.7% sand, 47.6% loam, 11.7% clay)	1.8	22	6.4	3.2×10^{7}
Melle B-horizon	sandy (84.8% sand, 10.3% loam, 4.9% clay)	0.6	20	6.0	2.0×10^{6}

[a]Heterotrophic plate counts were determined on R2A agar plates (Difco, Detroit, MI, USA).

Ternat, Pittem, and Melle and used immediately in the experiments without storage. All three soils originate from agricultural plots that had never been treated with 3-CA or 3,4-DCA, nor with any pesticide that was transformed into these compounds during degradation. Experiments were performed with soils sampled from both the A- (0–30 cm depth) and B-horizon (30–60 cm depth). The characteristics of the soils are presented in Table 2.

One hundred grams of fresh or sterile soil (sterilised by autoclaving twice for 20 min at 120°C and 1 bar, with a 1 day interval between treatments) was put into 500 ml glass microcosms. The soil was spiked with 50 mg kg^{-1} 3-CA or 3,4-DCA and inoculated with either 1 ml of one of the *R. eutropha* JMP228 inoculants (Table 1) suspended in MMN medium, or with 1 ml MMN medium. Bacterial inocula were grown overnight in 100 ml LB broth with or without 100 mg l^{-1} 3-CA, and the appropriate antibiotics. After washing, cells were resuspended in MMN medium and inoculated at a concentration of ca. 10^6 CFU g^{-1} soil, as determined by plate counts of the liquid cultures on to LB agar. After all amendments, the soil was mixed and the moisture content adjusted to 75% of the water holding capacity. Soil microcosms were placed at room temperature in the dark. All soil treatments were performed in duplicate and a summary of the different treatments is presented in Table 3.

2.5. Enrichment cultures and isolation of 3-CA and 3,4-DCA degrading strains

Soils that had been spiked with 3-CA were enriched for 3-CA degrading microorganisms, while 3,4-DCA treated soils were enriched for 3,4-DCA degraders. One enrichment culture was set up for each treated soil and consisted of 100 ml MMN medium to which 100 mg l^{-1} 3-CA or 25 mg l^{-1} 3,4-DCA together with 5 g of soil was added. The 250 ml glass Erlenmeyer flasks were shaken at 140 rpm and 28°C. When the substrates were no longer detected by HPLC analysis, an equal amount of product was added again to the enrichment flasks. As soon as this second dosage of chloroanilines was removed, a 1% inoculum was transferred to 100 ml fresh MMN medium that contained 200 mg l^{-1} 3-CA or 50 mg l^{-1} 3,4-DCA. After five consecutive 1% transfers to fresh medium, the final enrich-

Table 3
Summary of the different treatments of the different soils and number of chloroaniline degrading isolates obtained from each soil

Soil	Treatment[a]	Abbreviation	Total isolates[b]	Total degraders[b]
Ternat A-horizon	3-CA	Ternat-A-3CA	8	2
Ternat A-horizon	3-CA+*R. eutropha* JMP228*gfp*	Ternat-A-3CA-JMP	4	0
Ternat A-horizon	3-CA+*R. eutropha* JMP228*gfp* (pC1-3)	Ternat-A-3CA-JMP(pC1-3)	2	0
Ternat B-horizon	3CA	Ternat-B-3CA	7	1
Ternat B-horizon	3-CA+*R. eutropha* JMP228*gfp*	Ternat-B-3CA-JMP	5	0
Ternat B-horizon	3-CA+*R. eutropha* JMP228*gfp* (pC1-3)	Ternat-B-3CA-JMP(pC1-3)	2	0
Ternat A-horizon	3,4-DCA	Ternat-A-34DCA	7	0
Ternat A-horizon	3,4-DCA+*R. eutropha* JMP228n	Ternat-A-34DCA-JMP	6	2
Ternat A-horizon	3,4-DCA+*R. eutropha* JMP228n(pWDL7)	Ternat-A-34DCA-JMP(pWDL7)	3	0
Ternat B-horizon	3,4-DCA	Ternat-B-34DCA	4	0
Ternat B-horizon	3,4-DCA+*R. eutropha* JMP228n	Ternat-B-34DCA-JMP	3	0
Ternat B-horizon	3,4-DCA+*R. eutropha* JMP228n(pWDL7)	Ternat-B-34DCA-JMP(pWDL7)	11	2
Melle A-horizon	3-CA	Melle-A-3CA	10	3
Melle B-horizon	3-CA	Melle-B-3CA	no enrichment culture	0
Pittem A-horizon	3-CA	Pittem-A-3CA	11	1
Pittem B-horizon	3-CA	Pittem-B-3CA	7	1

[a]Abbreviations for carbon sources given in Table 1, all carbon sources added at 50 mg kg^{-1}.
[b]Numbers of isolates or degraders obtained.

ment cultures were plated on MMN agar with 300 mg l^{-1} 3-CA (3-CA enriched cultures) or on MMN agar with 50 mg l^{-1} 3,4-DCA (3,4-DCA enriched cultures). All plates contained 200 mg l^{-1} cycloheximide to inhibit fungal growth and were incubated for 14 days at 28°C. For each treatment, colonies with different morphologies were picked from the plates and streaked on the same medium as well as on to LB agar. The latter was used to confirm the purity of the strains and to better distinguish between different colony morphologies. Finally, pure colonies from the MMN plates were inoculated in 5 ml 1/10 LB broth with 100 mg l^{-1} 3-CA or 25 mg l^{-1} 3,4-DCA. After overnight growth, bacterial cultures were frozen at −80°C in a 20% (v/v) glycerol solution and in parallel 50 μl of these cultures were transferred to 5 ml MMN medium with 50 mg l^{-1} 3-CA or 25 mg l^{-1} 3,4-DCA. Next to these two media, the bacteria obtained from the Ternat soil were also transferred to MMN medium with 50 mg l^{-1} 3-CA and 1 g l^{-1} sodium pyruvate to select for bacteria that utilise 3-CA as a sole source of nitrogen. After shaking for 1 week at 140 rpm and 28°C, the remaining amount of 3-CA or 3,4-DCA was determined by HPLC analysis and 50 μl culture of the actively degrading cultures was again transferred to fresh MMN medium with 3-CA or 3,4-DCA. Bacterial isolates were scored positive for the degradation of 3-CA and 3,4-DCA when after 5 days growth was visually observed by an increase in the turbidity of the medium, 3-CA, 3,4-DCA or other ring intermediates were no longer detected by HPLC analysis and stoichiometric amounts of chloride were released.

2.6. Identification of isolates

The isolates were characterised by repetitive extragenic palindromic polymerase chain reaction (REP-PCR) as described by Rademaker and de Bruijn [24] and representatives were identified by partial (±460 bp) sequencing of the 16S rRNA genes, using the primer 16R519 (=PD) as described previously [25]. The FASTA programme [26] was applied to find the closest related sequences from the EMBL database.

2.7. Denaturing gradient gel electrophoresis

DNA templates were prepared from the sixth and final enrichment culture used to inoculate the agar plates, from colonies scraped from the agar medium after plating this final enrichment culture, and from purified isolates. Cells in 100 ml of the sixth and final enrichment culture were concentrated by centrifugation and the DNA was subsequently obtained through a previously described DNA extraction protocol [22]. To harvest the mixture of colonies from the MMN agar plates, the plates were scraped with a loop, washed with 2 ml of sterile water, and the bacterial suspension obtained was boiled for 10 min to release the DNA. The isolates were grown overnight in 5 ml 1/10 LB with 100 mg l^{-1} 3-CA. To prepare a DNA template of these strains, 200 μl of culture was boiled for 10 min. In all cases 1 μl of the template was used for PCR with the bacteria-specific 16S rRNA forward primer P63f and the reverse primer P518r, based on a universally conserved region, as previously described [27–29]. The PCR product contains a GC-clamp of 40 bases, added to the forward primer and has a total length of 531 bp (based on the reference *Escherichia coli* K12). PCR products were subjected to denaturing gradient gel electrophoresis (DGGE), based on the protocol described by Muyzer et al. [30] and El Fantroussi et al. [27]. In brief, PCR samples were run for 16 h at 45 V on a 6% (wt/vol) polyacrylamide gel with a denaturing gradient ranging from 45 to 60% (where 100% denaturant contains 7 M urea and 40% formamide). After electrophoresis the gels were stained with SYBR GreenI nucleic acid gel stain (1:10 000 dilution; FMC BioProducts, Rockland, ME, USA) and photographed.

2.8. Plasmid characterisation

A modified Kado and Liu plasmid extraction [8,31,32] was performed on pellets obtained after overnight growth of bacteria in 5 ml 1/10 LB with 100 mg l^{-1} 3-CA. *Eco*RI–*Pst*I, *Hin*dIII–*Bam*HI, and *Eco*RV–*Bgl*II restriction endonuclease digestions of plasmid DNA were performed according to the instructions of the enzyme supplier (Hoffmann-La Roche, Basel, Switzerland). Extracted and restricted plasmids were run on a 1% agarose gel, blotted onto Hybond-N nylon membranes (Amersham International, Little Chalfont, Buckinghamshire, UK) and used in Southern hybridisation performed at high stringency as described by Top et al. [32].

The incompatibility group of the plasmids was determined by PCR with the IncP primers *trfA1* (specific for IncP-1α plasmids) and *trfA2* (amplifies IncP-1α and IncP-1β plasmids) as described by Götz et al. [33] and as a template 1 μl of a 10 min boiled bacterial culture grown in 1/10 LB with 100 mg l^{-1} 3-CA. After verifying the length of the PCR product on a 1% agarose gel, PCR products were sequenced by IIT Biotech (Bielefeld, Germany). Sequences with a length between 180 and 215 bp were aligned to the National Centre for Biotechnology information database by using the BLAST, version 2.0, search programme [34]. The *trfA2* sequences have been deposited in the GenBank database under accession numbers AF467929–AF467940. The *trfA2* probe for the IncP-1β group was prepared by using the PCR digoxigenin (DIG) labelling mix (Hoffmann-La Roche) according to the instructions of the supplier and using the IncP-1β plasmid R751 [35] as template and the *trfA2* primers mentioned above.

Conjugation between 3-CA or 3,4-DCA degrading strains (donor), *R. eutropha* JMP228*gfp* (recipient) and *E. coli* CM404 (helper) was performed on 1/10 LB agar

Table 4
Isolates obtained from this study with their origin of isolation, degradation potential and plasmids

Isolate	Origin[a]	Acc. no.[b]	% Similarity of 16S rRNA gene fragments with nearest type strain (acc. no.)[c]	Degradation potential strain[d]	Plasmid encoded degradation of substrates[d]	Plasmid name	Plasmid group[e]	% Sequence similarity of *trfA2* with AF073901
Pseudomonas sp. TA12	Ternat-A-34DCA-JMP	AF461044	99.5 (AB021381)	3-CA^{NC}	–	pTA12	I	93
Pseudomonas sp. TA8	Ternat-A-34DCA-JMP	AF457652	99.8 (AF064461)	3-CA^{NC}	3-CA^{NC}	pTA8	IV	100
Acidovorax sp. TA2[f]	Ternat-A-3CA	AF457651	96.3 (Y18616)	3-CA^{NC}, 3,4-DCA^{NC}	3-CA^{NC}	pTA2	III	100
Acidovorax sp. TA35[f]	Ternat-A-3CA	AF457653	96.3 (Y18616)	3-CA^{NC}, 3,4-DCA^{NC}	3-CA^{NC}	pTA35	III	100
C. testosteroni TB1[g]	Ternat-B-34DCA-JMP(pWDL7)	AF457654	99.7 (M11224)	3-CA^{NC}, 3,4-DCA^{NC}	3-CA^{NC}, 3,4-DCA^{NC}	pTB1	II	100
C. testosteroni TB18[g]	Ternat-B-34DCA-JMP(pWDL7)	ND	ND	3-CA^{NC}, 3,4-DCA^{NC}	3-CA^{NC}, 3,4-DCA^{NC}	pTB18	II	100
C. testosteroni TB30	Ternat-B-3CA	AF457655	99.7 (M11224)	3-CA^{NC}, 3,4-DCA^{NC}	3-CA^{NC}	pTB30	III	99
Delftia sp. MA22	Melle-A-3CA	AF457648	97.4 (AF078774)	3-CA^{NC}	3-CA^{NC}	pMA22	IV	99
Delftia sp. MA34[h]	Melle-A-3CA	AF457649	97.3 (AF078774)	3-CA^{NC}	3-CA^{NC}	pMA34	IV	100
Delftia sp. MA14[h]	Melle-A-3CA	AF457647	97.3 (AF078774)	3-CA^{NC}	3-CA^{NC}	pMA14	IV	99
Achromobacter sp. PA4[i]	Pittem-A-3CA	ND	ND	3-CA^{NC}	3-CA^{NC}	pPA4	IV	100
Achromobacter sp. PB31[i]	Pittem-B-3CA	AF457650	97.7 (M22509)	3-CA^{NC}	3-CA^{NC}	pPB31	IV	100

[a]A and B, respectively A- and B-horizon; JMP, *R. eutropha* JMP228n; JMP(pWDL7), *R. eutropha* JMP228n(pWDL7).

[b]Accession number in the EMBL data library of the partial sequence of the 16S rRNA gene.

[c]The sequence similarity and the accession number of the closest matching type strain in the EMBL database was given. ND, no partial 16S rDNA sequence analysis was performed for these strains.

[d]3-CA, 3-chloroaniline; 3,4-DCA, 3,4-dichloroaniline. ^{N,C}Use of the compound as sole nitrogen source and sole carbon source, respectively.

[e]Plasmid groups were determined based on *Eco*RI–*Pst*I, *Hin*dIII–*Bam*HI, and *Eco*RV–*Bgl*II restriction digest patterns.

[f,g,h,i]Isolates with the same character are presumed to be identical based on very similar REP-PCR and plasmid patterns, 16S rRNA sequences, and plasmid encoded phenotypes.

as described by Mergeay and Springael [36]. Transconjugants were selected by streaking on MMN agar with 200 mg l^{-1} 3-CA, 2 g l^{-1} sodium pyruvate, 50 mg l^{-1} Km, and 100 mg l^{-1} Rif and tested for green fluorescence, the presence of a plasmid, growth on 3-CA and 3,4-DCA as a sole source of carbon (C), nitrogen (N) and energy, 3-CA or 3,4-DCA degradation, and release of stoichiometric amounts of chloride. The latter three parameters were tested in liquid MMN medium with 50 mg l^{-1} Km, and 50 mg l^{-1} 3-CA or 25 mg l^{-1} 3,4-DCA. The plasmid-free *R. eutropha* JMP228*gfp* recipient strain did not grow on or degrade 3-CA or 3,4-DCA.

2.9. Chemical analysis

3-CA and 3,4-DCA were extracted from duplicate soil samples by the addition of 3 ml methanol to 1 g of soil. After overnight shaking at 28°C the soil suspensions were centrifuged (10 min at 5000×*g*) and HPLC analysis was performed on the supernatant. This procedure was repeated for a second time on the remaining soil pellet, and the actual detectable chloroaniline concentration in the soil (mg kg^{-1} soil) was calculated based on the concentrations in the two methanol fractions. The extraction efficiency of this methanol procedure was 30 and 60% for respectively sterile A- and B-horizon of the Ternat soil. Other extraction procedures, such as extraction with water, ethylacetate, acetone and even a Likens–Nickerson steam distillation with dichloromethane [37] have been evaluated but were found to be even less efficient than the rather straightforward methanol extraction protocol. For bacterial cultures, the cells were removed by centrifugation (10 min at 5000×*g*) and the supernatant was subjected to HPLC and ion chromatograph (IC) analysis. The Summit HPLC system (Dionex, Wommelgem, Belgium) consisted of a Dionex Pump Series P580, a Dionex Autosampler Model ASI-100 (injection volume 20 μl), a STH585 Column oven (at 28°C), a Dionex UV/VIS Detector UVD 340S and a Chromeleon software system version 6.10. A Hypersil Green env column (150 mm×8 mm inner diameter; 5 μm particle size; Alltech, Deerfield, IL, USA), a mobile phase of CH_3OH/0.1% H_3PO_4 (70/30), a flow rate of 0.8 ml min^{-1}, and a UV detector set at 210 nm were used. The chloride concentration was determined by a Dionex ion chromatograph analyzer DX-600 (Dionex, Wommelgem, Belgium). The IC system consisted of a Dionex AS50 autosampler, a Dionex pump GP50, a Dionex ED50 Electrochemical Detector, an Ionpac AS9-HC column (250×4 mm inner diameter; 9 μm particle size; Dionex) with a Guard AG9-HC pre-column (80 mm×4 mm inner diameter; Dionex), a mobile phase of Na_2CO_3 (9 mM) with a flow rate of 1 ml min^{-1} and a PeakNet 6 software system version 6.10. Quantitative data of HPLC and IC analyses were obtained by comparing the peak areas of unknown concentrations with the peak areas of standards of known concentrations.

3. Results and discussion

3.1. Removal of 3-CA and 3,4-DCA from soils originating from different sites in Belgium

Within less than 2 weeks after the A- and B-horizon soils from three different sites (Ternat, Melle, Pittem, Table 3) had been spiked with 50 mg kg^{-1} 3-CA or 3,4-DCA, these compounds became undetectable by the HPLC method used (data not shown). One exception was the B-horizon from Pittem soil, where 3-CA was still detected at 5 mg kg^{-1} after 13 days, but had dropped below the detection limit by day 19. Additional microcosms with Ternat soils that were co-inoculated with *R. eutropha* JMP228 strains that contained either a 3-CA or a 3,4-DCA degradative plasmid (Tables 1 and 3), demonstrated that this inoculation did not accelerate the removal of these two compounds (data not shown). Other authors [7,38,39] have also observed this fast disappearance of chloroanilines from soil. Three possible reasons have been proposed: physical sorption (comprising surface adsorption, absorption, and migration in micro- and nanopores), chemical binding of the compounds to the soil [7,40], and degradation by indigenous soil bacteria [41]. To distinguish the contribution of biotic and abiotic processes to the disappearance of 3,4-DCA, parallel experiments with sterilised Ternat soil were performed. The concentrations of 3,4-DCA stabilised respectively in the A- and B-horizon at 15 and 31 mg kg^{-1} by day 13. This suggests that in 13 days ca. 70 and 40% of 3,4-DCA had either been transformed or become unavailable for extraction by abiotic factors in the A- and B-horizon, respectively. Since this same compound was however completely undetectable in the same non-autoclaved soil after that same incubation period, microbial processes such as biodegradation must have been in part responsible for the fast decline in detectable chloroaniline concentrations in the non-sterilised soils. These soils may thus contain single bacterial strains or microbial consortia that are able to degrade 3-CA and 3,4-DCA.

3.2. Isolation of 3-CA and 3,4-DCA degrading strains from the different soils

Enrichment cultures were set up for all 16 differently treated soils (Table 3) once the chloroanilines were no longer detected (day 14 for Ternat and Melle soil, and day 19 for Pittem soil). From all soils, except for the B-horizon of Melle, 3-CA enrichment cultures were obtained. 3,4-DCA degrading enrichment cultures were only set up for the Ternat soils – the only ones exposed to 3,4-DCA – and resulted in 3,4-DCA degradation. After plating the sixth and final 3-CA and 3,4-DCA enrichment cultures on MMN agar with the respective chloroaniline substrates as sole carbon (C) and nitrogen (N) source, all colonies with unique morphologies were tested for the

ability to degrade 3-CA and 3,4-DCA, present as the sole source of C and N in liquid MMN medium. Out of a total of 90 isolates, only 12 could use 3-CA as sole source of energy, C and N, and were obtained from all soils except the Melle B-horizon. Five of these 12 isolates could also degrade 3,4-DCA, and were all isolated from the Ternat soils (for a detailed distribution of isolates over the different treated soils, see Tables 3 and 4). The growth of the strains in liquid mineral medium with the chloroanilines as sole C and N source (as monitored by visual observation of the turbidity of the medium), the absence of accumulated ring intermediates during degradation as determined by HPLC analysis, and the release of stoichiometric amounts of chloride ions (data not shown) strongly suggest that the isolated strains are able to mineralise the molecules.

To verify whether the most dominant bacteria in the liquid enrichment cultures and on the MMN plates on which they were plated, had been isolated after purification of individual colonies, DGGE of 16S rRNA genes was applied on those different cultures. Most of the bacteria present in the enrichment cultures were found to be growing on the agar plates, and most of these were later isolated as single colonies (Fig. 1, data not shown). Since only a small fraction of the members of the enrichment cultures were found to be able to effectively degrade 3-CA and/or 3,4-DCA (only those represented in lanes 7 and 8 in Fig. 1), several cells were apparently able to grow on the MMN plates despite their inability to use the only added C and N source, i.e. 3-CA or 3,4-DCA. This can be explained by growth of these bacteria on impurities present in the agar plates, or by co-metabolism of the two chloroanilines, using impurities as carbon source. Degradation tests with isolates from the differently treated A- and B-horizon soils from the Ternat site (Table 3) indeed demonstrated that certain isolates (15 out of 62) could only degrade 3-CA in MMN medium when another C source, i.e. sodium pyruvate, was present, and thus 3-CA was only used as sole N source, probably by deamination of the aniline ring. Since we were particularly interested in isolates that can use 3-CA and 3,4-DCA as the sole C and N source, these 3-CA deaminating strains were not further examined.

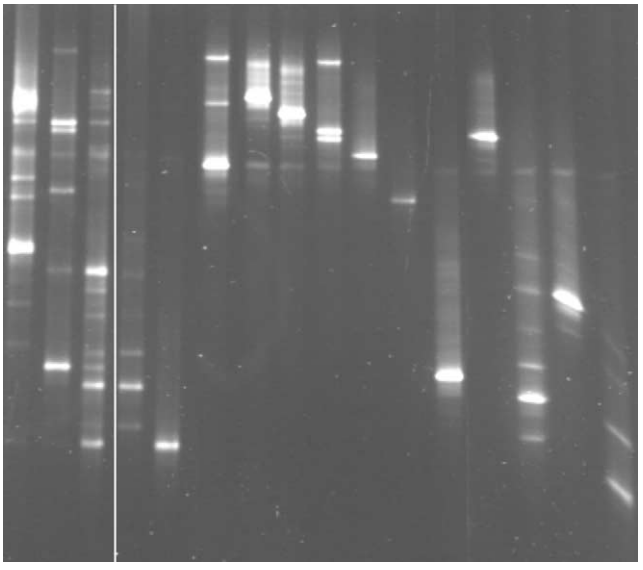

Fig. 1. DGGE analysis of plated enrichment cultures from Ternat A-horizon soil polluted with 3,4-DCA and inoculated with different *R. eutropha* JMP228n strains, and of the different isolates with unique DGGE patterns (12 out of 16) obtained from these enrichments. Lanes 1–3, DNA of a mixture of colonies growing on the isolation plates after plating the following enrichment cultures of Ternat A-horizon with 3,4-DCA: C, non-inoculated soil; J, soil inoculated with JMP228n; P, soil inoculated with JMP228n(pWDL7); lane 4, *R. eutropha* JMP228n; lanes 5–16, DNA of pure colonies of bacteria isolated from the differently treated soils. The arrow indicates a dominant bacterial species that was not obtained from the isolation plates. Bands in lanes 7, 8 and 14 respectively correspond to the 3-CA degrading strains *Pseudomonas* sp. TA8 and *Pseudomonas* sp. TA12, and *R. eutropha* JMP228n(pWDL7). Lanes 9, 10, 15, and 16 represent bacteria that can oxidatively deaminate 3-CA in the presence of 1 g l^{-1} sodium pyruvate.

Although some chloroaniline degrading strains were obtained from B-horizon soils (four isolates), most were found in the A-horizon (eight isolates). Most of the (chloro)aniline degrading strains that have been reported previously, have also been isolated from the top soil (A-horizon), except for one aniline degrading *Pseudomonas* strain that was isolated from a subsurface sediment (24 m below the surface) [18]. The higher organic matter content in an A-horizon, possibly including minor concentrations of natural chlorinated organic compounds [42], and higher probability of contact with anthropogenic chlorinated compounds may explain these findings. In a previous study, we also observed that the herbicide 2,4-dichlorophenoxyacetic acid was not at all degraded in B-horizon soil over a period of 3 weeks, while it disappeared within 2 weeks in the A-horizon of that same soil [43].

Five of our 12 isolates were able to degrade 3,4-DCA, while all of them degraded 3-CA. Until now, only four 3,4-DCA degrading bacteria have been described in literature [12–14]. The difficulty of growing bacteria on 3,4-DCA seems to be due to its toxicity [12]. Therefore, lower concentrations of 3,4-DCA compared to the other chlorinated anilines have to be used in enrichment cultures and degradation tests. Most bacteria can only degrade concentrations of 3,4-DCA up to 50 mg l^{-1}, although *Paracoccus denitrificans* 3CA can degrade concentrations of 3,4-DCA up to 150 mg l^{-1}. In comparison, the same strain can degrade concentrations of 3- and 4-CA up to 700 mg l^{-1} [12].

3.3. Identification of 3-CA and 3,4-DCA degrading strains

All strains that scored positive for the degradation of 3-CA or 3,4-DCA were identified via REP-PCR fingerprinting and partial 16S rDNA sequence analysis. A list of the 12 chloroaniline degrading bacteria with their origin of isolation and 3-CA and 3,4-DCA degradation potential

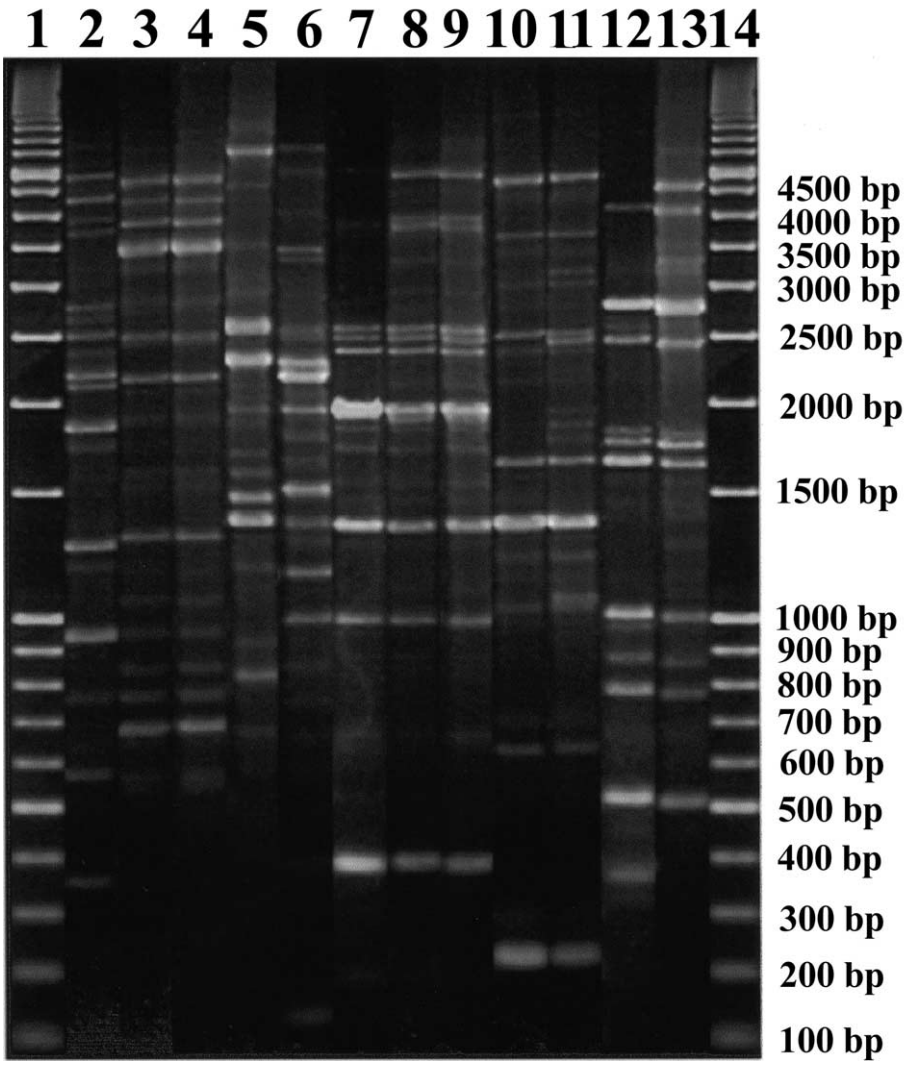

Fig. 2. Digitally reconstructed gel image of normalised REP-PCR profiles. Lanes 1 and 14, marker; lane 2, MA22; lane 3, MA34; lane 4, MA14; lane 5, TA8; lane 6, TA12; lane 7, TB18; lane 8, TB1; lane 9, TB30; lane 10, TA35; lane 11, TA2; lane 12, PA4; lane 13, PB31.

is presented in Table 4. Fig. 2 clearly shows that two isolates from Melle A-horizon (MA34 and MA14, lanes 3 and 4, respectively), yield an identical REP-PCR pattern, while the third isolate (MA22, lane 2) was clearly different. Nevertheless, all three isolates from Melle A-horizon showed nearly identical partial 16S rDNA sequences and were identified as *Delftia* sp., as the sequence similarity to the type species was $>79\%$. The REP-PCR results also showed that the three isolates from Ternat B-horizon soil (TB18, TB1 and TB30, corresponding to lanes 7–9, respectively) were genotypically very similar. The latter three isolates were identified as *C. testosteroni* as they displayed $>99\%$ 16S rDNA sequence similarity to the type strain. Furthermore, the two isolates from Ternat A-horizon soil amended with 3-CA (TA35 and TA2, corresponding to lanes 10 and 11, respectively) yielded very similar REP-PCR patterns and were identified as *Acidovorax* sp. In addition, two *Achromobacter* species with similar REP-PCR patterns were enriched from Pittem A- and B-horizon soil (PA4 and PB31, in lanes 12 and 13, respectively). Finally, some REP profiles were completely different from all other profiles (lanes 5 and 6). The corresponding strains (TA8 and TA12) were both identified as *Pseudomonas* sp., but with a relatively low internal sequence similarity (93.6%). In conclusion, the 12 chloroaniline degrading isolates seem to represent at least seven different strains, based on their REP-PCR patterns.

Although the collection of degraders was limited, identifications pointed out that the isolates could be grouped into five genera. Most isolates belong to the β subclass of the *Proteobacteria* and more particular to the family of the *Comamonadaceae*. Apparently isolates from soils collected at different locations belong to different genera while from the A- and the B-horizon of one particular soil strains were isolated that are either genotypically similar (e.g. Pittem) or completely different (e.g. Ternat). The diversity of chloroaniline degrading isolates was higher for the A-horizon (six different strains belonging to four genera;

Pseudomonas, *Acidovorax*, *Delftia* and *Achromobacter*) than for the B-horizon (two different strains belonging to only two genera; *Comamonas* and *Achromobacter*). Isolates from previous studies also mostly belonged to the β or γ subclass of the *Proteobacteria*. A lot of these chloroaniline degrading strains were identified as *Delftia*, *Comamonas* or *Pseudomonas* species [8,10,13].

3.4. Isolation and conjugative transfer of plasmids from the 3-CA and 3,4-DCA degrading strains

Since the degradation of chlorinated aromatic compounds is often plasmid encoded [44], the presence of such mobile genetic elements in the 3-CA and 3,4-DCA degrading strains was investigated. All 12 degrading isolates contained one plasmid that migrated to the same position in a 1% agarose gel. Based on *Eco*RI–*Pst*I, *Hin*dIII–*Bam*HI, and *Eco*RV–*Bgl*II restriction endonuclease digestions of plasmid DNA, these plasmids were divided into four clearly separated groups (Table 4 and Fig. 3). Group 1 consisted of plasmid pTA12 from *Pseudomonas* sp. TA12, which was isolated from Ternat-A-34DCA-JMP. Group 2 contained two plasmids that were obtained from the two identical *C. testosteroni* isolates TB1 and TB18 (Ternat-B-34DCA-JMP(pWDL7)) and from *R. eutropha* JMP228n(pWDL7), the strain that was inoculated in the Ternat soil. This suggests that plasmid pWDL7 has been transferred from the JMP228n strain to these *C. testosteroni* strains. Group 3 consisted of plasmids that were isolated from the two identical *Acidovorax* strains (Ternat-A-3CA) and from *C. testosteroni* TB30 (Ternat-B-3CA). Group 4 was the largest group and contained the previously isolated plasmid pC1-3 [8], plasmids from the three *Delftia* sp. isolates (Melle-A-3CA), *Pseudomonas* sp. TA8 (Ternat-A-34DCA-JMP), and the two *Achromobacter* strains (Pittem-A-3CA and Pittem-B-3CA). It seems that plasmids with the same restriction pattern are present in different soils and different genera. In addition, two apparently identical strains of *C. testosteroni* (TB1 and TB30) that were isolated from Ternat B-horizon after different treatments, contained two different plasmids (pTB1 and pTB30). Since pTB1 was found in the soil inoculated with JMP228n(pWDL7) and seems identical to pWDL7, this plasmid has probably been transferred from the donor into these *C. testosteroni* strains, and has displaced the indigenous plasmid pTB30 from that *C. testosteroni* strain. An alternative explanation is that both the inoculated plasmid pWDL7 and the indigenous plasmid pTB30 have transferred into the *C. testosteroni* strains during soil incubation or in the enrichment cultures.

To examine if these plasmids are involved in the degradation of 3-CA or 3,4-DCA, conjugation experiments were performed. The plasmids were transferred by triparental plate matings from the 3-CA or 3,4-DCA degrading strains to *R. eutropha* JMP228*gfp* with *E. coli* CM404, which contains the mobilising plasmid pRK2013, as a helper strain. All these plasmids, except pTA12 from *Pseudomonas* sp. TA12, seemed to encode total degradation of 3-CA. This was based on complete disappearance of the substrate and the absence of any ring structure in MMN medium inoculated with transconjugant cultures, as determined by HPLC analysis, and on the stoichiometric release of chloride ions. Only the plasmids belonging to group 2 encode degradation of 3,4-DCA (see Table 4), but they probably all represent plasmid pWDL7, which has been transferred from the inoculated JMP228n donor strain, as mentioned above. Under this assumption, no new 3,4-DCA degradative plasmids have been isolated in this study. The results show that plasmids play an important role in 3-CA degradation, and that transfer of plasmid pWDL7 seems to have occurred in the Ternat B-horizon soil that was inoculated with JMP228n(pWDL7).

This is the first report of plasmids that encode total degradation of 3-CA. Previous studies have shown that the total degradation of aniline [16,17,19,20] but not 3-CA is encoded by plasmids. Boon et al. [8] investigated the involvement of plasmids in aniline and 3-CA degradation in five strains that belong to the genera *Comamonas*

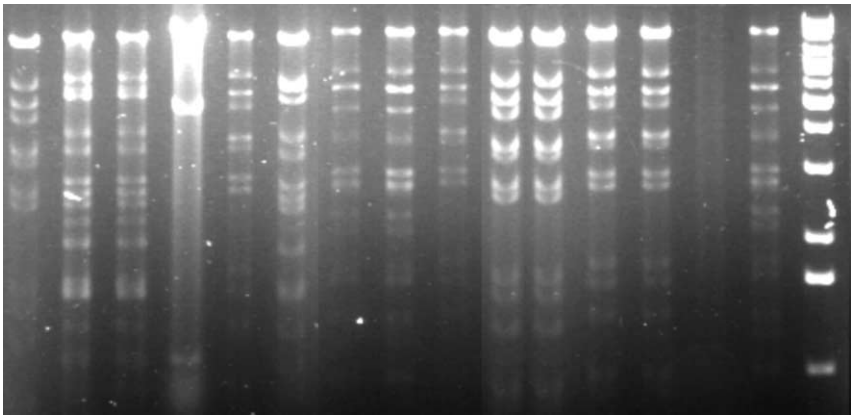

Fig. 3. *Eco*RI–*Pst*I restriction digestion analysis of plasmids present in the transconjugants of *R. eutropha* JMP228*gfp*. Lane 1, pC1-3; lane 2, pTA35; lane 3, pTA2; lane 4, pTA12; lane 5, pTA8; lane 6, pTB30; lane 7, pTB1; lane 8, pTB18; lane 9, pMA14; lane 10, pMA22; lane 11, pMA34; lane 12, pPA4; lane 13, pPB31; lane 14, JMP228*gfp*; lane 15, pWDL7; lane 16, 1 kb extended marker (Invitrogen, Life Technologies, Belgium).

and *Delftia*. Although all strains contained a plasmid, only one of these plasmids encoded only partial degradation of 3-CA (use of 3-CA as N source but not as C source, encoded by the plasmid in *D. acidovorans* CA28). This was observed using the same approach as in our study, i.e. by plate matings with *R. eutropha* JMP228*gfp* as recipient strain. None of the transconjugants in their study was able to degrade 200 mg l^{-1} 3-CA in the absence of pyruvate, as observed by HPLC analysis.

Most catabolic plasmids involved in degradation of chlorinated aromatics belong to the IncP-1 group of broad host range plasmids [44]. For the plasmids isolated in this study, PCR amplification was indeed obtained with the IncP primers *trfA2* (amplifying IncP-1α and IncP-1β plasmids), but not with the IncP primers *trfA1* (specific for IncP-1α plasmids). Sequencing of PCR fragments obtained with the *trfA2* primers revealed that the *trfA2* gene of most of the plasmids isolated in the present study had a similarity of 99–100% to the *trfA2* gene of plasmid pPS12-1 of the 1,2,4,5-tetrachlorobenzene degrading strain *Burkholderia* sp. PS12 [45] (Table 4). One exception was however the *trfA2* gene of *Pseudomonas* sp. TA12. This gene was also most similar to the *trfA2* gene of pPS12-1, but the percentage sequence similarity was only 93%. For all plasmids the *trfA2* sequence was 90% similar to that of the IncP-1β type plasmid R751 [35], except again for pTA12 (86%). In addition, Southern hybridisations of the digested plasmids under high stringency conditions with an IncP-1β-specific *trfA2* probe derived from plasmid pR751 confirmed that all plasmids belong to the IncP-1β family (data not shown).

TrfA is an initiator Rep protein involved in replication of IncP-1 plasmids, and its sequence has been used before to detect and compare IncP-1 plasmids [33]. Plasmid pPS12-1 has been shown to be an IncP-1β plasmid based on partial sequences of three replicon-specific regions, *trfA2*, k*orA* and *oriT* [45]. Just like for the plasmids in our study, the *trfA2* gene sequence of plasmid pPS12-1 showed 90–91% sequence identity to that of the IncP-1β plasmid R751. Interestingly, this plasmid pPS12-1 also codes for the degradation of a chlorinated aromatic compound, i.e. 1,2,4,5-tetrachlorobenzene, and was also found in a member of the β subclass of the *Proteobacteria*. The fact that all isolated 3-CA and 3,4-DCA degradative plasmids belong to the IncP-1β family demonstrates again [44] the importance of this group of broad host range plasmids in the degradation of chlorinated aromatic xenobiotics.

In this study several bacteria were isolated that are able to degrade 3-CA and 3,4-DCA in the absence of any other carbon and nitrogen source. Although they belong to different genera (*Comamonas*, *Delftia*, *Acidovorax*, *Achromobacter*, and *Pseudomonas*) most of them belong to the β subclass of the *Proteobacteria*. They all contained an IncP-1β plasmid that all, except one, encode 3-CA degradation, and in total, four different groups of plasmids were identified based on plasmid restriction patterns. This study has demonstrated that diverse bacterial species are able to degrade 3-CA and 3,4-DCA and has shown the importance of IncP-1β plasmids in the degradation of these compounds.

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