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Microbial Degradation of 3-Chloroanilne by two Bacterial Strains isolated from Common Effluent Treatment Plant

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Abstract The diversity has been examined of the plasmids and of the gene tdnQ, involved in oxidative deamination of aniline in three bacterial isolates that are able to metabolise both aniline and 3-chloroaniline (3-CA). Strains A and B were isolated in this study from a wastewater treatment plant and were identified as Comamonas testosterone and Delftia acidovorans, respectively. Strain C, identified as Delftia acidovorans, was isolated from a linuron-treated soil. Both Delftia and Comamonas belong to the family of the Comamonadaceae. All three strains possess a large plasmid of ca. 100 kb, but the plasmids from only 4 strains could be transferred to a recipient strain by selecting on aniline or 3-CA as sole source of carbon and/or nitrogen. Plasmid transfer experiments and Southern hybridization revealed that the plasmid of strain A encodes total aniline but not 3-CA degradation, while the plasmids of strains C and B were only responsible for the oxidative deamination of aniline. Using specific primers for the tdnQ gene, from Pseudomonas putida, the diversity of the PCR amplified fragments in the five strains was examined by denaturing gradient gel electrophoresis (DGGE). With DGGE, three different clusters of the tdnQ fragment could be distinguished. Sequencing data showed that the tdnQ sequences of A, C, B were very closely related, while the tdnQ fragment of BN3.1 and P. putida were only about 83% identical to the other sequences. Northern hybridization revealed that the tdnQ gene is only transcribed in the presence of aniline and not when only 3-CA is present.

Keywords: *DGGE, P. putida, deamination, comamonadaceae*

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1. Introduction

Chloroaniline is one of the chlorinated aromatic amines that have been extensively used in the industrial production of dyes, cosmetics, pharmaceutical products and herbicides (Kearney & Kaufmann, 1975; Latorre et al., 1984). It has been registered in the high production volume chemical program of the Organization for Economic Co-operation and Development (OECD, 1997). Not only distributed through the industrial production, 3-chloroaniline is also one of the primary intermediates predominantly generated by microbial transformation of phenylurea, acylanilide and phenylcarbamate herbicides (Zeyer & Kearney, 1982a; Haggblom, 1992). As a consequence of its intensive applications, it has been ubiquitous and accumulated in the environment including industrial effluent, sludge as well as agricultural soil. Due to its toxicity and recalcitrant properties, it has been considered as one of the important environmental pollutants (EEC, 1976; Federal Register, 1979). To dissimilate the toxic compound, bioremediation can be applied as one of the remediation technologies in which a decontamination process depends on the microbial biodegradability. Therefore, many efforts have been undertaken to isolate bacteria capable of biodegradation of chloroanilines (Surovtseva et al., 1985; Loidl et al., 1990). Although some microorganisms have been reported to have biodegradability towards chloroanilines, their limitation is that they are unable to grow on chloroanilines as sole sources of carbon and nitrogen (Helm & Reber, 1979; Reber et al., 1979; You & Bartha, 1982; Radianingtyas et al., 2003). Only recently, 3-chloroaniline-degrading Comamonas testosteroni strain I2gfp capable of utilizing 3-chloroaniline as a sole source of carbon and nitrogen was isolated (Boon et al., 2000). Nonetheless, under certain growth conditions, a few microorganisms have been reported to be able to grow on 3-chloroaniline as a sole carbon and nitrogen source. For instance, previous studies stated that the bacterial isolations were successful only after they were cocultivated or induced in the presence of aniline or another cosubstrate, e.g. glucose, for some periods of time (Zeyer & Kearney, 1982a; Radianingtyas et al., 2003; Travkin et al., 2003). This study describes the isolation

and characterization of three bacterial strains enriched and isolated from the contaminated soil of common effluent treatment plant with the capability of utilizing 3-chloroaniline as a sole carbon and nitrogen source. The present study was designed to investigate the genetic diversity of three different aniline- and 3-chloroaniline (3-CA)-degrading strains. We compared the involvement of the plasmids in these strains in the degradation of aniline and 3-CA, as well as the diversity of the *tdnQ* gene, one of the genes involved in oxidative deamination of aniline.

2. Materials & Methods

2.1. Bacterial Strains and Plasmids

Table 1. The bacterial strains and plasmids used in this study are listed

Strain or Plasmid	Characateristic	Source
Comamonas testosteroni A	AniNC, 3-CANC, RifR, HgR	This study
Delftia acidovorans C	AniNC, 3-CANC, RifR	This study
Delftia acidovorans B	AniNC, 3-CAN	This study
Ralstonia eutropha JMP228	RifR	Top E.M (1995)
Ralstonia eutropha JMP228gfp	RifR, KmR, GFP	This study
E. coli S17-1 λpir		Herrero M (1990)
Plasmids		
pNB2 (from strain A)	AniNC, HgR	This study
pNB1 (from strain C)	AniN	This study
pNB (from strain B)	AniN	This study
pUTgfp	AmpR, KmR, expresses GFP	Tombolone R. (1997)
pTDN1-3112	pUC19 + HindIII (2.59)-EcoRI (4.15) fragment, containing *tdnQ* of *P. putida* UCC22; AmpR	F. Fukumori (1997)

2.2. Media and Culture Conditions

The mineral medium MMN (Mineral Medium without Nitrogen and Carbon) is derived from mineral medium MMO (Stanier R., 1996) by elimination of all nitrogen. The MMN medium contained 1419.6 mg Na2HPO4, 1360.9 mg KH2PO4, 98.5 mg MgSO4, 5.88 mg CaCl2.2H2O, 1.16 mg H3BO4, 2.78 mg FeSO4.7H2O, 1.15 mg ZnSO4.7H2O, 1.69 mg MnSO4.H2O, 0.38 mg CuSO4.5H2O, 0.24 mg CoCl2.6H2O, 0.10 mg MoO3 and 3.2 mg EDTA in 1 L of distilled water. The liquid mineral medium was supplemented with 200 mg of aniline (Sigma-Aldrich Chemie, Steinheim, Germany) (MMN-A) or 3-CA (Fluka AG Chemische Fabrik, Buchs, Switzerland) (MMN-CA) per liter; for the solidified mineral medium, aniline and 3-CA were each used at a concentration of 500 mg/L. Sodium pyruvate (1000 mg/L of MMN medium) was added as an additional carbon source to MMN-A and MMN-CA, in order to select for bacteria by utilizing aniline or 3-CA as sole source of nitrogen (MMN-AP and MMN-CAP, respectively). LB medium containing 10 g of Bacto Peptone (Difco, Detroit, Mich.), 5 g Bacto Yeast Extract (Difco) and 5 g of NaCl in 1 L of demineralized water was used as a rich medium. These media were solidified with 2% agar for plate growth. Kanamycin (50 mg/L), rifampin (100 mg/L) and HgCl2 (10 mg/L) were added when media were cooled down to 50°C or lower.

2.3. Isolation of 3-CA-degrading microorganisms

Strain A, B & C was isolated from a wastewater treatment plant. Erlenmeyer flasks (0.25-liter capacity) containing 100 ml of activated sludge (4 g [dry weight]/L) or soil (5 g in 95 ml of MMN-CA medium) were used to select for 3-CAdegrading microorganisms over a 6-week period by adding 200 mg of 3-CA/liter at the beginning and once a week when less than 5 mg of 3-CA/liter was left in the flasks. The dry weight determination was performed by incubating a 50-ml sample at 105°C for 24 h and measuring the loss of weight after incubation (Greenber AE., 1992). Subsequently, a 0.5-liter Erlenmeyer flask containing 200 ml of MMN-CA medium (200 mg/liter) was inoculated with 2 ml of the enrichment culture. After 6 days, the second generation of the enrichment culture was transferred to fresh MMN-CA medium (1% inoculum) in a 0.5-liter Erlenmeyer flask. After 6 days of incubation, 100 µl of the culture was spread onto MMN-CA and MMN-CAP agar plates, which were incubated aerobically at 28°C for 1 week. Bacteria that were able to form colonies and that grew in liquid MMN-CA or MMN-CAP medium were regarded as 3-CAassimilating bacteria.

2.4. Cultivation of the Isolated Microorganisms

Overnight cultures in 5 ml of LB were used as inocula for degradation experiments. After 1 ml of culture was centrifuged for 5 min at 7,000 × *g*, washed, and resuspended in 1 ml of saline (0.85% NaCl), an inoculum (1% the final volume) was transferred to liquid MMN medium with the previously described concentrations of 3-CA and/or sodium pyruvate. All cultures were incubated aerobically at 28°C in the dark on a shaker (140 rpm).

2.5. Identification of the Isolates

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of whole-cell proteins was performed as previously described (Pot B., 1994). Briefly, cells were harvested from tryptic soy agar plates (BBL) after 48 h of incubation at 37°C. Protein extracts were prepared in a SDS- and beta-mercaptoethanol-containing buffer and separated on a discontinuous SDS polyacrylamide gel. The gel was then stained with Coomassie blue and scanned with an LKB 2202 Ultroscan laser densitometer (Sweden). The protein extract of *Psychrobacter immobilis* LMG 1125 was used as a standard for normalization. Numerical interpretation of the data was completed with the Gel Compar 4.1 software package (Applied Maths, Kortrijk, Belgium). DNA preparation and determination of the moles percent content guanine plus cytosine via high-pressure liquid chromatography (HPLC) were done as described by Logan et al. nonmethylated phage lambda DNA (Sigma) was used as the calibration standard. Total genomic DNA-DNA hybridizations were performed by the microplate method of Ezaki et al., using black MaxiSorp (Denmark) microplates and a HTS7000 bioassay reader (Perkin-Elmer, Norwalk, Conn.). The hybridization temperature was 55°C.

2.6. Methods for Plasmid DNA Extraction, Restriction Analysis, and Southern Hybridisation

Plasmid DNA was isolated by a modified version (Top E., 1990) of the alkaline extraction procedure for large plasmids (Kado CI., 1981). Restriction endonuclease digestion was done according to the instructions of the enzyme supplier (Switzerland). Southern hybridizations were performed at high stringency as described by Top et al. Digested plasmid DNA was separated by electrophoresis on a 0.7% agarose gel and blotted onto Hybond-N nylon membranes (Amersham International, Buckinghamshire, England). The *tdnQ* probe was prepared by PCR digoxigenin (DIG) labeling mix (Hoffmann-La Roche) according to the instructions of the supplier and using two primers designed in this study (see description of PCR amplification below) and vector pTDN1-3112 (Table 1) as a template. The IS*1071* probe was removed from vector pBRH4 (Peel MC., 1999) with *HindIII* and subsequently labeled with the DIG DNA random labeling kit. The *korA* probes for the IncP-1α and IncP-β groups were prepared by PCR labeling as previously described using plasmids RP4 (Datta N., 1971) and pJP4 (Don RH., 1981) as templates respectively.

2.7. Plate Matings

Biparental matings were performed by using LB agar plates with the donor and the recipient, *R. eutropha* JMP228*gfp*, grown separately overnight in LB. The first selection step for aniline or 3-CA-degrading transconjugants was done with liquid MMN-CA, MMN-CAP, MMN-A and MMN-AP media (5 ml in 20 ml tubes) supplemented with kanamycin (50 μg/ml). After the transconjugants showed growth in the liquid media (as observed by turbidity measurements), they were plated on the corresponding solid MMN media. Green fluorescence under UV light confirmed that potential transconjugant colonies were indeed JMP228*gfp*. Selection for transfer of HgR was performed directly on LB agar supplemented with HgCl2 (20 mg/L).

2.8. Northern Hybridization

C. testosteroni (A) was grown overnight at 28°C in LB, LB aniline (200 mg/liter), and LB-3-CA (200 mg/liter). Total RNA was extracted as described by Reddy et al. In brief, 10 ml of culture was centrifuged for 10 min. at 12,000 × g and 4°C. The pellet was resuspended in 10 ml protoplasting buffer (15 mM Tris, 0.45 M sucrose, 8 mM EDTA, 0.1% diethylpyrocarbonate [DEPC] [pH 8.0]), with addition of 80 μl of 50-mg/ml lysozyme and incubated on ice during 15 min. Subsequently, the protoplasts were centrifuged for 5 min. at 5,900 × g, and the pellet was resuspended in 0.5 ml of gram-negative bacterium lysing buffer (10 mM Tris, 10 mM NaCl, 1 mM sodium citrate, 1.5% SDS, 0.1% DEPC [pH 8.0]), incubated for 5 min. at 37 °C and chilled on ice. A 250-ml quantity of saturated NaCl (40 g NaCl / 100 ml of H2O) was added, and the solution was incubated on ice 10 min. and centrifuged at 12000 × g for 10 min. The supernatant was removed to a clean tube, 1 ml ice-cold 100% ethanol was added, and the RNA was precipitated on dry ice (30 min). Afterwards, the tube was centrifuged at 12000 × g for 15 min., and the pellet was rinsed in 70% ethanol, air dried, and dissolved in 100 μl of DEPC-treated water. Equal amounts of total RNA were loaded on a denaturing agarose gel with formaldehyde, and the gel was Northern blotted onto a Hybond-N nylon membrane (Amersham International, Buckinghamshire, England). Northern hybridization was done as described by Thomas.

2.9. Chemical Analysis

Supernatants of bacterial cultures were analyzed by reverse-phase HPLC after the cells were removed by centrifugation (10 min. at 5,000 × g). The HPLC system consisted of a Kontron liquid chromatograph with a DEGASYS DG-1310 system to degas the mobile phase, three Kontron 325 high-pressure pumps, a Kontron MSI 660 injector with a 20 μl loop, a Kontron DAD 495 diode-array detector, and a 450 MT2/DAD software system. An Alltima C18 column (250- by 8-mm inner diameter, 5-μm particle size; Alltech, Deerfield, Ill., USA) was used. The mobile phase consisted of CH3OH-0.1% H3PO4 (60:40), the flow rate was 0.75 ml/min, and the UV detector was set to 210 nm. Quantitative determination of aniline and 3-CA were done using an external standard ranging from 1 to 250 mg/liter. The detection limit was ca. 0.5 mg/L. Supernatants of bacterial cultures were analyzed for chloride content by ion chromatography after centrifugation at 5000 × g for 10 min. and filtering through a 0.45 μm filter. The DX-600 system (Belgium) consisted of a Dionex Pump GP50, a Dionex Autosampler Model AS50 (injection volume is 100 μl), a Dionex ED50 Electrochemical Detector and a PeakNet 6 software system version 6.10. Ionpac AS9-HC (250 mm × 4 mm ID; 9 μm particle size; Dionex) column and Ionpac CS12-HC (250 mm × 4 mm ID; 8 μm particle size; Dionex) were used for anion separation. The mobile phase consisted of Na2CO3 (9 mM) and methanesulfonic acid (20 mM) with a flow rate of 1 ml/min. Quantitative data were obtained by comparing the peak areas of unknown concentrations with the peak areas of standards of known oncentrations. Gas chromatography (GC)-mass spectrometry (MS) analyses were carried out with a model 2700 GC (Varian, Palo Alto, Calif.)-MAT112S (Finnigan, San Jose, Calif.) gas chromatograph-mass spectrometer equipped with a DB-1 capillary column (100% dimethylsiloxane; length, 30 m; internal diameter, 0.53 mm; film thickness, 5 μm). The temperature of the injector was 200°C, and that of the detector was 250°C. The oven temperature was programmed to increase from 40 to 220°C at a rate of 2°C/min. Helium was used as the carrier gas at a flow rate of 3.5 ml/min. Wavelength scans were recorded on a Kontron Uvikon Spectrophotometer, model 932.

2.10. PCR Amplification

For pure cultures, the template for PCR amplification was obtained by extracting total genomic DNA by the procedure of Bron and Venema. One microliter of genomic DNA solution was used in a PCR. The PCR mixture contained 0.5 μM (each) primer, 100 μM (each) deoxynucleoside triphosphate, 10 μl of 10x Expand High Fidelity PCR buffer and 2 U of Expand High Fidelity DNA polymerase (both from Switzerland), 400 ng of bovine serum albumin (Switzerland)/μl, and sterile water (Sigma-Aldrich Chemie, Steinheim, Germany) to a final volume of 50 μl. The *tdnQ* gene was amplified with

primers tdnQ1F (5'-TCC-CTG-CCT-GGA-GCC-CGAAAC-3') and tdnQ1R (5'-TCC-CGC-GCC-GTG-AGT-GAC-TG-3'). The latter were designed in this study on the basis of specific regions of the *tdnQ* sequence. The length of the expected amplified fragment was 384 bp. A GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA CGGGGGG-3') (Muyzer C., 1993) was attached to the 5' end of the tdnQ1F primer. PCR was performed with a Perkin-Elmer 9600 thermal cycler as follows: 94°C for 5 min, and then 30 cycles of 92°C for 1 min, 53°C for 1 min, and 72°C for 2 min. A final extension was carried out at 72°C for 10 min. For incompatibility group determination, *korA* primers specific for incompatibility group IncP-1 were used, and PCR amplification was performed as described previously (Gotz A., 1996). The REP-PCR was done according to Versavolic et al. (Versalovic J., 1991) to distinguish identical isolates.

2.11. DGGE

Denaturing gradient gel electrophoresis (DGGE) based on the protocol of Muyzer et al. was performed with a D Gene System (Bio-Rad, USA). PCR samples were loaded onto 8% (wt/vol) polyacrylamide gels in 1 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA [pH 7.4]). The polyacrylamide gels were made with a denaturing gradient ranging from 50 to 80% (where 100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was carried out for 5 h at 60°C and 180 V. Then, the gels were stained with SYBR GreenI nucleic acid gel stain (1:10,000 dilution; FMC BioProducts, Rockland, Maine, USA) and photographed (Boon N., 2000).

2.12. DNA Cloning and Sequencing

Putative *tdnQ* gene fragments were cloned by using a TOPO TA cloning kit (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's instructions. DNA sequencing was carried out Banagalore Genei (India). Analysis of DNA sequences and homology searches were completed with standard DNA sequencing programs and the BLAST server of the National Center for Biotechnology Information using the BLAST algorithm (5), and using the BLASTN and BLASTX programs for the comparison of a nucleotide query sequence against a nucleotide sequence database and a nucleotide query sequence translated in all reading frames against a protein sequence database, respectively.

3. Results

3.1. Isolation and Identification of 3-CA-Metabolizing Bacteria

In order to obtain single strains of 3-CA degrading bacteria from the enrichment cultures, which had been growing with 3-CA as sole carbon and nitrogen source as described above, the culture, was plated on MMN medium. The purified colonies were tested in liquid MMN-CA or MMN CAP medium with 3-CA as sole source of carbon, nitrogen and energy. Strains A, B and C were isolated as new 3-CA-metabolizing bacteria from activated sludge of common effluent treatment plant (A and B) and from linuron-treated soil (C), respectively. Two of these strains, strain A and C, (Table 1) are able to use aniline and 3-CA as sole sources of carbon and nitrogen. When aniline and 3-CA were used as sole carbon sources, all strains could degrade the compounds between 40 and 75 h (Figure 1). On LB agar plates, the strain A showed a phenotypic instability, resulting in two types of colonies with different morphology. Further purification of both types of colonies continued to yield a mixture of the both types. When aniline and 3-CA were used as sole nitrogen sources and sodium pyruvate was used as an additional carbon source, the degradation of both compounds was already completed between 14 and 24 h. No aromatic intermediates were observed by HPLC analysis. Strain B8c, on the contrary, grew with aniline as a sole carbon source but not with 3-CA (data not shown). However, it grew in MMN-CAP medium with 3-CA as a sole N source and formed a brown intermediate (Figure 2). This result was corroborated by the detection of an aromatic intermediate by HPLC analysis (Figure 2). The mass spectrum of this product, analyzed by GC-MS, was consistent with the structure of 4-chlorocatechol (Hickey, WJ., 1990). The molecular ion (M) at *m/z* 144 showed the characteristic 3:1 M/M + 2 isotope ratio of a single Cl atom. Major fragment ions had a *m/z* ratios of 126, 98, and 63 (Figure 3). The accumulation of 4-chlorocatechol in the culture of strain B is consistent with the inability of this strain to use 3-CA as carbon source, and indicates that the strain can only transform 3-CA into 4-chlorocatechol. During the growth of A in MMN medium (both liquid and solid) with 3-CA as the sole carbon, nitrogen and energy source, a yellow coloration of the medium developed and disappeared again after prolonged incubation. In order to quantify the yellow intermediary product, a wavelength scan of the medium between 200 and 400 nm was performed at different times of incubation of the growth culture (Figure 5). During the first 48 hours, no visual changes were observed and no 3-CA was degraded. After 48 h, the culture ended the lag-phase and the medium began to become yellow (Figure 4). One day later, the cells were in the logarithmic phase and the intensity of the yellow color was high. Until 77 h, this metabolite accumulated and at the same time the concentration of 3-CA decreased equally. Once the absorption peak at 380 nm had disappeared (82 h) (Figure 4 and Figure 5), the total 3-CA was metabolized. After 82 hours, an equimolar amount of chloride ions was released during the degradation of 3-CA by strain I2 (data not shown). The absorption maxima of the yellow intermediate at different pHs were examined. At pH 2, 7 and 12 the maximum absorption of the yellow intermediary product were at 323 nm, 380 nm and 378 nm, respectively.

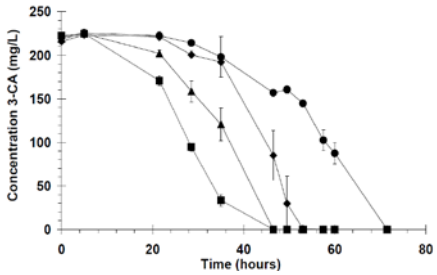

Figure 1. Degradation 0f 3-CA in MMN-CA as sole source of carbon, nitrogen, and energy in C. testosterone (♦), D. acidovorans (▲), C (●) and BN3.1 (■). Data points are averages for duplicate cultures

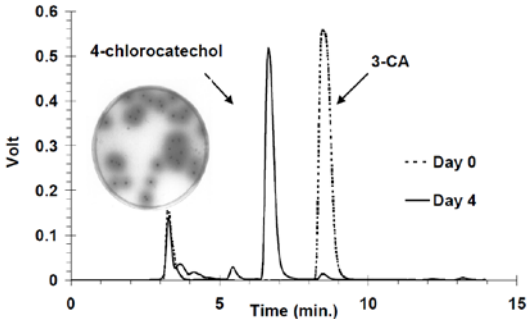

Figure 2. HPLC chromatogram for MMN-CAP medium, incubated with *D. acidovorans* B at day 0 and at day 4. The inset shows strain B grown on MMN-CAP plates. The colonies are surrounded by a brown color

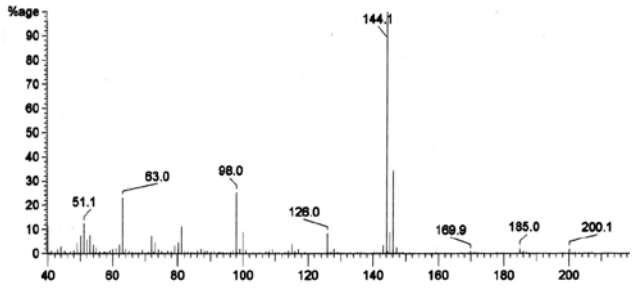

Figure 3. GC-MS mass spectrum of the brown intermediate, obtained after incubating MMN-CAP with *D. acidovorans* B

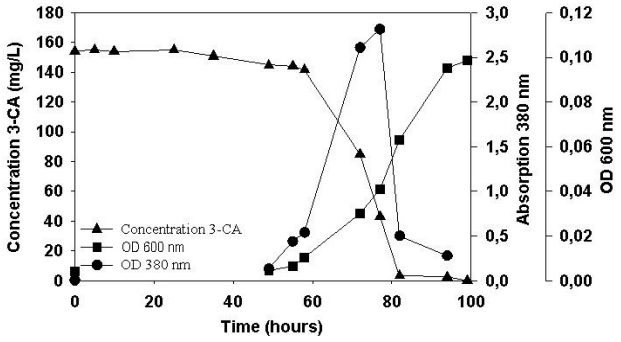

Figure 4. Growth of C. testosterone A in MMN + 3-CA during 4 days. Degradation of 3-CA(♦) in relation to the cell growth (■) and accumulation of te intermediate (peak at 380 nm) (●) is shown. The inset showa the coloration of the MMN-CA medium after 0 hours (left) and after 80 hours (right is shown)

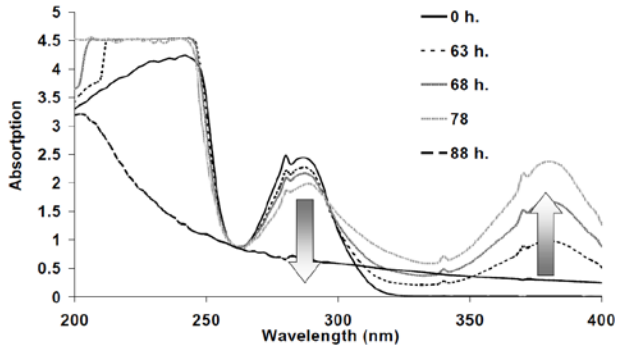

Figure 5. Wavelength scans during the growth of *C. testosteroni* A in MMN + 3-CA during 4 days. The arrows indicate the changes at a particular wavelength in function of time

3.2. Identification of 3-chloroaniline Metabolizing Bacteria

Strain A had a nucleotide composition of 62 mol% guanosine + cytosine and was identified via FAME analysis (using the commercial MIS-database) as *Comamonas testosteroni*. In DNA-DNA hybridization experiments, the strain showed a DNA reassociation of 76% with *Comamonas testosteroni* LMG 1800T (Marcus P., 1956), and was therefore identified as *Comamonas testosteroni*. Strain B has a nucleotide composition of 66.6 mol% G+C and showed 92% DNA reassociation when hybridized with LMG 1226T (292), the type strain of *Comamonas acidovorans*, which was recently accommodated in the new genus *Delftia* as *Delftia acidovorans* (332). Much lower DNA reassociation values of 28 and 32% were found when strain B was hybridized with *Comamonas testosteroni* LMG 1800T (Tamaoka J., 1987) and *Comamonas terrigena* LMG 1253T (De Vos p., 1985), respectively. Since strains B and C showed identical SDS-PAGE patterns of whole cell proteins, both isolates were unambiguously identified as *Delftia acidovorans*.

3.3. Involvement of the Plasmids in the Degradation

Extraction of plasmids from strains C, A, B revealed that they all contained a plasmid with a size of ca. 100 kb, designated pNB1, pNB2, pNB respectively. An *EcoR*I-*Pst*I digest of the plasmids revealed different restriction patterns (Figure 6A). All plasmids yielded an amplification product after PCR with the *korA* primers, which are specific for the IncP-1 group of broad-host-range plasmids. Southern hybridization of these *korA* PCR products with RP4 (IncP-1α) - and pJP4 (IncP-1β)-generated probes revealed that plasmids pNB1, pNB2, pNB hybridized with the IncP-1β-derived probe; pNB also hybridized with the IncP-1α-derived probe; and pB1 did not hybridize with either of the two probes, although an amplification product had been obtained (data not shown). Four of the five plasmids clearly belong to the IncP-1 group; three of them appear to be IncP-1β plasmids. To determine if some of the catabolic genes were plasmid encoded, conjugative transfer of the aniline or 3-CA degradation phenotype from strains A, C, B.1 to the recipient strain *R. eutropha* JMP228*gfp* was examined. From these mating experiments different plasmid-encoded functions could be derived, as summarized in Table 1 and Table 2. All transconjugants, except for that from the mating with *D. acidovorans* BN3.1 used aniline as nitrogen source; however, only JMP228*gfp* (pNB2) degraded aniline completely in MMN-A medium and thus used it as sole carbon source as well. As determined by HPLC, the other transconjugants, which can use aniline only as nitrogen and not as carbon source, transformed only 50% of the added aniline in MMN-A medium. This result could have been due to the toxic effect of accumulated intermediates, such as catechol. A brown color, usually caused by polymerization products of catechol, was indeed observed in these cultures. (Figure 8). When the three types of transconjugants were grown in MMN-AP, the medium colored brown, probably due to the formation and accumulation of catechol and its polymerization products. This result was not observed when transconjugants JMP228*gfp* (pC1-1) and JMP228*gfp* (pC1-3) were grown in the MMN-CAP. Plasmid extraction with *EcoR*I-*Pst*I-digestion and PCR with the *korA*-primers for IncP-1 plasmids revealed the presence of

the different plasmids in the JMP228*gfp* transconjugants. The plasmids in the donors and the respective transconjugants had identical restriction patterns (data not shown). Also no differences in restriction patterns between pC1-1, pC1-2 and pC1-3 were visible. Conjugation between *D. acidovorans* BN3.1 and *R. eutropha* JMP228*gfp* did not yield any transconjugants that were able to metabolize aniline or 3-CA. *C. testosteroni* I2, which was the only mercury resistant strain, could transfer this mercury resistance to JMP228*gfp*, indicating that the resistance gene is also located on plasmid pNB2 (Table 1 and Table 2). If the aniline- and/or 3-CA-degradative genes located on the plasmids have enough similarity with some of the cloned and sequenced *tdn* genes of *P. putida* UCC22 involved in the oxidative deamination of aniline (F. Fukumori., 1997), we should be able to confirm their localization on the plasmids by hybridization. Therefore, primers were designed and a probe was developed for one of the genes, *tdnQ*. Restriction digestion of plasmids pNB2, pNB1, pNB and pC1, showed a clear hybridization signal of a 1.4-kb fragment after Southern hybridization with the *tdnQ*-probe (Figure 6B). Only with plasmid pB1 no hybridization signal was obtained. The probe derived from IS*1071* also hybridized with a 1.4-kb fragment (Figure 6C). This probe also hybridized to an additional 2.7-kb fragment of plasmid pNB2 as well as to a large fragment (ca. 17 kb) of plasmid pB1. The large fragments of plasmids pNB is due to incomplete digestion (Figure 6C). The data show that *tdnQ*-like genes, very similar to *tdnQ* of *P. putida* UCC22, are located on all four plasmids that could transfer the capacity to deaminate aniline and/or 3-CA by conjugation.

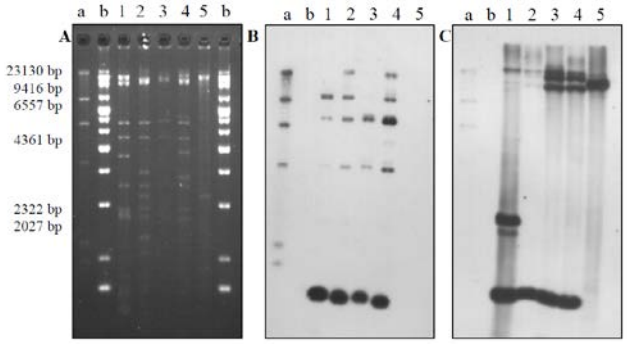

Figure 6. Restriction digestion analysis and Southern hybridization. (A) Analysis on a 0.7% agarose gel of *Eco*RI-*Pst*I-digested plasmids. (B and C) Hybridization with *tdnQ* (B) and IS*1071* (C). Lane 1, *C. testosteroni* A (plasmid pNB2); lane 2, *D. acidovorans* C (plasmid pNB1); lane 3, *D. acidovorans* B (plasmid pB8c); lane 4, *D. acidovorans* CA28 (plasmid pC1); lane 5, *D. acidovorans* BN3.1 (plasmid pB1); lane a, DIG-labeled Marker II (Hoffmann-La Roche); lane b, 1-kb extended marker. The Southern blot in panel C was obtained from a gel different from that shown in panel A and B, but containing the same DNA samples

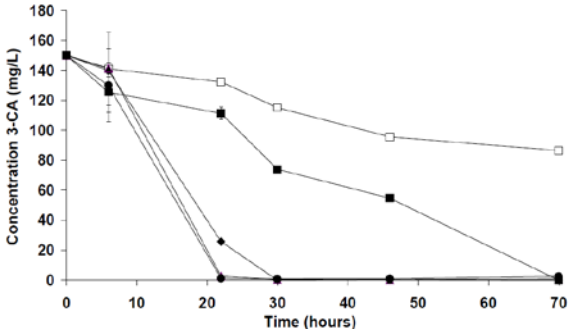

Figure 7. Degradation of 3-CA in MMN-CAP as sole source of nitrogen by D. acidovorans CA28 (♦); JMP228gfp (□); JMP228gfp (pC1-1) (▲); JMP228gfp (pC1-2) (■) and JMP228gfp (pC1-3) (●). Data points are averages of duplicate cultures and error bars represent standard deviations

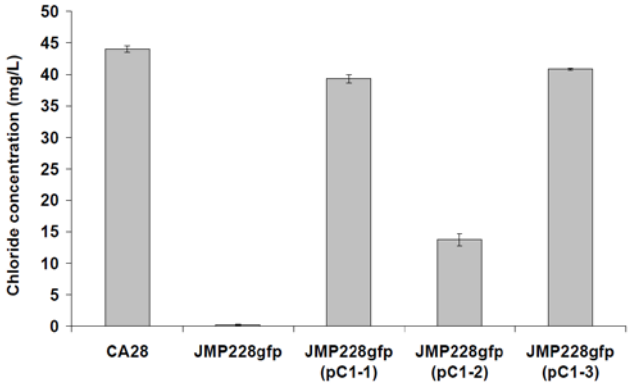

Figure 8. Chloride release after 70 hours if incubation of 3-CA in MMN-CAP as sole source of nitrogen by D. acidovorans CA28 (♦); JMP228gfp (□); JMP228gfp (pC1-1) (▲); JMP228gfp (pC1-2) (■) and JMP228gfp (pC1-3) (●). Data points are averages of duplicate cultures and error bars represent standard deviations

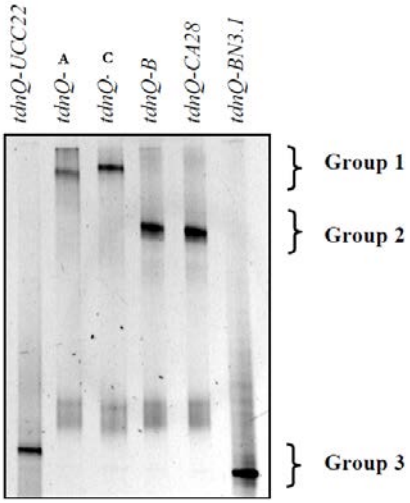

Figure 9. DGGE analysis of *tdnQ* fragments of different strains capable of degrading aniline and 3-CA

Table 2. Plasmid encoded properties, determined by conjugation experiments with the different isolates and *R. eutropha* JMP228*gfp*. The composition of the different MMN media are listed in the material and methods section

Isolate (plasmid)	MMN-CA	MMN-CAP	MMN-A	MMN-AP	Hg
A(pNB2)	-	-	+	+	+
C(pNB1)	-	-	-	+	-
B(Pnb8C)	-	-	-	+	-
CA28(pCl)	-	+	-	+	-
BN3.1(pBl)	-	-	-	-	-

+= growth, -= no growth; Hg: mercury resistant

Table 3. Levels of nucleotide and amino acid sequence identities for the 384-bp amplified portion of the *tdnQ* genes of aniline- and 3-CA-degrading bacteria and for database sequences

Strain	%Nucleotide or amino acid identity with:[a]						
	C. testosterone A	D. acidoborans C	D. acidoborans B	D. acidoborans CA28	D. acidoborans BN3.1	p. putida UCC22 b	Acinetobacter sp YAA b
C. testosterone A	-	95/95	88/88	95/95	88/91	89/91	68/81
D. acidoborans C	98	-	91/92	98/98	92/95	91/95	69/82
D. acidoborans B	94	95	-	93/93	86/89	86/89	67/81
D. acidoborans CA28	98	98	96	-	92/94	92/97	68/81
D. acidoborans BN3.1	83	84	80	84	-	99/100	70/84
p. putida UCC22[b]	83	83	81	84	99	-	69/84
Acinetobacter sp YAA[b]	NSS	NSS	NSS	NSS	NSS	NSS	-

[a]Single entries indicate nucleotide identities. Double entries indicate amino acid identities/positives. NSS, no significant similarity was found.
[b]Sequences were obtained from GenBank.

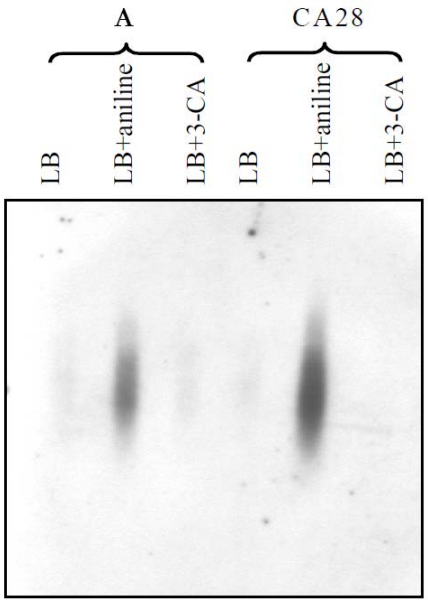

Figure 10. Hybridization with *tdnQ* of the total RNA of *C. testosteroni* A and *D. acidovorans* CA28, grown in LB, LB-aniline and LB-3-CA

3.3. Comparison of Partial *tdnQ*-Sequences.

To investigate the diversity of the *tdnQ*-like genes in the five strains, PCR amplification with *tdnQ* primers, including one GC clamp, was performed. All the anilineand 3-CA-metabolizing strains A, C, B, as well as the positive control (the vector pTDN1-3112) yielded a PCR amplification product of the expected length of 384 bp. An initial comparison of the sequences of the amplified fragments was done via DGGE analysis. A 50% to 80% gradient of denaturing agents resulted in the best separation of the fragments of the different strains. The *tdnQ* fragments were clearly not identical and could be classified in three groups (Figure 9). The first group, *tdnQ* from *C. testosteroni* I2 (*tdnQ-I2*) and from *D. acidovorans* C (*tdnQ-C*), was denatured at rather low denaturant concentrations (upper part in the gel) and a very small difference in migration between the two fragments was observed. The second group, *tdnQ* from *D. acidovorans* B (*tdnQ-B*) was localized at higher concentrations of denaturing agents (middle of the gel) and seemed to migrate at the same rates. The fragments of the original *tdnQ* gene of *P. putida* (*tdnQ-UCC22*) both migrated to the bottom of the gel at the highest denaturant concentration and thus formed the third group. While the difference in migration positions between the last two groups of PCR fragments was large, there was only a small difference between the first and the second groups. To confirm the sequence differences of these *tdnQ* fragments, all 5 PCR-products (without GC clamp) were cloned and subsequently sequenced. Analysis of the DNA sequences is summarized in Table 3. The partial *tdnQ* genes of the five strains were related to the partial *tdnQ* gene of *P. putida* UCC22. While the sequence of the *tdnQ-BN3.1* fragment was nearly identical to that of the *tdnQ-UCC22* fragment, the sequences of the *tdnQ-A*, *tdnQC*, *tdnQ-B* fragments were only 80-84% similar to that of the *tdnQUCC22* fragments. When the partial sequences were translated to amino acid sequence level, all the sequences were found to be highly similar to the amino acid sequence of the Tdn Qprotein of *P. putida* UCC22 (F. Fukumori., 1997) and less similar to a component of an aniline dioxygenase (glutamine synthetase like protein) of *Acinetobacter* sp. strain YAA (Fujii T., 1997) (Table 3). Only the last 306 bp of the 384 bp *tdnQ* fragment was translated into the partial protein structure of 102 amino acids. The comparison of the DGGE and sequencing results showed that the DGGE approach can be useful for specifically amplifying and analyzing *tdnQ*-like genes from mixed cultures.

3.4. Differential Expression of *tdnQ*

In order to investigate the role of *tdnQ* in the degradation of 3-CA, strains *C. testosteroni* I2 and *D. acidovorans* CA28 were grown in LB, LB -aniline, and LB-3-CA. No traces of aniline and 3-CA could be detected at the time of cell collection, prior to RNA extraction. This result indicates that at least one or several genes involved in the initial transformation steps had been transcribed. Total RNA was blotted and hybridized with the *tdnQ*-probe (Figure 10). With both strains, only the RNA that was extracted from the cells grown with aniline hybridized with the *tdnQ*-probe. These results suggest that under the conditions used here the *tdnQ* gene in these strains is induced by aniline or its metabolites (F. FUkumori., 1997) but not by 3-CA or its metabolites. This notion implies that oxidative deamination of 3-CA in

these strains may involve different genes than those responsible for aniline degradation.

4. Discussion

Several bacterial species are known to degrade 3-CA (Brunsbach FR., 1993, Latorre., Loidl., 1990, Surovtseva., 1985, Surovtseva., 1996, Zeyer., 1982). Strains A, C and B, isolated and described in this study, are three new strains of the species *Comamonas testosteroni* and *Delftia acidovorans* that are able to metabolize aniline and 3-CA. *C. testosteroni* A, and C show similar metabolic capacities. *D. acidovorans* B is not able to use 3-CA as sole carbon source and thus unable to degrade 3-CA completely. This situation leads to accumulation of 4-chlorocatechol in the medium. During the dioxygenation of 3-CA, theoretically two different intermediates may be formed, e.g., 3-chlorocatechol and 4-chlorocatechol (Latorre., 1984). The mass spectrometry results in this study, together with data from the literature (Loidl., 1990, Schukat., 1983, Zeyer., 1985), suggest that *D. acidovorans* B degrades 3-CA preferably up to 4-chlorocatechol. *D. acidovorans* C showed no accumulation of chlorinated catechols, probably because of the high level of activity of a chlorocatechol dioxygenase (Loidl., 1990). Strain A, isolated and described in this study, is is able to use 3-chloroaniline as the sole source of carbon and nitrogen. This species, which is often isolated from activated sludge, is resistant to starvation (McClure., 1989) and has been reported to be involved in the degradation of many aromatic products, such as (chloro)phenols (Arai., 1998, Bae., 1997), p-toluenesulfonic acid (Balashov., 1997), polychlorinated biphenyls (Barriault., 1993) In pure culture, complete degradation was achieved at 3-CA concentration of 200 mg/L and was coupled with quantitative liberation of chloride. During the degradation of 3-CA, a yellow intermediate accumulated temporarily, which was further metabolized, thus indicating total metabolism of the aromatic amines. The chlorinated catechols seem to usually be degraded by a modified *ortho*-cleavage pathway (Hinteregeer., 1992). In our study, the λmax values at the different pHs were very similar. This comparison suggests that the degradation of 3-CA by *C. testosteroni* A also occurs by means of a distal *meta*-cleavage pathway for chlorocatechol. This is in contrast with the case for most other chloroaniline degraders, which have been shown to degrade 3-CA via modified *ortho*- leavage pathway (132, 340). Only one study, by Surovtseva et al., mentioned a *meta*-cleavage of monochloroanilines by *Alcaligenes faecalis*; however, it is not clear if this cleavage was metabolic or co-metabolic. Recently Walter Reineke (personal communication) determined that our strain A is able to use 4-chlorocatechol. This information together with the detection of the yellow chlorohydroxymuconic semialdehyde would indicate that the strain employs a proximal or distal *meta*-cleavage pathway (data not shown). All the strains that were investigated harbor a large plasmid. However, no differences in plasmid restriction patterns of the different transconjugant colonies could be shown. The cause of these differences is currently not yet understood. When these transconjugants were grown in MMN-CAP, no formation of chlorocatechol was noticed. However, complete mineralization of 3-CA apparently did not occur, since 3-CA could not be used as carbon source. This result suggests that an aliphatic intermediate accumulated after ring cleavage and dechlorination. This aliphatic intermediate would likely have a six-carbon backbone because release of any carbon should support some growth. It is clear that none of the plasmids codes for complete 3-CA mineralization. Plasmid pNB2, on the other hand, was the only plasmid that confers complete degradation of aniline in *R. eutropha* JMP228*gfp*, allowing the strain to use the compound as sole carbon source. This plasmid of *C. testosteroni* A is thus a new aniline catabolic plasmid, which also encodes mercury resistance. The observation that 3 plasmids could transfer the ability to use aniline but not 3-CA as sole nitrogen source suggests that the genes carried on these plasmids are insufficient for the oxidative deamination of 3-CA. This suggestion leads to the hypothesis that other, asyet-unknown chromosomally located genes are required for deamination of 3-CA. In order to confirm the involvement of the plasmids in aniline and/or 3-CA degradation, hybridization experiments were performed with a *tdnQ* probe. This particular gene was chosen as a representative of the *tdn* genes for several reasons. First, after primers were designed for *tdnQ* and *tdnA*, based on their sequences in the database, the only set that yielded amplification with the 5 strains was the set of *tdnQ* primers. An additional advantage was that the cloned *tdnQ* gene of *P. putida* UCC22 was provided to us by F. Fukumori, allowing us to make a *tdnQ* probe by PCR labeling. The other genes, such as *tdnA2* and *tdnT*, were smaller and did not allow effective primer design. Out of the five plasmids, the four that were able to transfer the ability to use aniline as a nitrogen source also hybridized with the *tdnQ* gene. Only plasmid pB1 of *D. acidovorans*, which could not transfer the ability to transform aniline and/or 3-CA, did not yield a hybridization signal. This result suggests that either plasmid pB1 does not carry the catabolic genes, or it carries catabolic genes involved in aniline or 3-CA with lower sequence similarity to *tdnQ*, and it is not conjugative. All plasmids in this study belong to the IncP-1 incompatibility group, and most of them could be assigned to the IncP-1β subclass by *korA* primers and probes. The incompatibility group and host range of several other catabolic plasmids are still not known. Interestingly, most plasmids involved in degradation of chlorinated aromatics and for which the incompatibility group has been determined seem to belong to the IncP-1 group (often even IncP-1β), known to contain plasmids with a very broad host range (301). Examples are pJP4 (2, 4-dichlorophenoxyacetic acid and 3-chlorobenzoic acid), pAC25 (3-chlorobenzoic acid), pBR60 (3-chlorobenzoic acid) and others (Top EM., 2000). In our study, the only plasmid which yielded a *korA* PCR product that did not hybridize with the IncP-1α- or the IncP-1β-derived probe was plasmid pB1 from *D. acidovorans*. This result could mean that plasmid pB1 belongs to incompatibility group other than IncP-1, with a more restricted host range. Interestingly pB1 is also the only one of the five plasmids that did not allow conjugative transfer of the 3-CA- or aniline-transforming phenotype and which did not hybridize with the *tdnQ* probe. Results of recent studies have shown that a variety of catabolic genes and operons

are flanked by insertion elements (Di Gioia., 1998). IS*1071* is an insertion sequence that has been found to bracket the class II transposable element Tn*5271*, first described for the 3- and 4-chlorobenzoate-degrading strain *Alcaligenes* sp. strain BR60 (Nakatsu., 1991). Fulthorpe and Wyndham observed that after the introduction of this host strain in lake water and sediment microcosms exposed to 4-chloroaniline, IS*1071* was mobilized into different strains and was found in a plasmid unrelated to the donor, pBRC60. Also, in our study, insertion sequences strongly related to IS*1071* were detected on the plasmids of the aniline- and 3-CA-degrading strains, probably on the same restriction fragment as the *tdnQ* gene. This finding suggests that in our strains, *tdnQ* is flanked by an insertion sequence fragment of the group IS*1071*. Furthermore, Fujii et al. found the transposase gene sequence of Tn*1000* on the aniline catabolic plasmid pAS185 of *Acinetobacter* sp. strain YAA. These findings suggest that during bacterial evolution, the genes responsible for aniline degradation have been spread by horizontal transfer aided by transposons, such as Tn*5271*. The additional hybridization signal of a 2.7-kb fragment of plasmid pNB2 with the IS*1071* probe could be related with the plasmid-encoded mercury resistance. This observation corroborates with the findings of Pearson et al., who observed that class II transposase genes are often associated with mercury resistance genes (*mer* genes). The classification of *tdnQ*-like gene fragments in three groups, based on their rates of migration in the DGGE gel (Figure 9), did not correspond entirely with the degree in sequence similarity between the cloned fragments (Table 3). This situation is to be expected, since fragments with different DNA sequences may sometimes end up at the same location in the DGGE gel, while in many other cases, a 1-bp difference can be sufficient to separate two sequences (Felske., 1999). A comparison of DGGE and sequencing data demonstrates, however, that there was sufficient variation at the DNA sequence level to separate the different *tdnQ* like genes in the DGGE gel. This DGGE approach, applied to the total DNA from various environmental habitats, could be especially useful for further investigation of the diversity of *tdnQ*-like genes and other catabolic genes in microbial communities without prior cultivation of the degrading organisms. Interestingly, different *tdnQ* sequences were found in strains of the same species, while almost identical sequences were detected in 2 strains of different genera (*tdnQ-A* and *tdnQ-C* or *tdnQ-BN3.1* and *tdnQ-UCC22*). These results suggest again that horizontal gene transfer has played a role in the evolution of (chloro) aniline-degrading bacteria. None of the obtained *tdnQ* nucleotide sequences was related to the sequence of the aniline dioxygenase gene (glutamine synthetase-like protein) *of Acinetobacter sp.* YAA (Fujii., 1997), while there was a good relationship at the level of the amino acid sequence. The *tdnQ*-primers were probably too specific to detect possible genes responsible for oxidative deamination of 3-CA in the strains. Work to identify the latter genes and their diversity within chloroaniline-degrading bacteria warrants further research. In the present study and in previous reports (Latorre., 1984), the relationship between the degradation of aniline and its chlorinated analogue, 3-CA has been mentioned. However, it is not clear if the genes and enzymes responsible for the transformation of aniline and 3-CA into chlorocatechol (oxidative deamination) are also different. Some anilinedegrading bacteria were able to transform 3-CA into chlorocatechol, but these bacteria needed aniline or glucose as a cosubstrate and the cells had to be preincubated with aniline (Reber., 1979, Schukat., 1983). On the one hand, evidence in support of the hypothesis that the oxidative deamination of aniline and its chlorinated analogue is performed by the same enzyme, was given by the work of Latorre et al. (Latorre., 1984). The authors obtained 2-chloroaniline-, 3-CA-, and 4-chloroaniline-degrading bacteria by natural gene exchange between an aniline- or toluidinedegrading *Pseudomonas* strain and chlorocatechol assimilating *Pseudomonas* sp. B13. Hybrid organisms were isolated through cocultivation of the parent strains in a chemostat as well as through conjugation on solid media in the presence of chloroanilines as selective substrate. This information suggests the existence of at least two different sets of enzymes, one that can transform only aniline and another that can transform both aniline and 3-CA. In our study, *C. testosteroni* A and *D. acidovorans* B and C could transfer the genes encoding the oxidative deamination of aniline, while genes encoding the oxidative deamination of 3-CA could not be transferred. These findings, together with the differential transcription of the *tdnQ*-mRNA (Figure 10), strongly suggest that two different sets of genes are involved in the oxidative deamination of aniline and 3-CA. This work has shown that the catabolic plasmids and the *tdnQ* genes involved in oxidative deamination of aniline in five strains of the family *Comamonadaceae* are quite diverse. We described a new plasmid encoding complete aniline degradation and two plasmids that code for the partial oxidative deamination of aniline. The importance of IncP-1 plasmids and insertion sequence elements in the spread of catabolic genes was confirmed. Increasing the understanding of new catabolic plasmids for future studies on the bioaugmentation of polluted environments is warranted.

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