***Arthrobacter chlorophenolicus* sp. nov., a new species capable of degrading high concentrations of 4-chlorophenol**

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A micro-organism was isolated from soil which could grow on high concentrations [up to 350 p.p.m. (2.7 mM)] of 4-chlorophenol (4-CP). The isolate, designated strain A6^T, was obtained from a soil suspension that had been selectively enriched with gradually increasing concentrations of 4-CP. Strain A6^T could also grow on several other *para*-substituted phenols. Characterization of strain A6^T with respect to chemical, biochemical and morphological properties, 16S rDNA sequencing and DNA–DNA hybridization indicated that the isolate is a novel species within the genus *Arthrobacter* for which the name *Arthrobacter chlorophenolicus* sp. nov. is proposed. The type strain is DSM 12829^T.

Keywords: *Arthrobacter chlorophenolicus* sp. nov., 4-chlorophenol, biodegradation, soil enrichment

INTRODUCTION

Bioremediation exploits the catabolic diversity of micro-organisms to transform contaminants into less harmful compounds. In this process, one can either use the micro-organisms already present at the site of contamination (intrinsic bioremediation) or inoculate with micro-organisms having special catabolic properties (bioaugmentation). Inoculation may be warranted when the intrinsic micro-organisms present at the location are not able to degrade the compound because of lack of suitable enzymes, or when the pollutant is so toxic that the intrinsic micro-organisms are inhibited (Timmis & Pieper, 1999; Vogel, 1996).

Bioaugmentation, however, is not a straightforward technique, as there are many parameters to consider to ensure inoculant survival and efficacy (Liu & Suflita, 1993; van Veen *et al.*, 1997; Vogel, 1996). To assess the potential success of bioaugmentation, knowledge is required about inoculant survival and activity in the contaminated environment and how it is affected by external variables such as soil humidity and temperature (Liu & Suflita, 1993; Vogel, 1996). Knowledge is also required about the chemical properties of

the pollutant itself and its bioavailability (Liu & Suflita, 1993; Vogel, 1996). The more that is known about these parameters, the easier it is to optimize bioaugmentation strategies and therefore to increase the chance of successful clean-up.

One pollutant which is a candidate for bioremediation is 4-chlorophenol (4-CP). 4-CP is a toxic and recalcitrant compound which is formed, for example, from chlorination of waste water, in pulp mills, from breakdown of herbicides such as 2,4-dichlorophenoxyacetic acid (Pritchard *et al.*, 1987) and from anaerobic degradation of more highly chlorinated phenols, such as pentachlorophenol and 2,4,6-trichlorophenol (Madsen & Aamand, 1992; Woods *et al.*, 1989). The toxicity of 4-CP is reflected in the fact that although several microbial strains can degrade 4-CP, such as certain members of the genera *Pseudomonas*, *Alcaligenes* and *Arthrobacter* (Häggblom, 1990, 1992), degradation is usually limited to relatively low concentrations in the range of 20–100 p.p.m. (0.2–0.8 mM). This toxicity severely limits the potential for bioremediation of high concentrations of 4-CP, for example, at locations where the compound has accumulated over a period of time. Several other problems may also arise in a bioaugmentation scenario. For example, 4-CP degradation might be restricted to co-metabolism in the presence of an inducer, such as phenol (Hill *et al.*, 1996), or dead-end meta-

Abbreviation: 4-CP, 4-chlorophenol.

The GenBank accession number for the 16S rDNA sequence of strain A6^T is AF102267.

bolites may accumulate (Ihn *et al.*, 1989; Wieser *et al.*, 1994). Thus, when selecting a micro-organism for bioaugmentation, one needs to consider these potential problems. These hindrances may be overcome by genetically modifying existing strains, or by using selective enrichment to obtain adapted isolates with the desired catabolic properties.

Our aim was to isolate a micro-organism capable of degrading high concentrations of 4-CP for bioaugmentation of 4-CP contaminated soil. From a 4-CP enrichment inoculated with soil, an isolate was obtained which was able to use high concentrations of 4-CP as sole source of carbon and energy. We have characterized the isolate as a novel species within the genus *Arthrobacter* and we propose the name *Arthrobacter chlorophenolicus* sp. nov.

METHODS

Culture conditions. Strain A6^T was grown at 23–28 °C in a minimal medium (GM) containing (g l⁻¹): K₂HPO₄, 2.1; KH₂PO₄, 0.4; NH₄NO₃, 0.5; MgSO₄·7H₂O, 0.2; CaCl₂·2H₂O, 0.023; FeCl₃·6H₂O, 0.002 (Alexander & Lustigman, 1966). When higher cell densities were needed, yeast extract was added to a final concentration of 0.1–0.3 % (w/v) as indicated. A stock solution of 4-CP (Acros) was prepared in distilled water at a concentration of 5–10 mg ml⁻¹ and stored in the dark until use. For plates, 150 or 200 p.p.m. 4-CP was used and agar was added to a concentration of 1.5 %. GMY plates consisted of GM medium with 0.3 % yeast extract. In liquid culture, strain A6^T was routinely grown at a 4-CP concentration of 150 or 250 p.p.m., either shaken at 125 r.p.m. or without shaking. The temperature growth range was determined in LB broth.

Escherichia coli DH5α was grown in LB broth or on LB plates at 37 °C, with 50 µg ampicillin ml⁻¹ when necessary.

Enrichment and isolation of a 4-CP-degrading isolate. Two grams of soil (Aridic haplustoll, sandy loam, pH 7.5, 68.6 % sand, 16.1 % silt, 15.3 % clay) was added to 300 ml GM medium containing 50 p.p.m. 4-CP. When 30–50 % of the initial concentration had disappeared, as determined by spectrophotometric analysis (described below), new 4-CP was added. The addition of 4-CP was repeated over several cycles, up to a concentration of 350 p.p.m. (2.7 mM). 4-CP-degrading bacteria were isolated from the enrichment culture on GM agar plates containing 300 p.p.m. 4-CP and 0.3 % yeast extract hardened at a slant with a top layer of GM agar, creating a diffusion gradient of 4-CP. 4-CP-resistant bacteria were isolated by cross-streaking against the 4-CP gradient on the gradient plates. Colonies resistant to high concentrations of 4-CP were purified by successive streaking on GM agar containing 200 p.p.m. 4-CP.

4-CP degradation analyses. The capability of strain A6^T to degrade different concentrations of 4-CP was determined in liquid cultures. Strain A6^T cells were pregrown in GM medium with 150 p.p.m. 4-CP to stationary phase. The cells were harvested and inoculated into Erlenmeyer flasks containing 250 ml GM medium with a 4-CP concentration of 150–375 p.p.m. Triplicate treatments were performed for each 4-CP concentration level, except the 350 p.p.m. level which was performed in duplicate. Growth was measured by determining OD₆₀₀ with a Beckman DU 640 spectrophotometer.

The 4-CP concentration in soil enrichments and liquid cultures was determined by pelleting cells and any soil particles by centrifugation, measuring the absorbance of the supernatant at 280 nm with a Beckman DU 640 spectrophotometer and relating the value to a standard curve prepared in sterile GM medium.

Growth of strain A6^T on other aromatic compounds and pigment formation. Strain A6^T was grown to exponential phase with 4-CP as sole carbon source in GM medium. The cells were pelleted, washed twice and OD₆₀₀ was adjusted to 0.03 in fresh GM medium. Triplicate 100 ml cultures were set up with this cell suspension and the compounds to be tested as substrates were added to a final concentration of 100 p.p.m. Controls were prepared by using cell suspensions without substrate and uninoculated medium with substrate. The cultures were incubated at 28 °C with shaking and OD₆₀₀ was measured periodically. Cultures were monitored for at least three weeks. Growth on a substrate was inferred from OD₆₀₀ measurements and in some cases also by monitoring the decrease in concentration of the substrate compound using spectrophotometry.

The ability of strain A6^T to use 4-nitrophenol as sole source of nitrogen was tested in GM medium without nitrogen. The cultures were treated as above, except that an additional control was used in which strain A6^T was inoculated into nitrogen-free GM medium containing glucose to verify that 4-nitrophenol, and not traces of nitrogen in the medium, was indeed the nitrogen source.

Pigment production from different aromatic compounds by strain A6^T was investigated in liquid cultures. The cultures were prepared as described above. When the aromatic compound to be tested could be used for growth, as determined above, the cultures were incubated without shaking. For investigation of pigment production from compounds that could not be used for growth, yeast extract was added to 0.1 % (w/v) to serve as a growth substrate and the cultures were incubated with shaking. The production of pigment was checked visually and also spectrophotometrically by scanning in the range of 200–800 nm with a Beckman DU 640 spectrophotometer.

Stability of the 4-CP-degrading phenotype. Cultures of strain A6^T were grown in triplicate in LB broth and upon reaching stationary phase the cultures were repeatedly transferred to fresh LB medium. After 10 consecutive transfers, the cells were plated on LB medium and the ability to grow on 4-CP was tested by transferring cells to 4-CP plates (150 p.p.m.) and fresh LB plates using sterile toothpicks.

Plasmid preparation. Three methods were used to attempt to isolate plasmids from strain A6^T: standard alkaline lysis minipreps (Sambrook *et al.*, 1989), horizontal Eckhardt gels (Eckhardt, 1978) and a method developed for isolating plasmids from *Arthrobacter oxydans* (Brandsch & Decker, 1984). Plasmids were isolated from *E. coli* using the Wizard Miniprep kit (Promega).

Metabolic characteristics and motility. BIOLOG tests were performed by Kirsten S. Jørgensen at the Finnish Environment Institute, Helsinki, Finland, as follows. The bacteria were pregrown twice on BUGM medium and inoculated to a GP MicroPlate (BIOLOG). The plates were read at 4 and 24 h. No identification could be made at 4 h and the results reported are from the 24 h readout (BIOLOG GP database release 3.50).

Growth on additional substrates of taxonomic importance other than those tested by the BIOLOG system was assessed

by growth in liquid cultures and/or on plates with the compound in question as sole carbon source at concentrations in the range 0.2–0.5%. Formation of nicotine blue pigment was investigated on plates consisting of GM medium with 0.2% (v/v) nicotine and either 5 or 0.5 g l⁻¹ of yeast extract.

Motility was tested by the hanging drop method. For a description, see Meynell & Meynell (1970).

Cellular fatty acid analysis. Whole-cell fatty acids were analysed by M. Salkinoja-Salonen at the Department of Applied Chemistry and Microbiology, University of Helsinki, Finland, as described by Väisänen *et al.* (1994).

Cell wall analysis. Cell wall diamino acids were analysed by TLC (Staneck & Roberts, 1974). For detailed analysis of the peptidoglycan, purified cell wall preparations were obtained by the method of Schleifer & Kandler (1972). The amino acids and peptides in cell wall hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates using the solvent systems described by Schleifer & Kandler (1972).

16S rDNA analysis. Genomic DNA preparation and standard cloning procedures were performed according to Sambrook *et al.* (1989). 16S rDNA from strain A6^T was PCR-amplified with the primers rP2 and fD1, modified from Weisburg *et al.* (1991) by removing the linker sequences. Genomic DNA (100 ng) was used in the PCR reaction together with 2.5 U *Pfu* DNA polymerase (Stratagene). The resulting blunt-ended fragment of approximately 1500 bp was ligated into *Sma*I-digested pUC19 (Pharmacia) and the resulting construct was transformed into *E. coli* DH5 α . The outer parts of the insert were sequenced using the commercial primers M13 and M13 reverse (Amersham Pharmacia Biotech). To obtain the entire sequence, primer fD1 (described above) was used, in addition to the following specifically designed primers: SF3, 5'-CACCACCTGGGGAATATTGCA-3'; SF5, 5'-AG-ACTGGAATTCCTGGTGTA-3'. The sequencing reaction was performed using unlabelled primers and the dye termination method (Big Dye kit; Perkin-Elmer) and the DNA sequence was determined on an ABI 377 sequencer (Perkin-Elmer Applied Biosystems). One 350 bp stretch was previously sequenced by Frans deBruijn (Department of Microbiology and NSF centre for Microbial Ecology, Michigan State University, East Lansing, MI, USA) and this sequence was also included in the analysis. The resulting sequence was 1446 bp (excluding the primer sequences), corresponding to positions 28–1493 of rDNA (*E. coli* numbering; Brosius *et al.*, 1978).

To find out which micro-organisms were most closely related to strain A6^T, the 16S rDNA sequence was used to search the RDP II database (Ribosomal Database Project, Michigan State University, USA; Maidak *et al.*, 1994) and subjected to an NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) BLAST search. Using the program CLUSTAL W 1.7, the 16S rDNA sequence of strain A6^T was aligned to the 16S rDNA of other *Arthrobacter* species and to closely related micro-organisms of other genera. *Mycobacterium tuberculosis* was included as an outgroup. For the following phylogenetic analysis, the PHYLIP package was used (Felsenstein, 1993). A pairwise distance matrix was created from the aligned sequences with the program DNADIST, using the corrections for superimposed substitutions of Jukes & Cantor (1969). Only homologous positions were used and sites where the alignment was ambiguous were excluded from the analysis. The program NEIGHBOR was subsequently used to create a phylogenetic tree, using the neighbour-joining method. Bootstrapping

was performed using SEQBOOT, DNADIST, NEIGHBOR and CONSENSE (100 replicates) (Felsenstein, 1985).

DNA base composition. The G + C content of the genome of strain A6^T was determined by the melting temperature of the genomic DNA, courtesy of Vigdis Torsvik, Department of Microbiology, University of Bergen, Norway. Two parallel tests of the sample were performed, together with two controls, *E. coli* and '*Micrococcus lysodeikticus*', and the G + C content was calculated according to Mandel *et al.* (1970).

DNA–DNA hybridization. DNA from *Arthrobacter oxydans* DSM 20119^T, *Arthrobacter polychromogenes* DSM 20136^T and strain A6^T was isolated by chromatography on hydroxyapatite by the procedure of Cashion *et al.* (1977). DNA–DNA hybridization was carried out according to De Ley *et al.* (1970) with modifications as described by Huß *et al.* (1983) and Escara & Hutton (1980) using a Gilford System 2600 spectrophotometer equipped with a Gilford 2527-R thermoprogammer and plotter. Renaturation rates were computed by the program TRANSFER.BAS (Jahnke, 1992).

RESULTS AND DISCUSSION

Isolation of a 4-CP-degrading micro-organism

A soil suspension was gradually enriched with 4-CP by successively increasing the 4-CP concentration from 50 to 350 p.p.m. over a period of 165 d. At the end of the adaptation process, the microbial community in the enrichment could degrade a significantly higher concentration of 4-CP than at the start (350 p.p.m. compared to 50 p.p.m.) and the rate of degradation increased from 8 p.p.m. d⁻¹ for the initial substrate addition to 33.2 p.p.m. d⁻¹ for the final addition (data not shown).

Micro-organisms that could grow on 4-CP as sole carbon source were isolated from the enrichment culture. One isolate, hereafter called strain A6^T, was particularly efficient at 4-CP degradation and was studied further as described below.

The increased tolerance to 4-CP of the enrichment culture demonstrates the usefulness of adaptation processes for selecting micro-organisms with desirable traits; in this case, growth on high concentrations of 4-CP. Kramer & Kory (1992) used a similar approach. They inoculated a 4-CP enrichment culture with sediment and were able to isolate two bacterial strains which could grow on 100 p.p.m. 4-CP in pure culture.

4-CP degradation by strain A6^T

Strain A6^T was able to grow on up to 350 p.p.m. 4-CP in liquid culture (Fig. 1a), using the compound as sole carbon and energy source. The lag phase preceding growth increased with higher concentrations, probably due to toxicity effects. Strain A6^T also completely removed the 4-CP in the medium during growth (Fig. 1b). The onset of degradation was correlated with the initiation of growth.

When 4-CP was completely removed from the cultures, the OD₆₀₀ started to decline (Fig. 1a, b). This was not due to a decrease in cell numbers, as determined by

Fig. 1. (a). Growth of strain A6^T on different concentrations of 4-CP (p.p.m.): ○, 150; ●, 200; □, 250; ■, 320; △, 350; ▲, 375. (b) Removal of different concentrations of 4-CP from culture medium by strain A6^T. Symbols as in (a). Data points are means of three replicates except for the 350 p.p.m. treatment which is in duplicate.

microscopic enumeration, but a transition from a rod morphology to a smaller coccus cell morphology upon entry into stationary phase (data not shown).

To our knowledge, 350 p.p.m. is the highest substrate concentration of 4-CP known to be utilized as sole carbon source by a pure microbial culture. By comparison, the highest 4-CP concentrations previously reported to be degraded were up to 230 p.p.m. by *Comamonas testosteroni* JH5 (Hollender *et al.*, 1997) and up to 206 p.p.m. by *Arthrobacter ureafaciens* CPR706 (Bae *et al.*, 1996). In these studies, vitamins or trace amounts of yeast extract, respectively, were routinely added to the culture medium. Chitra *et al.* (1995) reported a *Pseudomonas pictorum* HU174 strain able to degrade up to 300 p.p.m. 4-CP in 30 h, but glucose was used as an additional carbon source. None of these carbon or nutrient additions were necessary in our study, since strain A6^T only requires a mineral salts medium to grow on 4-CP.

The ability to degrade 4-CP appears to be a stable trait of strain A6^T. The organism was serially transferred on

Table 1. Growth and pigment production by strain A6^T on different aromatic compounds

Compound	Growth	Pigment production
4-Chlorophenol	+	+
2-Chlorophenol	—	+
3-Chlorophenol	—	+
2,4-Dichlorophenol	—	+
2,4-Dichlorophenoxyacetic acid	—	—
Phenol	+	+
4-Bromophenol	+	+
4-Nitrophenol*	+	+
4-Fluorophenol	+	+
4-Methylphenol	—	—
4-Chlorobenzoate	—	—

* Also serves as sole nitrogen source.

non-selective medium (LB), then plated on LB and thereafter tested for the ability of the cells to grow on 4-CP as sole carbon source. The test was performed on cells from the ninth and the tenth transfers for a combined total of 389 colonies. All of these colonies retained the ability to grow on 4-CP, thereby demonstrating that the 4-CP-degrading phenotype was not lost under these non-selective conditions. This is indicative of a chromosomally encoded trait. Furthermore, no plasmid DNA was ever detected from strain A6^T using a variety of plasmid isolation protocols. Although the lack of a detectable plasmid is not in itself proof of its absence, the evidence leans towards a chromosomally encoded 4-CP phenotype.

Growth on other aromatics and pigment production

In addition to 4-CP, strain A6^T could grow on a number of other *para*-substituted phenolic compounds (Table 1). 4-Nitrophenol could also be used as sole source of nitrogen. Of the chlorophenols tested, strain A6^T could only use 4-CP for growth, further demonstrating a specificity for the *para* substitution. Growth was restricted to aromatic compounds that are phenols, e.g. 4-chlorobenzoate did not support growth (Table 1).

Specificity for the *para* position was also found by Bae *et al.* (1996) who described the *Arthrobacter ureafaciens* CPR706 strain mentioned above. In addition to 4-CP, it also degrades 4-bromophenol, 4-nitrophenol, 4-fluorophenol and 4-iodophenol, but not 2-chlorophenol or 3-chlorophenol. Specificity has also been seen for other positions on the aromatic ring, for example *Pseudomonas* sp. B13 is able to degrade 3-chlorobenzoate, but not 2- or 4-chlorobenzoate. This is due to the narrow substrate specificity of benzoate dioxygenase, the initial enzyme in the pathway. (Dorn *et al.*, 1974; Reineke & Knackmuss, 1978). One possible explanation for the preference for the *para* position exhibited by strain A6^T is that during the

enrichment on 4-CP, enzymes were recruited, via gene acquisition or mutation, that conferred the ability to degrade a specific range of phenols. For example, Shimp & Pfaender (1987) saw that an aquatic microbial community that had been adapted to phenol as sole carbon source acquired an increased ability to also degrade 4-CP, 3-methylphenol and 3-aminophenol.

Strain A6^T formed pigments from most of the phenolic compounds tested, even those that did not support growth (Table 1) (when testing compounds which could not be used for growth, yeast extract was added to the medium). 4-Methylphenol was the only phenol tested from which pigment was not formed. Interestingly, this compound was also the only *para*-substituted phenol tested which strain A6^T could not grow on. Pigments were only produced from growth substrates when the cultures were incubated without shaking. A possible explanation for this observation could be that pigments are formed under conditions of low oxygen availability, as would be the case in a liquid culture incubated without shaking, whereas when oxygen is plentiful, the phenols are completely degraded. The pigments were initially orange to red in colour, varying somewhat with the substrate for pigment formation, and eventually turning black. The black pigments were insoluble in water and formed a precipitate. The pigments could consist of accumulating intermediate(s) in the 4-CP degradation pathway (Häggbloom, 1992). Further characterization of the pigments is under way.

Cellular and metabolic characteristics of strain A6^T

Strain A6^T was characterized as Gram-positive with a rod-coccus life cycle. Spores were not formed, the strain was obligately aerobic and catalase was generated. The cells were motile and the motility was strongly influenced by the type of growth medium; the level of motility increased in rich medium such as LB.

Tests of different growth substrates on plates and/or in liquid culture showed that strain A6^T was able to grow on acetate, ethanol, glycerol, creatine, uric acid, succinate, glucose, tyrosine, ascorbic acid, nicotine and weakly on creatinine. Niacin and methanol were not utilized. Blue pigment was not formed, neither on carbohydrate-peptone agar nor on nicotine-yeast extract agar.

A BIOLOG test was performed to investigate which compounds strain A6^T could use for respiration. The following compounds were utilized for respiration: dextrin, glycogen, D-fructose, D-galactose, D-gluconic acid, α -D-glucose, maltose, maltotriose, D-mannose, D-melezitose, D-melibiose, D-psicose, D-raffinose, D-ribose, stachyose, sucrose, turanose, acetic acid, β -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α -ketoglutaric acid, D-lactic acid methyl ester, L-malic acid, methyl pyruvate, pyruvic acid, *N*-acetyl-L-glutamic acid, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, L-pyroglutamic acid, L-serine and putrescine. The following substrates were

not used for respiration: inulin, α -cyclodextrin, β -cyclodextrin, Tween 40, Tween 80, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, amygdalin, L-arabinose, L-arabitol, arbutin, L-fucose, D-galacturonic acid, α -D-lactose, β -methyl-D-galactoside, α -methyl-D-glucoside, β -methyl-D-glucoside, α -methyl-D-mannoside, salicin, sedoheptulosan, D-tagatose, γ -hydroxybutyric acid, α -ketovaleric acid, lactamide, 2,3-butanediol, 2-deoxyadenosine, inosine, thymidine, adenosine-5'-monophosphate, thymidine-5' monophosphate, uridine-5'-monophosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate and D-L- α -glycerol phosphate.

The pattern generated was a good match with *Arthrobacter histidinovorans*, the only *Arthrobacter* species in the BIOLOG database.

Chemotaxonomic analyses

The cell wall diamino acid was found to be lysine. This finding together with the observation of a rod-coccus life cycle conclusively defined the isolate as an *Arthrobacter* species (Keddie *et al.*, 1986). Species of some other genera, such as *Brevibacterium*, *Rhodococcus* and *Aureobacterium*, also have a rod-coccus life cycle, but in these genera either ornithine or *meso*-diaminopimelic acid is the cell wall diamino acid (Keddie *et al.*, 1986).

The peptidoglycan of strain A6^T contained Ala, Ser, Thr and Lys in a molar ratio of 2.0:1.0:1.0:1.0. Enantiomeric analysis of cell wall amino acids resulted in detection of D-Ala, L-Ala, L-Ser, L-Thr and L-Lys. From these results and from two-dimensional TLC peptide patterns of partial hydrolysates of cell walls (data not shown), it was concluded that strain A6^T contains a peptidoglycan of type A3 α (Schleifer & Kandler, 1972) with a L-Lys-L-Ser-L-Thr-L-Ala interpeptide bridge (A11.234 DSMZ catalogue, 1998). This interpeptide bridge is unique to two members of the genus *Arthrobacter*; *Arthrobacter oxydans* and *Arthrobacter polychromogenes*. The acyl type was acetyl.

The cellular fatty acid content was consistent with that of the genus *Arthrobacter*, with anteiso-pentadecanoic acid (15:0 anteiso) predominating (for details, see the species description below). By contrast, in the related genus *Brevibacterium*, both 15:0 anteiso and 17:0 anteiso predominate (Funke & Carlotti, 1994). However, the fatty acid content gave little information as to which species the isolate belonged to, since the fatty acid content is very similar between species in the genus *Arthrobacter*. Several authors have found it impossible to distinguish between different *Arthrobacter* species based on fatty acid data (Funke *et al.*, 1996; Koch *et al.*, 1995; Kodama *et al.*, 1992).

Genetic analyses

The melting temperature of the genomic DNA of strain A6^T was found to be 82.4 °C, giving a G+C content of 65.1 mol%. This value is characteristic for

Fig. 2. Phylogenetic tree constructed from the 16S rDNA sequences of strain A6^T and its 24 closest relatives. *Mycobacterium tuberculosis* was used as an outgroup. The numbers at the forks show how many times the group to the right of the fork occurred among the 100 trees generated in a bootstrap analysis. Only values > 50 are shown. The bar represents 1 expected substitution per 100 nt. The culture collection accession number and the GenBank accession number (in parentheses) is indicated for each strain.

Arthrobacter species, which typically have a G+C content in the range of 59–70% (Keddie *et al.*, 1986).

The 16S rDNA of strain A6^T was cloned and sequenced, revealing that the closest relatives were *Arthrobacter polychromogenes* and *Arthrobacter oxydans*, both with a sequence similarity to strain A6^T of 97.5% (with gaps included and treated as a fifth nucleotide). Such a value is typical for interspecies differences in the genus *Arthrobacter* (Funke *et al.*, 1996). Stackebrandt & Goebel (1994) note that at a sequence homology of about 97.5%, it is unlikely that two organisms are related at the species level, this percentage being indicative of moderately related species.

A phylogenetic tree was constructed based on the 16S rDNA sequence of strain A6^T and those of its closest relatives, using *Mycobacterium tuberculosis* as an outgroup (Fig. 2). Bootstrap analysis showed that many branching points could not be considered statistically significant, which has also been found by other authors (Funke *et al.*, 1996; Koch *et al.*, 1995).

Strain A6^T clustered with *Arthrobacter oxydans* and *Arthrobacter polychromogenes* in the phylogenetic tree (Fig. 2), in accordance with the finding that strain A6^T had the same peptidoglycan interpeptide bridge as these two species.

According to the phylogenetic tree, strain A6^T is also closely related to *Arthrobacter psychrolactophilus*. However, strain A6^T appears to be less related to this organism than to *Arthrobacter oxydans* and *Arthrobacter polychromogenes*, since the similarity of the 16S rDNA between strain A6^T and *Arthrobacter psychrolactophilus* is lower (96.0%, gaps treated as a fifth nucleotide).

To decide whether strain A6^T should be classified as the type strain of a novel *Arthrobacter* species or whether it should be affiliated to either *Arthrobacter oxydans* or *Arthrobacter polychromogenes*, DNA–DNA reassociation values were determined for strain A6^T and type strains of the two described species. While the latter two strains share 66% similarity, strain A6^T is significantly less related to either of them,

Table 2. Distinguishing characteristics between strain A6^T and its closest relatives

Data are from this study, Keddie *et al.* (1986), Loveland-Curtze *et al.* (1999), Schippers-Lammertse *et al.* (1963) and Sguros (1955). NA, Not applicable.

Trait	Strain A6 ^T	<i>A. oxydans</i>	<i>A. polychromogenes</i>	<i>A. psychrolactophilus</i>
Serine in cell wall	Yes	Yes	Yes	No
Colony pigmentation on peptone-carbohydrate medium	Pearl grey	Pearl grey to yellow	Blue	Cream to yellow
Growth on nicotine	Yes	Yes	No	No
Colony pigmentation on nicotine-yeast extract agar	Pearl grey	Blue	NA	NA
Growth in mineral salts medium without biotin	Yes	No	No	Yes
Growth at 37 °C	Yes	Yes	Yes	No
Temperature range	3–37 °C	Not reported	10–37 °C	0–30 °C
Motile	Yes	No	No	No
Isolation source	Soil	Tobacco leaves and air	Airborne infection	Soil

sharing 52% and 41% similarity with *Arthrobacter oxydans* DSM 20119^T and *Arthrobacter polychromogenes* DSM 20136^T, respectively.

DNA–DNA hybridization is thought to be the best method to determine relationships between strains, and when the reassociation values are less than 70%, the strains are considered to belong to different species (Wayne *et al.*, 1987). Thus, both 16S similarity values and DNA–DNA reassociation values obtained for strain A6^T, *Arthrobacter oxydans* DSM 20119^T and *Arthrobacter polychromogenes* DSM 20136^T cluster the latter two strains while strain A6^T is more distantly related. These findings support the description of strain A6^T as the type strain of a new *Arthrobacter* species.

Comparison of strain A6^T with phylogenetic neighbours

In addition to differences at the DNA level, strain A6^T can also be distinguished from its closest relatives based on a number of other characteristics (Table 2). The finding from DNA sequence data that strain A6^T is more closely related to *Arthrobacter oxydans* and *Arthrobacter polychromogenes* than to *Arthrobacter psychrolactophilus* is supported by the finding that strain A6^T has the same type of peptidoglycan composition as the two former species, while *Arthrobacter psychrolactophilus* differs in lacking serine. Interestingly, strain A6^T, like *Arthrobacter oxydans*, is capable of growth on nicotine, although strain A6^T was not isolated from any nicotine-containing environment. However, strain A6^T differs from *Arthrobacter oxydans* in that it does not form a blue pigment on nicotine-yeast extract agar. Neither does strain A6^T form the blue colony pigment on carbohydrate-peptone medium, a feature typical of *Arthrobacter polychromogenes*. The temperature growth range also differs between the strains (Table 2). Strain A6^T grows

at a comparatively wide range of temperatures (3–37 °C). *Arthrobacter psychrolactophilus* is not able to grow at 37 °C and the lowest reported growth temperature of *Arthrobacter oxydans* is 10 °C. Furthermore, strain A6^T and *Arthrobacter psychrolactophilus*, unlike *Arthrobacter oxydans* and *Arthrobacter polychromogenes*, can grow in mineral salts medium with ammonium or nitrate as sole nitrogen source, while the latter two require vitamins such as biotin. Another distinguishing characteristic is motility, since strain A6^T is the only one of these strains which is motile. Finally, the sources of isolation differ. Whereas *Arthrobacter oxydans* and *Arthrobacter polychromogenes* were isolated from tobacco leaves and air, respectively, strain A6^T and *Arthrobacter psychrolactophilus* were isolated from soil.

Since species of the genus *Arthrobacter* are among the most common bacteria in soil (Keddie *et al.*, 1986), it is not surprising that our soil isolate was found to be an *Arthrobacter* species. Soil is generally considered to be a harsh environment with fierce competition for resources. Members of the genus *Arthrobacter* appear well adapted to this habitat, as they are resistant to both starvation and desiccation, and are capable of utilizing a wide range of carbon sources (Ensign, 1970; Keddie *et al.*, 1986). The nutritional versatility of this genus is also reflected in the fact that they have been found to degrade several other pollutants in addition to 4-CP, for example chlorinated phenoxyalkanoic herbicides such as 2,4-D (Häggbloom, 1992), polychlorinated biphenyls (Abramowicz, 1990) and petroleum hydrocarbons (Leahy & Colwell, 1990).

Conclusions

We have isolated a micro-organism which is promising for bioremediation of 4-CP since it degrades unusually high concentrations of the compound. Moreover, no

additional growth factors are required and the 4-CP degradation phenotype is stable. All of the available taxonomic evidence indicates that strain A6^T is a novel species within the genus *Arthrobacter* and we propose the name *Arthrobacter chlorophenolicus* sp. nov.

Description of *Arthrobacter chlorophenolicus* sp. nov.

Arthrobacter chlorophenolicus (chlor.o.phen.o'li.cus. M.L. adj. *chloro* containing chlorine, N.L. n. *phenol* phenol; M.L. adj. *chlorophenolicus* relating to chlorophenols).

Cells are Gram-positive and display a rod-coccus life cycle. The cocci are ~0.75 µm in diameter. The rods are ~0.5–0.7 µm wide, while the length varies between 1 and 4 µm, depending on stage in the life cycle and composition of the growth medium. Agar colonies are circular, smooth and pearl grey in colour. Spores are not formed. Growth occurs with a suitable carbon source in mineral salts medium, no additional growth factors are needed. Red to black pigment is formed from several phenolic compounds, only during incubation with shaking from phenols that can be used for growth, and both with and without shaking for phenols that cannot be used for growth. Catalase-positive. Motile. Obligately aerobic. Grows at 3–37 °C, optimum between 20 and 30 °C. Grows on acetate, ethanol, glycerol, creatine, uric acid, succinate, glucose, tyrosine, ascorbic acid, nicotine and weakly on creatinine. Niacin and methanol are not utilized. A BIOLOG test showed that the following compounds were utilized for respiration: dextrin, glycogen, D-fructose, D-galactose, D-gluconic acid, α-D-glucose, maltose, maltotriose, D-mannose, D-melezitose, D-melibiose, D-psicose, D-raffinose, D-ribose, stachyose, sucrose, turanose, acetic acid, β-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, D-lactic acid methyl ester, L-malic acid, methyl pyruvate, pyruvic acid, *N*-acetyl-L-glutamic acid, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, L-pyroglutamic acid, L-serine and putrescine. The following substrates were not used for respiration: inulin, α-cyclodextrin, β-cyclodextrin, Tween 40, Tween 80, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, amygdalin, L-arabinose, L-arabitol, arbutin, L-fucose, D-galacturonic acid, α-D-lactose, β-methyl-D-galactoside, α-methyl-D-glucoside, β-methyl-D-glucoside, α-methyl-D-mannoside, salicin, sedoheptulosan, D-tagatose, γ-hydroxybutyric acid, α-ketovaleric acid, lactamide, 2,3-butanediol, 2-deoxyadenosine, inosine, thymidine, adenosine-5'-monophosphate, thymidine-5' monophosphate, uridine-5'-monophosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate and D-L-α-glycerol phosphate. The G+C content is 65.1 mol%. The cellular fatty acid content is as follows: 15:0 anteiso, 66%; 16:0 iso, 10%; 15:0 iso, 7%; 17:0 anteiso, 5%; 14:0 iso, 4%; 16:0, 3%; 14:0, 2%; 16:1 iso h, 1%; 16:1ω7c, 1%; anteiso 17:1ω9c, 1% (only values ≥ 1% are reported). The cell wall diamino acid is

lysine. The peptidoglycan type is A3α (Schleifer & Kandler, 1972), with an L-Lys-L-Ser-L-Thr-L-Ala interpeptide bridge. *Arthrobacter chlorophenolicus* A6^T was isolated from soil at Fort Collins, CO, USA. The type strain (A6^T) has been deposited at the DSMZ as DSM 12829^T.

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