African Journal of Biotechnology Vol. 10(55), pp. 11567-11573, 21 September, 2011
Available online at http://www.academicjournals.org
DOI: 10.5897/AJB10.759
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Characterization of 1,2-dichloroethane (DCA) degrading bacteria isolated from South African waste water

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Accepted 13 April, 2011

1,2-Dichloroethane (DCA), a potential carcinogen that is toxic to both terrestrial and aquatic ecosystems, is one of the most widely produced chemicals in the modern world. It is used as a metal degreaser, solvent, chemical intermediate and fuel additive. Contamination of the environment with DCA results from accidental spills and poor handling. To date, several bacterial isolates, capable of utilizing this compound as a sole carbon and energy source, have been identified in the northern hemisphere. This report focused on the isolation and characterization of bacterial isolates in the southern hemisphere that are capable of degrading DCA. Samples obtained from a waste water treatment plant in Durban, South Africa were batch cultured in minimal medium containing DCA and repeatedly sub-cultured every five days over a 25 day period. A halogen release assay was performed in order to determine whether individual isolates possessed dehalogenase activity. Confirmation of DCA utilization by bacterial isolates that were positive for dehalogenase activity was done by sub-culturing back into minimal medium containing DCA. It was found that five isolates possessed an identical hydrolytic dehalogenase gene following the design of primers based on known hydrolytic dehalogenase genes. Analysis of 16S rDNA sequences indicated that, all the South African isolates belonged to the genus *Ancylobacter* and were different from each other.

Key words: 1,2- dichloroethane, halogenated hydrocarbon, xenobiotic, dehalogenase.

INTRODUCTION

Halogenated organic compounds constitute one of the largest groups of environmental pollutants as a result of their widespread use as herbicides, insecticides, fungicides, solvents, hydraulic and heat transfer fluids, plasticizers and intermediates for chemical synthesis. Due to their toxicity, bioconcentration and persistence, the ubiquitous distribution of halogenated compounds in the biosphere has caused public concern over the possible effects on the quality of life (Fetzner and Lingens, 1994).

The biosphere contains a multitude of halogenated organic compounds, more than 2400 of which have been identified as occurring naturally; however, those constituting the bulk quantities are synthesized industrially (Hill et al., 1999). Many halo-organic compounds have been categorized as priority pollutants, even though a wide range of bacterial species that can degrade such substances and in many cases utilize them as sole sources of carbon and energy have been isolated in laboratory culture (Fetzner and Lingens, 1994). Being able to withstand the recalcitrance of halo-organic compounds in the biosphere, microbial catabolism is clearly a major latent route by which these compounds may be detoxified and recycled. Therefore, there is the need to understand much more about the process of microbial adaptation involved in the degradation process in order to harness this potential (Hill et al., 1999).

The suspected carcinogenic solvent 1,2-dichloroethane (DCA) is the most abundant chlorinated groundwater pollutant (Hunkeler et al., 2005). DCA is a xenobiotic compound used in the degreasing of metals as well as in the production of various plastics. This compound is not known to be degraded naturally in the environment and its low solubility in water allows it to contaminate groundwater supplies, thus, affecting all forms of life.

Currently, several bacterial isolates have been dis-

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Table 1. Hydrolytic dehalogenase producing-bacteria used in this study.

Bacterial strain	Source
P. pavonacae 170 formerly *P. cichorii* 170	D. B. Janssen*
R. erythropolis NCIMB13064 formerly *R. rhodochrous* NCIMB13064	D. B. Janssen*
A. aquaticus AD25	D. B. Janssen*
A. aquaticus AD27	D. B. Janssen*
X. autotrophicus GJ10	D. B. Janssen*

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covered that are capable of DCA utilization as a sole carbon and energy source (Janssen et al., 2005).

Microorganisms are very versatile, diverse and possess the ability to easily adapt to new environments which makes them the most suitable candidates to redirect xenobiotic compounds into biogeochemical cycles. In recent years there has been a flurry of research activities to isolate new microorganisms that possess unusual catabolic activities that can be used to degrade anthropogenic compounds. However, some compounds have been shown to be unusually recalcitrant that is, microorganisms either do not metabolize these compounds or do not possess the necessary enzymes to transform these compounds (Eyers et al., 2004).

This study focused on the characterization of DCA degrading bacteria from different waste water treatment works in the KwaZulu-Natal region, South Africa by 16S rDNA sequencing. It also described the design of primers that may be used to PCR amplify specific hydrolytic dehalogenase genes as well as the hydrolytic dehalogenase genes that these new DCA degrading isolates possess.

MATERIALS AND METHODS

Bacterial strains used in this study

Table 1 lists known hydrolytic dehalogenase producing bacteria that were used as controls in this study.

Sample collection

The Northern Sewage Treatment Works (Durban, KwaZulu-Natal, South Africa) was identified as one of the waste water facilities that handled large quantities of industrial waste water. Samples were collected in sterile schott bottles from various treatment points which included the settling tank, activated sludge treatment, secondary clarifier and anaerobic sludge digester. Samples were also collected from SAPPI aerated paper treatment works and SAPPI bleach plant (South African Pulp and Paper Industry, KZN). All samples were transported on ice and stored at 4°C until further use.

Media and growth conditions

Minimal salts medium (MMV) was prepared per liter as follows: 1.36 g KH_2PO_4; 5.37 g $Na_2HPO_4.12H_2O$; 0.5 g $(NH_4)_2SO_4$; 0.2 g $MgSO_4.7H_2O$ (sterilized by autoclaving at 121°C for 15 min) and 5 ml of a trace metal solution (added after sterilization). The trace metal solution contained per liter: 530 mg $CaCl_2$; 200 mg $FeSO_4.7H_2O$; 10 mg $ZnSO_4.7H_2O$; 10 mg H_3BO_3; 10 mg $CoCl_2.6H_20$; 4 mg $MnSO_4.5H_2O$; 3 mg $Na_2MoO_4.2H_2O$ and 2 mg $NiCl_2.6H_2O$. The trace metal solution was filter sterilized and stored at 4°C. Prior to inoculation, MMV was supplemented with 1 ml vitamin stock solution which contained per liter: 12 mg biotin; 1 g choline chloride; 1 g calcium (D)-pantothenate; 2 g i-inositol; 1 g nicotinic acid; 1 g pyridoxine chloride; 1 g thiamine chloride; 0.2 g *p*-aminobenzoic acid and 0.01 g cyanocobalamin (Janssen et al., 1984). Prior to inoculation, the pH of the medium was adjusted to 7. This medium was supplemented with 5 mM DCA (final concentration) which served as the carbon and energy source. The volume of the complete medium was adjusted to 1 L with deionised water. 50 ml of the minimal salts medium was then aliquoted into 250 ml schott bottles with air tight viton rubber seals. Each bottle was inoculated with 1% inoculum from each of the sources. Bottles were incubated at 30°C with shaking. Fresh medium was inoculated with 1% inoculum from these bottles every five days over a 25 day period.

Isolation of pure cultures

Following 25 day enrichment, 100 µl of culture was spread over minimal salts agar medium. The agar medium (1L) was prepared by first sterilizing 1.36 g KH_2PO_4; 5.37 g $Na_2HPO_4.12H_2O$; 0.5 g $(NH_4)_2SO_4$; 0.2 g $MgSO_4.7H_2O$ together with 12 g bacteriological agar. The medium was allowed to cool to approximately 45°C before the trace metal and vitamin solutions were then added and poured into 90 mm Petri plates. After inoculation, the plates were inverted and 20 µl of DCA was added to filter paper discs placed on the inside surface on the lid (Janssen et al., 1989). This allowed for the creation of a DCA atmosphere. Plates were sealed with cellophane tape and incubated at 30°C up to five days until colonies appeared. Single colonies were transferred to Luria Bertani (LB) agar (10 g tryptone, 5 g yeast extract and 12 g bacteriological agar per liter) plates without the addition of NaCl as the presence of NaCl produces false results in the halide release assay.

Halide release assays

In order to determine whether the isolates possessed the ability to release free halide from DCA, a simple qualitative colorimetric assay was performed. To a standard clear round bottom microtitre plate 100 µl of 50 mM Tris-sulphate (pH7.5) containing 5 mM 1,2-dibromoethane was added. Individual colonies were picked up using a needle and emulsified into the substrate solution. The plate was incubated for 1 h at 30°C. Following incubation, 100 µl of 0.25

Table 2. Primers designed for the amplification of hydrolytic dehalogenases.

Primer name	Gene used in alignment	Sequence of primer* (5' – 3')
DHM	*dhlA* and *dhmA*	F: GGCGAGCCCACCTGGAGYTAC R: GWMKYGTCRGGGAARGGCGC
LIN	*linB* and *dhaA*	F: CTGTGGCGCAAYATCATSCCG R: GAGGAAGGGCTCGCGATAGKSGKC

*IUPAC ambiguity code used; B = C, G, or T; D = A, G, or T; K = G or T; M = A or C; N = A, C, G, or T; R = A or G; S = C or G; W = A or T; Y = C or T.

M ferric ammonium sulfate ($Fe(NH_4)(SO_4)_2.12H_2O$) solution in 6 M nitric acid was added followed by one drop of a saturated solution of mercuric thiocyanate in ethanol. The development of a red colour is indicative of the presence of free halide (Janssen et al., 1989). *Xanthobacter autotrophicus* GJ10 was used as a positive control while *Escherichia coli* DH5α was used as a negative control in this assay.

Comparison of bacterial growth

Individual isolates were re-inoculated into the minimal salts medium containing 5 mM DCA and growth was monitored spectrophotometrically to confirm the utilization of DCA as a sole carbon and energy source. This was achieved by first growing the individual isolates in 100 ml LB broth overnight at 30°C with shaking. Cells were then harvested by centrifugation at 10 000 x *g* for 10 min. Cells were then washed once in an equal volume of saline (0.87 g NaCl/l, pH 7) and finally, re-suspended in 20 ml saline. The optical density of the cell suspension was then, adjusted to 0.05 at 600 nm. Schott bottles (250 ml), containing 50 ml MMV together with 5 mM DCA with a viton sealed hole in the cap through which sample can be withdrawn were then, inoculated with 500 µl cell suspension. The optical density at 600 nm was monitored at various time intervals until stationary phase was reached.

Dehalogenase gene identification

Primer design

PCR primers were designed based on the sequences of known hydrolytic dehalogenase genes. Sequences were downloaded from the NCBI Database and aligned using the DNAMAN DNA analysis software (Lynon Biosoft). The DNA sequences of the conserved *dhlA* gene from *X. autotrophicus* GJ10 (accession number: M26950) and *A. aquaticus* AD27 and AD25, the *linB* gene of *Sphingomonas paucimobilis* UT26 (accession number: D14594), the conserved *dhaA* gene from *Pseudomonas pavonacae* 170 (accession number: AJ250371) and *Rhodococcus erythropolis* NCIMB13064; and the *dhmA* gene from *Mycobacterium tuberculosis* H37Rv (accession number: Z77724) were aligned. It was found that the *dhaA* and *linB* genes shared 56% identity therefore, these were aligned to form the LIN primer set. The remaining two genes *dhlA* and *dhmA* were found to have 50% identity therefore; these were aligned to form the DHM primer set. The primer design was based on regions showing a significant level of identity and suitably far apart to generate a PCR product (Table 2). The *dhlA* gene of *X. autotrophicus* GJ10 and *A. aquaticus* AD27 are identical (van den Wijngaard et al., 1992) and were used as controls for the amplification of the *dhlA* gene. The *dhaA* genes of *P. pavonacae* 170 and *R. erythropolis* NCIMB13064 are identical (Poelarends et al., 1998) and were used as controls for the *dhaA* gene. The expected size of the PCR product in the control bacteria using either primer set was 450 bp. Primers were synthesized by Inqaba Biotech (South Africa).

DNA amplification

Total DNA was isolated using the QIAamp DNA mini kit (Qiagen). PCR mixtures (50 µl) contained 10 ng DNA, 100 pmol of each primer, 100 µM deoxynucleoside triphosphates (dNTPs), 1 x Super-therm *Taq* polymerase buffer and 0.5U super-therm *Taq* polymerase (Southern cross biotech) and brought to volume with sterile de-ionised water.

PCR was performed using the PE Applied Biosystems GeneAmp PCR System 9700 (Perkin Elmer) programmed to perform an initial denaturation at 94°C for 5 min and 30 cycles consisting of 94°C for 2 min, 60°C for 1 min and 72°C for 2 min followed by a final extension step of 72°C for 5 min. The PCR conditions were the same for both primer sets. DNA from *X. autotrophicus* GJ10 was used as a positive control for the DHM primer set (*dhlA*) and DNA from *P. cichorii* 170 was used as positive control for the LIN primer set (*dhaA*). PCR products were identified by agarose gel electrophoresis on a 2% TAE agarose gel (Sambrook et al., 1989). Gels were documented and analysed using the Chemi Genius Bio-Imaging system (Syngene).

Bacterial identification

16S rDNA sequencing

The 16S rDNA regions were amplified by PCR as described by Marchesi et al. (1998). Following amplification, PCR products were visualized on a 2% agarose gel. PCR products were cleaned using the QIAquick PCR cleanup kit (Qiagen), ligated to the pGEM-T easy vector system I (Promega) and electro-transformed into electro-competent *E. coli* DH5α cells. Transformant colonies were selected on LB agar (10 g tryptone, 5 g yeast extract, 5 g NaCl and 12 g bacteriological agar per liter) plates containing 100 µg/ml ampicillin. Plasmid DNA was isolated using the high pure plasmid isolation kit (Roche) and sequenced by Inqaba Biotech from both directions using the T7 and SP6 primers. DNA sequences were edited and aligned using DNAMAN DNA analysis software (Lynon Biosoft). The BLAST program (Altschul et al., 1997) was used to screen DNA databases for sequences that share similarity with new sequence information. DNA sequences were aligned using Genedoc version 2.4 (Nicholas and Nicholas, 1997).

RESULTS

Isolation of halide releasing bacteria

A total of 187 bacterial isolates were screened using the

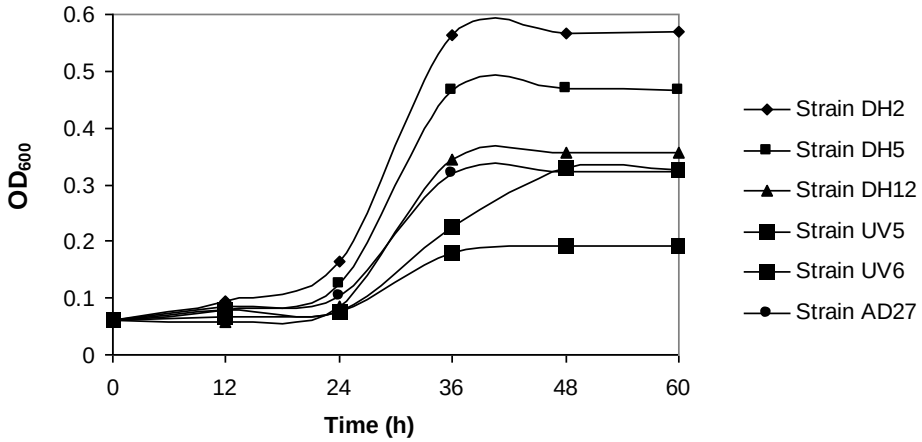

Figure 1. Bacterial growth curves of the different isolates grown in minimal salts medium containing 5 mM DCA. *A. aquaticus* AD27 was used as a control organism.

halide release assay. These isolates appeared as single colonies on the minimal salts agar plates and were subsequently transferred to LB agar plates without NaCl before being screened. Distinct colonies were chosen from the different sources based on colour, size of colony and colony morphology. Five isolates from different point sources produced a positive result for the halide release assay. Three isolates (DH2, DH5 and DH12) were isolated from different points at the northern waste water works, whilst the two other isolates (UV5 and UV6) were isolated from different points at the SAPPI waste water treatment facility.

Comparison of bacterial growth

Figure 1 represents the growth curves of the different isolates in a minimal salts medium containing 5 mM DCA as a carbon source. The increase in the absorbance over time indicated that, these isolates have the ability to utilize DCA as carbon and energy source. *A. aquaticus* AD27, a known degrader of DCA which was used as a control did not perform as well as isolates DH2, DH5 and DH12. All of the isolates showed a relatively long lag period of approximately 24 h and stationary phase was generally reached after 36 h (except for isolate UV5 which has a longer exponential phase and the stationary phase is only reached after 48 h). The longer lag might possibly be due to poor assimilation of the carbon source or poor utilization of one of the intermediates along the catabolic route.

Dehalogenase gene identification

The DHM and LIN PCR primer sets (Table 2) were used in order to identify the hydrolytic dehalogenase produced by the South African isolates. The 450bp region of the *dhaA* gene was successfully amplified in *P. pavonacae* 170 (lane 9) and R. erythropolis NCIMB13064 (lane 10), using the LIN primer set (Figure 2). However,no products were observed for the South African isolates, as well as *X. autotrophicus* GJ10 and *A. aquaticus* strains AD25 and AD27 (lanes 1 to 8) using the same primer set This may indicate that the South African isolates do not possess a dehalogenase gene that is related to the *dhaA*. Furthermore, since the LIN primer set did not amplify any part of the *dhlA* gene of *X. autotrophicus* GJ10 and *A. aquaticus* strains AD25 and AD27 (lanes 1 to 8) this suggests that, this primer set was specific for the *dhaA* gene.

Figure 3 shows the amplification of the 450 bp region of the *dhlA* gene of *X. autotrophicus* GJ10 and *A. aquaticus* AD27 (which were used as controls) with the DHM primer set (lanes 6 to 8). A PCR product of similar size was also obtained for the South African isolates (lanes 1 to 5). As expected, no products were observed for *P. pavonacae* 170 (lane 9) and *R. erythropolis* NCIMB13064 (lane 10) with the same primer set. However, the amplification of a region of similar size within the genomes of the South African isolates of *A. aquaticus* may indicate that, these isolates possessed the *dhlA* gene since the DHM primer was specific for the 450 bp region within the *dhlA* gene.

Bacterial identification

16S rDNA sequencing

Figure 4 shows the amplification of the 16S rDNA region of the five South African isolates as well as *A. aquaticus* AD27. Amplicons of the expected size of approximately 1300 bp, as reported by Marchesi et al. (1998), were produced. The 16S rDNA amplicons were cloned into the pGEM-T easy vector system (Promega), transformed into

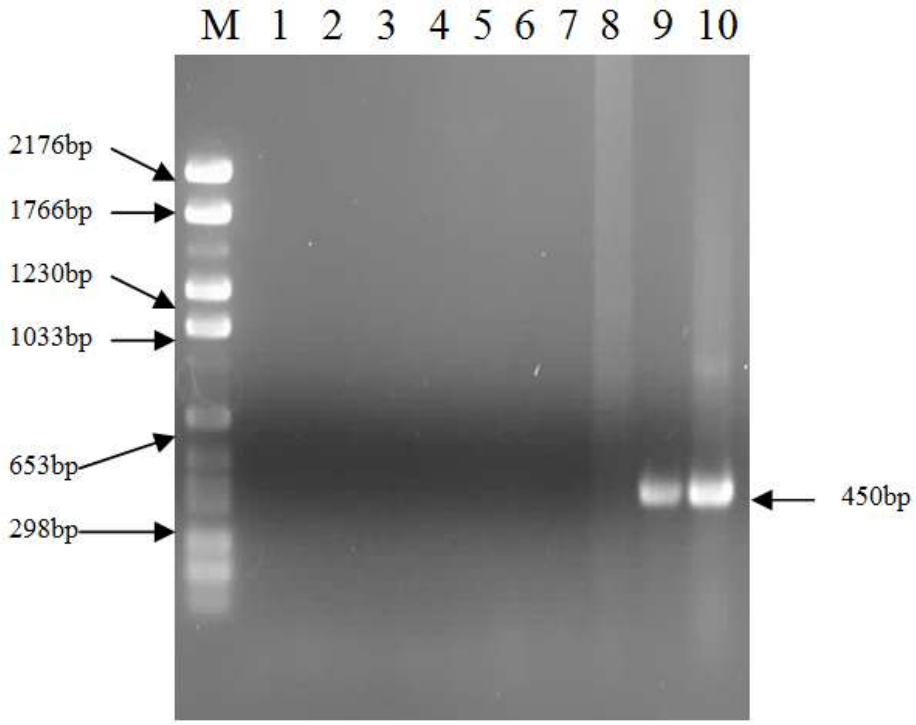

Figure 2. PCR amplification using the LIN primer set. M, Marker VI; Lane 1, DH2; lane 2, DH5; lane 3, DH12; lane 4, UV5; lane 5, UV6; lane 6, *A. aquaticus* AD25, lane 7, A. *aquaticus* AD27; lane 8, *X. autotrophicus* GJ10; lane 9, *R. erythropolis* NCIMB13064 and lane 10, *P. pavonacae* 170.

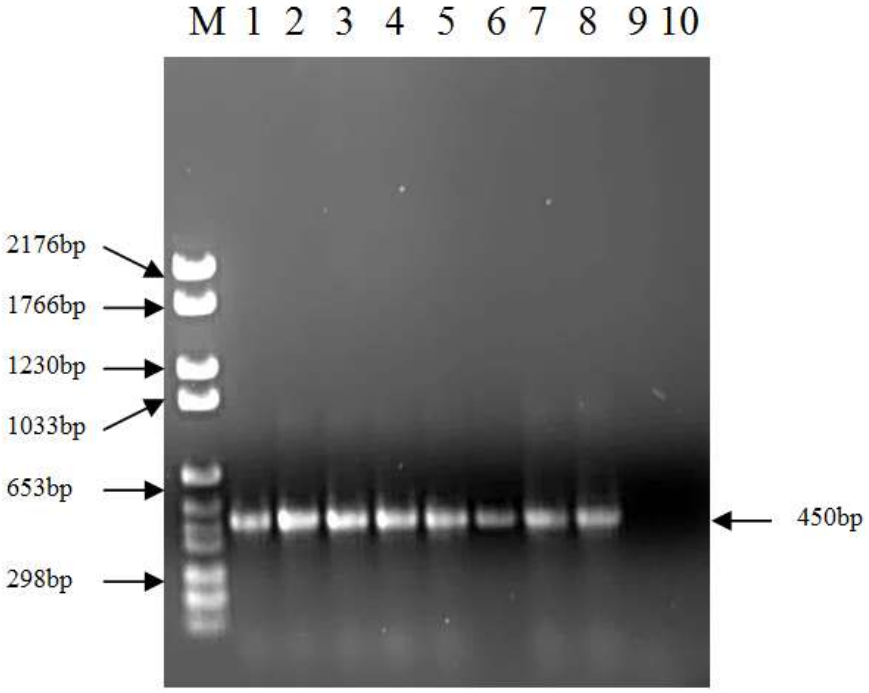

Figure 3. PCR amplification using the DHM primer set. M, Marker VI; Lane 1, DH2; lane 2, DH5; lane 3, DH12; lane 4, UV5; lane 5, UV6; lane 6, *A. aquaticus* AD25, lane 7, A. *aquaticus* AD27; lane 8, *X. autotrophicus* GJ10; lane 9, *R. erythropolis* NCIMB13064 and lane 10, *P. pavonacae* 170.

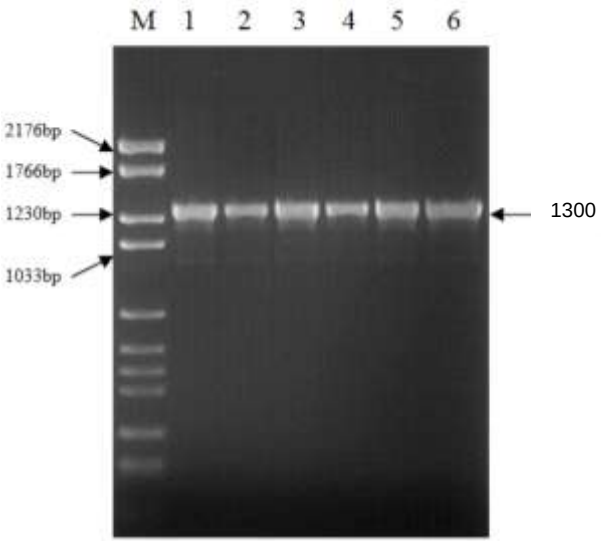

Figure 4. PCR amplification using the 16S rDNA primer set. M, Marker VI; Lane 1, DH2; lane 2, DH5; lane 3, DH12; lane 4, UV5; lane 5, UV6; lane 6: *A. aquaticus* AD27.

E. coli DH5αF' and positive transformants were selected and inoculated into LB medium containing ampicillin. Plasmid preparations with the 16S rDNA amplicons were sequenced by Inqaba Biotech (results not shown). Following BLAST analysis of edited sequence information, all of the isolates were shown to belong to the genus *Ancylobacter*. NCBI accession numbers for the amplified 16S rDNA se-quences were as follows: FJ572205 (isolate DH2); FJ572206 (isolate DH5); FJ572207 (isolate DH12); FJ572208 (isolate UV5) and FJ572209 (isolate UV6).

DISCUSSION

In this study waste water treatment plants handling chemical effluent were used as sources for the isolation of bacteria capable of utilizing DCA as a sole carbon and energy source. The successful isolation of five isolates (DH2, DH5, DH12, UV5 and UV6) that were positive for a halide release assay indicated that, DCA degrading bacteria were indeed present within the different waste treatment facilities in the southern hemisphere.

Effluent treatment plants are a source of great biodiversity in which microorganisms co-exist as a dynamic community with each microbe playing a sometimes small but pivotal role in the degradation process (Rani et al., 2008). The bleaching of pulp by the pulp and paper industry involves the oxidation of wood pulp with elemental chlorine or chlorine oxide making this industry a major source of chlorinated organics. The waste water generated from this industry generally contains sugars, polysaccharides, organic acids, resin acids, lignin transformation products and a variety of chlorinated derivatives that is generally treated in aerated stabilization basins or lagoons making it the ideal source of chlorinated hydrocarbon degrading bacteria (Fulthorpe et al., 1992). Isolates DH2, DH5 and DH12 were isolated from different points within the northern waste water works (KZN, South Africa) that handles the treatment of industrial effluent whereas, isolates UV5 and UV6 were isolated from the waste water treatment facility at SAPPI (South Africa) indicating that, each of these facilities is a potential reservoir of microorganisms that have the capacity to effectively degrade DCA.

The isolation procedure used in this study entailed first enriching DCA utilizing bacteria in a minimal salts broth medium supplemented with vitamins and DCA, followed by plating on a similar agar medium but replacing DCA incorporation into the medium with a DCA atmosphere. This two stage procedure ensured the selection of bacteria only capable of utilizing DCA as a carbon and energy source. The halide release assay initially developed by Bergmann and Sanik (1957) and later modified by Janssen et al. (1989), provides a simple means of detecting liberated chloride ions in solution. Although, this assay was used to qualitatively detect free halide, it can be modified to quantify the liberated chlorine ions.

In order to confirm that these isolates were indeed capable of utilizing DCA as sole carbon and energy source, pure cultures were re-inoculated into the mineral salts medium with DCA as the sole carbon source. For this experiment, a known DCA degrader *A. aquaticus* AD27 (van der Wijngaard et al., 1992) was used as a positive control. The results indicated that, three of the South African isolates (DH2, DH5 and DH12) were capable of better growth compared with *A. aquaticus* AD27 in the minimal medium containing 5 mM DCA.

The ability of the three South African isolates to grow better in the presence of DCA when compared with *A. aquaticus* AD27, could be attributed to the fact that the northern waste water works (KZN, South Africa) routinely handles large quantities of toxic industrial effluent which may give these isolates an adaptive advantage (Jain et al., 2005) when compared with *A. aquaticus* AD27 which was isolated from the brackish water sediment following enrichment with DCA as a growth substrate (van den Wijngaard et al., 1992).

PCR primers used for the amplification of regions within hydrolytic dehalogenase genes were designed based on currently available sequences of the known hydrolytic dehalogenase genes. It was found that the LIN primer set selectively amplified the *dhaA* gene of *P. pavonacae* 170 and *R. erythropolis* NCIMB13064 and not the *dhlA* gene of *X. autotrophicus* GJ10 and *A. aquaticus* strains AD25 and AD27. On the other hand, the DHM primer set amplified the *dhlA* gene of *X. autotrophicus* GJ10 and *A. aquaticus* strains AD25 and AD27 and not the *dhaA* gene of *P. pavonacae* 170 and *R. erythropolis* NCIMB13064.

The presence of a 450 bp amplicon using the DHM primer set in the South African isolates indicated that, these isolates may possess a hydrolytic dehalogenase gene similar to the *dhlA* gene of *X. autotrophicus* GJ10 (Janssen et al., 1989) and *A. aquaticus* strains AD25 and AD27 (van den Wijngaard et al., 1992) or *dhm* gene of *M. tuberculosis* H37Rv (Jesenska et al., 2002).

The use of primers to amplify part of the 16S rDNA region of bacteria for identification purposes has become a common practice and is used often (Marchesi et al., 1998). BLAST analysis of the 16S rDNA sequence between nucleotides 63 and 1387 showed that, while all isolates belonged to the genus *Ancylobacter* they were different from the previously characterized *A. aquaticus* AD27 (van den Wijngaard et al., 1992). Multiple sequence alignment of the five South African isolates as well as *A. aquaticus* AD27 indicated that, all the isolates had base substitutions at various points along the gene, thus, indicating that the five isolates as well as *A. aquaticus* AD27 are not identical to each other.

The first monooxygenase mediated degradation of DCA was demonstrated by Stucki et al. (1983). The isolated bacterium, *Pseudomonas* sp. strain DE2 was able to grow on DCA as a sole source of carbon and energy. Later, *Pseudomonas* sp. strain DCA1 was isolated by Hage and Hartmans (1999) which was found to catalyze the initial step DCA metabolism using a monooxygenase. The oxidation reaction which was found to be the first step in DCA degradation by both *Pseudomonas* sp. strains DE2 and DCA1 was in contrast to the hydrolytic dehalogenation of DCA by *X. autotrophicus* GJ10 (Janssen et al., 1984).

Pseudomonas sp. strains DE2 is capable of aerobic utilization of DCA as well as several other short chain aliphatic compounds as sole sources of carbon and energy. The gene coding for the hydrolytic dehalogenase (*dhlA*) capable of cleaving the carbon-chlorine bond was later cloned and sequenced (Janssen et al., 1989). It was later discovered that three isolates of *A. aquaticus* (AD20, AD25 and AD27) also possessed the *dhlA* gene and were also capable of catalysing the breakdown of short chain aliphatic compounds (van der Wijngaard et al., 1992).

The successful isolation and identification of bacterial isolates capable of DCA degradation from waste water treatment facilities would suggest that, these facilities possess an enormous reservoir of unexploited microbes. In this study, three of the isolates tested were found to degrade DCA more efficiently than the previously characterized isolate *A. aquaticus* AD27 and thus, have the potential to be used in future bioremediation strategies.

ACKNOWLEDGEMENT

The authors acknowledge the National Research Foundation of South Africa for the financial assistance.

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