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Expression of the 2,4-D degradative pathway of pJP4 in an alkaliphilic, moderately halophilic soda lake isolate, *Halomonas* sp. EF43

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Abstract The broad host range plasmid pJP4, which carries genes for the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid, and 3-chlorobenzoic acid, was used in conjugation experiments with mixed cultures enriched from water and sediment samples from an alkaline pond in the area of Szegedi Fehértó, a soda lake in south Hungary. pJP4-encoded mercury resistance was used as a selection marker. One of the transconjugants, the alkaliphilic, moderately halophilic strain EF43, stably maintained the plasmid and was able to degrade 2,4-D and 3-chlorobenzoate under alkaline conditions in the presence of an additional carbon source such as pyruvate, benzoate, or α-ketoglutarate, indicating that the degradative genes of pJP4 were expressed in this strain. However, it was unable to grow on these chloroaromatic substrates when the substrate was the sole source of carbon and energy. Chemostat cultivation experiments revealed that the 2,4-D degradation rate during growth on benzoate or pyruvate was limited by the low activity of chlorocatechol-degrading enzymes, particularly chloromuconate cycloisomerase. Strain EF43 was identified as *Halomonas* sp. on the basis of 16S rRNA sequencing and additional taxonomic studies. 16S rRNA sequence analysis revealed that strain EF43 is closely related to typical soda lake isolates belonging to the genus *Halomonas*.

Key words Alkaliphiles · Halophiles · *Halomonas* · 2,4-D · Degradation of chloroaromatic compounds · Conjugative transfer · pJP4

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Introduction

Alkaliphilic bacteria have been extensively studied with regard to their biotechnologically relevant extracellular enzymes (for reviews, see Grant et al. 1990; Horikoshi 1996) and as model organisms to solve bioenergetic problems (for reviews, see Krulwich et al. 1988; Krulwich and Guffanti 1992; Hicks and Krulwich 1995; Krulwich 1995). However, little is known about their capabilities of detoxifying xenobiotics. One special problem in this context is toxic residues in pesticide factories and microbial decontamination of building rubble after their demolition, since aqueous eluates from building rubble are highly alkaline. Microbes degrading xenobiotics in such inhospitable environments must be adapted both to extreme pH values and toxic substrates. There are a few examples of bacterial strains isolated from contaminated building rubble that are capable of degrading xenobiotics such as phenoxyalkanoic acid herbicides (Hoffmann et al. 1996; Ehrig et al. 1997; Mertingk et al. 1998; Müller et al. 1999) or chlorophenols (Müller et al. 1998) under alkaline conditions. However, bacteria isolated from naturally occurring alkaline environments and degrading xenobiotics have rarely been described (Maltseva et al. 1996; Kanekar et al. 1999). The most stable naturally occurring alkaline environments are soda lakes with pH values up to 11.5. They are located in areas characterized by a unique combination of geological, geographical, and climatic conditions that diminish the significant buffering capacity of atmospheric CO_2 by the evaporative concentration of sodium carbonate (Grant and Tindall 1986; Grant 1992).

Genes for the degradation of xenobiotics are often located on transmissible plasmids. Whereas the horizontal intergeneric exchange of degradative genes and the dissemination of catabolic plasmids as a means of enhancing the degradative potential of microbial communities are well described for soil ecosystems (Short et al. 1991; Neilson et al. 1994; Hong et al. 1995; Ingham et al. 1995; DiGiovanni et al. 1996; Top et al. 1998; Newby et al. 2000), little is known about these mechanisms in alkaline environments or about the taxa with degradative capabilities in such ecosystems. One of the best characterized catabolic plasmids is the broad host range

IncP1 plasmid pJP4 from *Ralstonia eutropha* JMP134, which encodes the pathway for the degradation of the chlorinated aromatic compounds 2,4-dichlorophenoxyacetate (2,4-D), 2-methyl-4-chlorophenoxyacetate (MCPA), and 3-chlorobenzoate, and confers resistance to mercury ions (Pemberton et al. 1979; Don and Pemberton 1981). Although it is known to be transmissible in bacteria of various genera, the taxonomic range on which pJP4 confers the ability to grow on 2,4-D or 3-chlorobenzoate is narrower than its host range (Don and Pemberton 1981; Friedrich et al. 1983; Neilson et al. 1994; Feng et al. 1994; DiGiovanni et al. 1996; Newby et al. 2000). The 2,4-D degradation pathway of pJP4 is well understood, the enzymes participating in the pathway have been purified and characterized (Pieper et al. 1988; Kuhm et al. 1990; Fukumori and Hausinger 1993; Seibert et al. 1993; Farhana and New 1997), the genes encoding these enzymes (*tfd* genes) have been localized, cloned, and sequenced (Don et al. 1985; Streber et al. 1987; Perkins et al. 1990; Matrubutham and Harker 1994; Kasberg et al. 1995; Leveau et al. 1998), and the regulatory mechanisms of *tfd* gene expression have been elucidated (You and Ghosal 1995; Leveau and van der Meer 1996; Filer and Harker 1997; Leveau et al. 1999; Pérez-Pantoja et al. 2000; Laemmli et al. 2000).

We used the plasmid pJP4 as a model system to elucidate the adaptive potential of bacteria from pristine alkaline environments to toxic substrates such as 2,4-D. Our objective was to ascertain whether conjugative transfer of plasmid pJP4 into soda lake isolates enables the 2,4-D degradative trait to be established in these isolates under alkaline conditions.

Materials and methods

Sample collection and enrichment

Water and sediment samples were collected from an alkaline pond in the area of Szegedi Fehértó, a soda lake near Szeged in south Hungary. The chemical parameters of the water sample were as follows: pH 9.1; conductivity 6.34 mS/cm; Na^+ 1,499 mg/l; HCO_3^- 1,371 mg/l; CO_3^{2-} 393 mg/l; Cl^- 470 mg/l.

Alkaline mineral medium (AMM), pH 10 (modified according to Maltseva et al. 1996) was prepared by mixing sterile component A (20 g Na_2CO_3 and 20 g $NaHCO_3$ in 300 ml H_2O), component B [10 g NaCl, 3 g KCl, 0.5 g NH_4NO_3, 0.3 g $(NH_4)_2SO_4$, 0.4 g $MgSO_4$, 0.3 g NaH_2PO_4, and trace elements (Imhoff and Trüper 1977) in 700 ml H_2O], and the vitamin solution DSM 141. Enrichment cultures for 2,4-D-degrading bacteria were grown in AMM supplemented with 100 mg/l yeast extract or 5 mM α-ketoglutarate, and 0.5 mM 2,4-D. 2,4-D concentration was measured by HPLC. Aerobic chemoorganotrophic bacteria were enriched in AMM supplemented with 10 mM succinate as a carbon source. Cultivation was performed in 500 ml shaking flasks on a rotary shaker at 200 rpm and 30°C.

Mating and screening of transconjugants

Equal volumes of the succinate-grown enrichment cultures and LB-grown cultures of *Escherichia coli* HB101 harboring pJP4 were harvested, washed in 100 mM Tris-HCl, pH 8.5, and spread together on R2A agar plates buffered with 100 mM Tris-HCl, pH 8.5, and containing 1% (w/v) NaCl. Control plates were inoculated with either the donor or the recipient. After overnight incubation at 30°C, cells were harvested from the plates, diluted in AMM, and spread on AMM agar plates containing 10 mM succinate and 0.1 mM $HgCl_2$. After 2–3 days' incubation at 30°C, the resulting mercury-resistant colonies were picked and transferred onto fresh selection plates. The biomass of the presumptive transconjugants was boiled in sterilized water and centrifuged, and then 5 µl of the supernatants was transferred onto a nylon membrane together with control DNA from *Ralstonia eutropha* JMP134 (pJP4) and *R. eutropha* JMP222 (a plasmid-free derivative of JMP134). A pJP4-specific *tfdC* probe was prepared and digoxygenin-labeled as described by Kleinsteuber et al. (1998). Dot blot hybridization with the *tfdC* probe was performed at 55°C, and hybridization and detection were done as described elsewhere (Kleinsteuber et al. 1998). *tfdC*-positive clones were further screened for the presence of plasmid pJP4 using the plasmid minipreparation method described by Müller et al. (1998). Plasmid DNA was separated in 0.6% agarose gels, blotted onto nylon membrane using standard procedures (Sambrook et al. 1989), and hybridized at 60°C to a digoxygenin-labeled *tfdB* probe, which had been prepared by polymerase chain reaction (PCR) from pJP4 DNA by applying *tfdB*-specific primers (Vallaeys et al. 1996). PCR, purification, and labeling of the PCR products, as well as hybridization and detection, were performed as described by Kleinsteuber et al. (1998).

Degradation tests

To screen the degradation properties of transconjugants, cells grown on succinate overnight were harvested by centrifugation, washed in AMM, and resuspended in 3 ml fresh AMM (pH 10) with 0.5 to 1 mM 2,4-D, 50 mg/l yeast extract, and, additionally, 5 µg/l ferrous sulfate. For degradation tests at pH 8.5, component A of AMM was omitted and Tris-HCl, pH 8.5, was added to a final concentration of 100 mM, and NaCl was added to an equimolar sodium ion concentration. After 6–10 days' incubation at 30°C, the decrease in 2,4-D concentration in comparison to a sterile control was determined by HPLC.

Further degradation tests with strain EF43 were performed in 100-ml batch cultures at 30°C and 200 rpm with alkaline R2A medium (buffered with 20 g/l Na_2CO_3 and 20 g/l $NaHCO_3$) or AMM, supplemented with 0.5 mM 2,4-D or 3-chlorobenzoate, respectively. Additional carbon sources were added to a final concentration of 5 mM. The concentration of 2,4-D or 3-chlorobenzoate was measured by HPLC and compared with the sterile controls.

Chemostat cultivation and growth analysis

Continuous cultivation was performed in a 1-l laboratory fermenter under aerobic conditions at 30°C and pH 9.0. AMM without component A but containing 35 g/l NaCl instead was used. Either 10 mM sodium benzoate or 15 mM sodium pyruvate was supplied as the growth substrate, and for each growth substrate, experiments were done both with and without 5 mM 2,4-D and with and without 5 mM 3-chlorobenzoate. The biomass concentration was determined by measuring the optical density at 700 nm (OD_{700}) and by the dry mass determination of washed cells. Off-gas was monitored by a Maihak UNOR 6N and OXOR 6N analyzer (Maihak, Hamburg, Germany) at a gas flow rate of 70 l/h. Total inorganic carbon (TIC) and organic carbon (TOC) were determined from the cell-free supernatant of the culture broth by a high-TOC analyzer (Elementar, Hanau, Germany) at 1,050°C in TIC/nonpurgeable organic carbon mode. The carbon content of biomass was obtained from a CHN-1000 analyzer (Leco, St. Joseph, MI, USA) from washed cells dried overnight at 105°C.

The pH-dependency of growth was determined from continuous cultivation using the method of Esener et al. (1981). The culture was chemostatically precultivated on benzoate at a rate of 0.35 h^{-1} at pH 8.5. Maximum growth rates were derived from biomass decay characteristics at supercritical dilution rates. The maximum rates at the various pH values were consecutively determined after having exposed the culture to the individual pH values for about 2 h by continuously feeding the growth medium at appropriate rates.

The stability of plasmid pJP4 during chemostatic cultivation was verified by plasmid preparation using the Nucleobond AX100 kit (Macherey-Nagel, Düren, Germany), applying the protocol for the isolation of low copy number plasmids.

Determination of enzyme activities

2,4-D/α-ketoglutarate dioxygenase was determined according to Fukumori and Hausinger (1993) by colorimetrically measuring the liberation of 2,4-dichlorophenol after reaction with 4-aminoantipyrin. Catechol 1,2-dioxygenases I and II were determined using the method of Nakazawa and Nakazawa (1970) by determining the formation of the *cis,cis*-muconic acid derivatives using catechol or 3,5-dichlorocatechol, respectively, as the substrate. Catechol 2,3-dioxygenase was determined by the method of Nozaki (1970) by monitoring the formation of 2-hydroxymuconic acid semialdehyde. *cis,cis*-Muconate cycloisomerase was assayed according to Schmidt and Knackmuss (1980) by applying 3,5-dichlorocatechol and the partially purified type II catechol 1,2-dioxygenase (kindly provided by M. Schlömann, University of Stuttgart) in order to generate the 2,4-dichloro-*cis,cis*-muconic acid derivative in a precultivation phase; the activity was calculated by using an extinction coefficient of 9,700 l/mol cm (Dorn and Knackmuss 1978). Benzoate dioxygenase and 2,4-dichlorophenol hydroxylase were measured respirometrically in the presence of 1 mM substrate. The enzyme activities were measured at 30°C and are given in μmol/min mg protein (U/mg).

Microbiological characterization and identification

Gram behavior was determined by the KOH test (Ryu 1938). The GC content of DNA was measured by HPLC according to Mesbah et al. (1989). Cellular fatty acids were determined after methylation by gas chromatography as described by Loffhagen et al. (1995) using the MIS automated microbial identification system (MIDI, Barksdale Professional Center, Newark, NJ, USA) after growth of the strain on R2A agar (3.5% NaCl) at 28°C. Substrate utilization patterns and additional metabolic characteristics were determined using the Biolog automated microbial identification system (Biolog, Hayward, CA, USA), and the API 20 identification kit (Biomérieux, Marcy-l'Ecole, France) according to the suppliers' recommendations with the difference that the cells were resuspended in 3% (w/v) NaCl solution.

The salt dependency of growth was determined in R2A medium containing various concentrations of NaCl in the range of 0%–25% (w/v), corresponding to 0.02–4.3 M total sodium ion concentration. Specific growth rates under various salt concentrations were analyzed during exponential growth in batch cultures by linear regression of the logarithm of OD_{700} with time.

Sequencing of the 16S rDNA was performed as described by Müller et al. (1999). The BLASTN program (http://www.ncbi.nlm.nih.gov/blast/; version 2.0; Altschul et al. 1990) was used to search for similar sequences in the nucleotide sequence databases, and the Sequence Match tool (version 2.7) was used to search for similar sequences in the Ribosomal Database Project II (http://rdp.cme.msu.edu/html/; Maidak et al. 1999). The sequence of the 16S rDNA of strain EF43 will appear in the GenBank, EMBL, and DDBJ nucleotide sequence databases under accession No. AF280796. Phylogenetic analysis was performed using the ARB package (http://www.arb-home.de/). A phylogenetic tree was reconstructed from the 16S rRNA sequences of strain EF43, its 38 closest relatives within the ARB database, and two additional 16S rRNA sequences from GenBank (Acc. Nos. AJ133761, AF140007). The sequences of strain EF43 and the two additional sequences were aligned to the 16S rRNA sequences of the ARB database by the ARB editor, the alignment was corrected manually, and a tree was reconstructed from all 41 sequences using the fastDNAml tool.

Results

Conjugative transfer of pJP4 into soda lake alkaliphiles and characterization of transconjugants

Aerobic chemoorganotrophic bacteria growing on succinate as the sole source of carbon and energy were enriched

from the water and sediment samples under alkaline conditions (pH 10). Direct enrichments of 2,4-D-degrading bacteria did not even succeed in the presence of α-ketoglutarate as an additional carbon source. The succinate-grown mixed bacterial cultures were mated with *E. coli* HB101 as donor of the plasmid pJP4 using the plasmid-encoded mercury resistance as the selection marker. Transconjugants were selected on mercury-containing AMM agar (pH 10), where *E. coli* is unable to grow. Although mercury-resistant bacteria were already present in the recipient mixed cultures as indicated by control plating, the number of colonies growing on the transconjugant selection plates was significantly higher. In order to confirm that mercury-resistant colonies were actually transconjugants, they were screened by dot blot hybridization with a pJP4-specific *tfdC* probe. In total, 842 clones were tested, of which 762 clones were *tfdC*-positive.

Plasmid profiles of 454 transconjugants were analyzed by isolating the plasmid DNA from succinate-grown cultures, agarose gel electrophoresis, and Southern hybridization with a pJP4-derived *tfdB* probe to detect homology of plasmid bands to pJP4. Of the 454 transconjugants, 403 clones carried a plasmid of about 80 kb, which corresponds to the size of pJP4. All these plasmids hybridized to the *tfdB* probe under stringent conditions. No plasmid was detected in 27 clones, and 24 clones showed plasmid bands smaller than 80 kb. One of these plasmids hybridized to the *tfdB* probe under stringent conditions, indicating that it was a deletion derivative of pJP4. However, with the other smaller plasmids no *tfdB* signals were detected, indicating that they were not pJP4 derivatives. Nevertheless, it cannot be ruled out that the catabolic region of pJP4 was deleted in these clones. Thirty-five isolates exhibited multiple plasmid bands of various sizes, not hybridizing even to the entire pJP4, indicating that these isolates carry indigenous plasmids.

Only with 32 of the 762 transconjugants tested was a decrease of at least 10 mg/l 2,4-D, in comparison with the sterile control, detected after 6–10 days' incubation at 30°C. No significant differences in the degradative behavior of the isolates were detected at pH 10 or pH 8.5. None of the transconjugants was able to degrade the 2,4-D completely or to grow on 2,4-D as the sole carbon source, although no toxic intermediates such as 2,4-dichlorophenol were detected. However, a significant decrease in 2,4-D was observed with a few isolates during growth on α-ketoglutarate or pyruvate. One of these isolates, strain EF43, was further investigated regarding its degradative properties as well as its microbiological and physiological characteristics.

Taxonomic characterization of strain EF43

After growth on alkaline R2A agar, strain EF43 forms round convex colonies of 1–2 mm diameter, faintly bright and white to cream-colored. The cells are strongly motile, straight rods and have a length of 1.4–1.7 μm and a diameter of 0.8 μm. They are Gram-negative and show positive reactions for oxidase, catalase, and nitrate reduction. The following composition of cellular fatty acids was determined: 10:0 (4.7%), 12:0 (4.8%), 12:0 3OH (9.4%), 16:1 ω7c (12.8%), 16:0 (12.5%), 17:0 cyclo (2.7%), 18:1 ω7c (49.9%), and 19:0 cyclo ω8c (3.2%). The GC content of the DNA was 67.5 mol%. Concerning the substrate utilization pattern, the Biolog automated microbial identification system identified strain EF43 as *Halomonas aquamarina* with a similarity index of 0.550. Positive reactions were obtained with respect to the utilization of α-ketoglutaric acid, DL-lactic acid, succinamic acid, mono-methylsuccinic acid, succinic acid, β-hydroxybutyric acid, L-glutamic acid, L-proline, L-serine, and L-alanine.

Strain EF43 is able to grow over a wide range of salt concentrations: growth was observed in R2A medium containing 1%–15% (w/v) NaCl. No growth was observed above 20% NaCl. Sodium ions are required for growth. The optimal sodium ion concentration for growth was 0.4–0.8 M. The influence of pH on the growth of strain EF43 was determined in mineral medium at optimal sodium ion concentration (about 0.6 M NaCl) and with benzoate as the growth substrate. The strain has a broad pH optimum (8.5–10). Although the specific growth rate significantly decreases below pH 8 and above pH 10.6, in rich medium (R2A) it also grows at neutral pH. According to its growth characteristics with respect to salt content and pH value, strain EF43 is an alkaliphilic, moderately halophilic bacterium.

The nucleotide sequence of the nearly complete 16S rRNA gene (1488 bp) was determined. As calculated by BLAST, the 16S rRNA sequence of strain EF43 shows 97% similarity to the 16S rRNA sequences of Crater Lake isolate 12C1 (Acc. No. X92135) and of Lake Elmenteita isolate WE5 (Acc. No. X92140), two soda lake isolates from the East African Rift Valley (Duckworth et al. 1996), and 97% similarity to the 16S rRNA sequences of *Halomonas* sp. isolate Co12 (Acc. No. AJ133761), a marine isolate, and of *Halomonas* sp. ML-185 (Acc. No. AF140007), a soda lake isolate from Mono Lake, California. The closest relative in the Ribosomal Database Project was *Halomonas salina*^{T} ATCC 49509 with a similarity score of 0.848. A phylogenetic tree was reconstructed by the maximum likelihood method from the 16S rRNA sequences of strain EF43 and its 38 closest relatives from the ARB database and of strains *Halomonas* sp. isolate Co12 and *Halomon*as sp. ML-185 (Fig. 1). The tree clearly shows that strain EF43 clusters within the genus *Halomonas* and that it is closely related to the soda lake isolates *Halomonas* sp. ML-185 (Mono Lake isolate), Crater Lake isolate 12C1 and Lake Elmenteita isolate WE5, as well as to some other East African soda lake isolates (Duckworth et al. 1996). The 16S rRNA sequence of strain EF43 contains all 15 signature features characteristic of the family *Halomonadaceae* and the additional 4 signature features characteristic of the genus *Halomonas* (Dobson and Franzmann 1996). By virtue of its taxonomic characteristics and the phylogenetic analysis, strain EF43 was identified as *Halomonas* sp.

Degradative properties and physiological characteristics of strain EF43

Degradation tests in batch cultures revealed that strain EF43 is able to degrade 2,4-D and 3-chlorobenzoate during

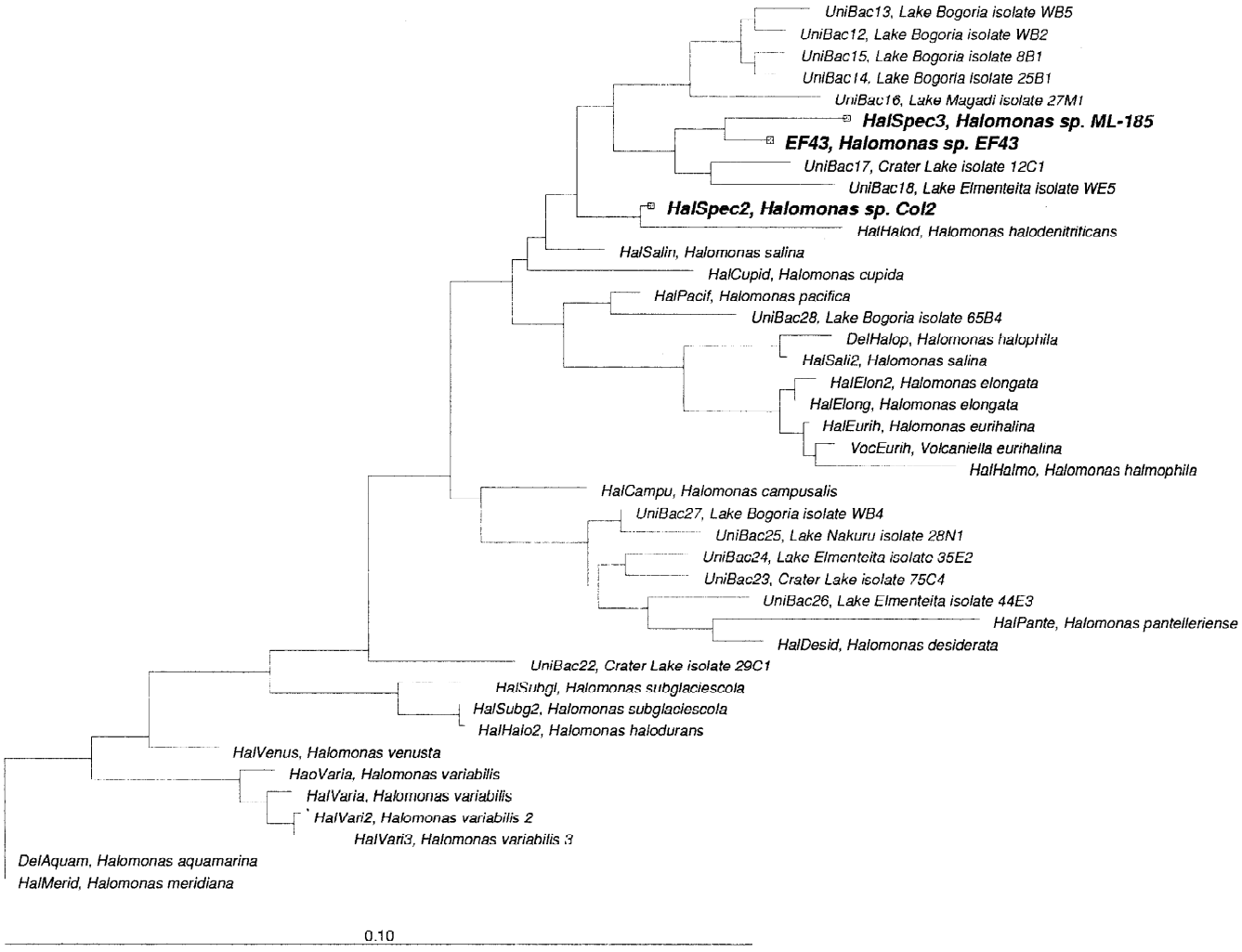

Fig. 1. Phylogenetic tree showing the relationship between strain EF43 and its closest relatives. The 16S rRNA sequences added to the ARB database are shown in *bold*

growth on pyruvate, α-ketoglutarate, or benzoate (Figs. 2A, 3A). In the presence of pyruvate as growth substrate, the degradation of 0.5 mM 3-chlorobenzoate was virtually complete within 24 h (Fig. 3A). By contrast, during growth on acetate or succinate as well as in the absence of an additional carbon source, only slight or no degradation was measured (Figs. 2B, 3B). Degradation of 3-chlorobenzoate was completely inhibited when the strain grew on succinate (Fig. 3B). Although no intermediates such as 2,4-dichlorophenol were detected during the degradation of 2,4-D, during the degradation of 3-chlorobenzoate, the media became brown colored, which might have been due to the accumulation of an oxidized polymer of the intermediate 3-chlorocatechol.

The results obtained with batch incubation were confirmed by chemostat cultivation of strain EF43 on 2,4-D or 3-chlorobenzoate with benzoate or pyruvate as the growth substrates. A considerable amount of the chloroaromatics was utilized under these conditions in the presence of either of the substrates. In the case of benzoate, the amount was 77% of the carbon from 2,4-D as follows from the carbon balances, i.e., from the difference between the total carbon fed during growth on benzoate or benzoate + 2,4-D (2.0 mMolC/h) and the difference between the TOC values during growth on benzoate or benzoate + 2,4-D (0.46 mMolC/h) (Table 1). The utilization of 2,4-D had little effect on the biomass gain; moreover, the overall growth yield on the basis of C-moles was significantly diminished in the presence of 2,4-D as compared with that on growth substrate alone (Table 1). Utilization of the growth substrates proceeded with rather low efficiency: from the biomass yield, the efficiency of energy transduction with benzoate and pyruvate was calculated to have an apparent P/O coefficient of 1.29 and 1.15, respectively (for more details on the calculations see Müller and Babel 1994).

Steady states were characterized by rather high stationary 2,4-D concentrations even at comparatively low growth rates (the maximum growth rate was $\mu_{max} = 0.45\ h^{-1}$ for benzoate at pH 9). In addition, a product appeared in HPLC measurements with both UV and RI (refractive index) detection, which coeluted in HPLC measurements with the product formed by incubating 3,5-dichlorocatechol with catechol 1,2-dioxygenase type II. However, whether this compound is identical to 2,4-dichloro-*cis,cis*-muconate remains

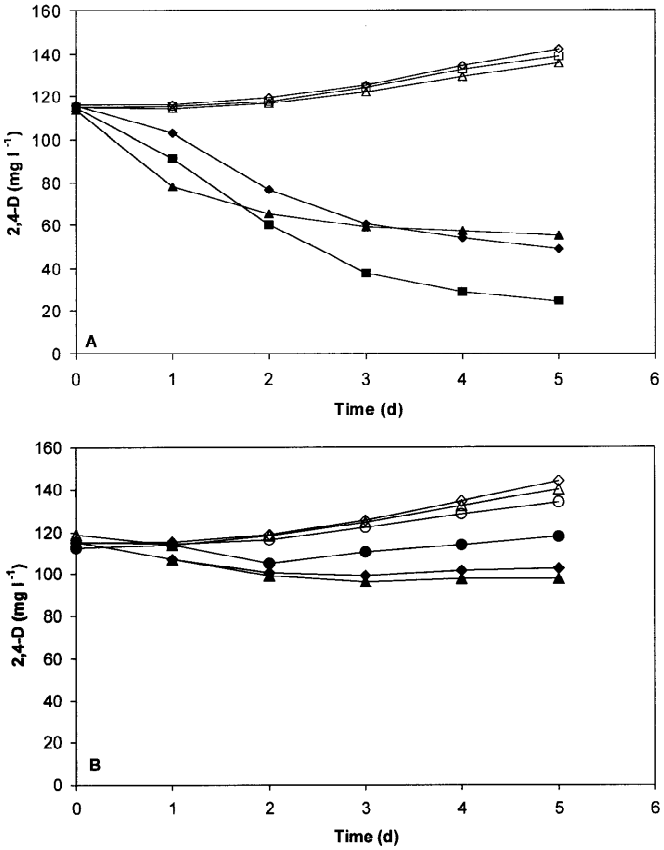

Fig. 2A,B. Degradation of 2,4-dichlorophenoxyacetate (*2,4-D*) by strain EF43 in alkaline mineral medium (pH 10) in the presence of various carbon sources (*open symbols*: sterile control; *filled symbols*: strain EF43). **A** 2,4-D degradation during growth on 5 mM α-ketoglutarate (*solid triangles*), 5 mM benzoate (*solid diamonds*), and 5 mM pyruvate (*solid squares*). **B** 2,4-D degradation without an additional carbon source (*solid circles*), during growth on 5 mM succinate (*solid diamonds*), and during growth on 5 mM acetate (*solid triangles*)

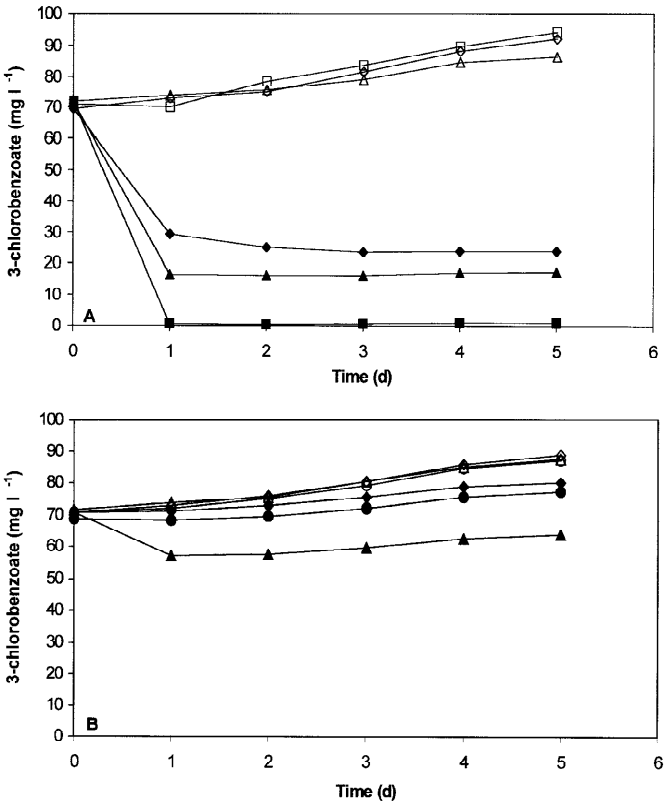

Fig. 3A,B. Degradation of 3-chlorobenzoate by strain EF43 in alkaline mineral medium (pH 10) in the presence of various carbon sources (*open symbols*: sterile control; *filled symbols*: strain EF43). **A** 3-chlorobenzoate degradation during growth on 5 mM α-ketoglutarate (*solid triangles*), 5 mM benzoate (*solid diamonds*), and 5 mM pyruvate (*solid squares*). **B** 3-chlorobenzoate degradation without an additional carbon source (*solid circles*), during growth on 5 mM succinate (*solid diamonds*), and during growth on 5 mM acetate (*solid triangles*)

Table 1. Growth parameters and distribution of carbon during chemostat cultivation of strain EF43 on heterotrophic substrates and substrate mixtures

Substrate(s)	D (h^{-1})	Dry wt (g/l)	Y (Cmol/Cmol)	2,4-D (mM)	DCP	C_{feed} (mMolC/h)	$C_{Dry\ wt}$ (mMolC/h)	CO_2	TOC	TIC	Σ	%
10 mM Benzoate	0.05	0.623	0.313	–	–	3.5	1.06	1.81	0.12	0.46	3.45	98.6
10 mM Benzoate+5 mM 2,4-D	0.05	0.648	0.215	1	→ 0	5.5	1.06	2.25	0.58	1.38	5.27	95.8
15 mM Pyruvate	0.05	0.426	0.27	–	–	2.25	0.5	0.68	0.4	0.5	2.08	92.4
15 mM Pyruvate+5 mM 2,4-D	0.035	0.519	0.182	0.55	→ 0	2.975	0.46	1.475	0.45	0.49	2.875	96.6

Y, growth yield coefficient; C_{feed}, substrate carbon fed; 2,4-D, 2,4,-dichlorophenoxyacetate; TOC, organic carbon in cell-free supernatant of fermentation broth; TIC, inorganic carbon in cell-free supernatant of fermentation broth, corrected by concentrations of carbonate trapped from atmospheric CO_2 at pH 9; DCP, 2,4-dichlorophenol

to be proved by more detailed analyses. No accumulation of 2,4-dichlorophenol nor β-ketoadipate was detected by the application of references in HPLC analyses. A possible accumulation of chlorodienlactone or chloromaleylacetate was not investigated because these compounds were not available for application. The liberation of products during growth on substrate mixtures was supported by TOC measurements in the supernatants, which showed that the amount of carbon derived from stationary 2,4-D levels was lower than the total amount found in the culture broth (Table 1). The concentrations of benzoate and pyruvate themselves were below the detection limits in these cultures.

Enzyme activities with regard to 2,4-D metabolism were determined from chemostat cultures. From the results

Table 2. Enzyme activities (in μmol/min mg protein) for aromate degradation during growth on individual substrates and substrate mixtures

Enzyme	Assay substrate	Growth substrate(s) benzoate/2,4-D	Benzoate/ 3-CBA	Benzoate	Pyruvate/ 2,4-D	Pyruvate	JMP134[b]
2,4-D/α-ketoglutarate dioxygenase	2,4-D	0.127	0.06	<0.02	0.658	<0.02	0.105
Phenol hydroxylase	Phenol	0[a]	n.d.	n.d.	0[a]	0[a]	0.13
	2,4-Dichlorophenol	0.114	n.d.	n.d.	0.034	0.002	0.23
Catechol 1,2- dioxygenase	Catechol	12.1	6.145	5.0	4.9	0	0.49
	3,5-Dichlorocatechol	0.066	0.145	0	0.742	0	0.45
cis-cis-Muconate cycloisomerase	*cis,cis*-Muconate	0.195	0.6	0.475	0.26	n.d.	0.008
	2,4-Dichloro-*cis,cis*-muconate	0.046	0.03	0.0075	0.11	n.d.	0.39
Catechol 2,3- dioxygenase	Catechol	0	0	0	0	0	<0.001
Benzoate dioxygenase	Benzoate	0.322	0.12	0.124	n.d.	n.d.	

n.d., not determined; 3-CBA, 3-chlorobenzoic acid
[a] Inhibition of endogenous respiration after adding phenol
[b] After growth on 2,4-D (data from Pieper et al. 1988)

shown in Table 2, it is evident that high 2,4-D/α-ketoglutarate dioxygenase activities were expressed during cultivation in the presence of 2,4-D. In combination with benzoate, this activity was similar to that found in the natural host of pJP4, *R. eutropha* JMP134, during growth on 2,4-D, but was more than fivefold that after growth on pyruvate. High chlorocatechol 1,2-dioxygenase activity was found for growth on pyruvate in the presence of 2,4-D. By contrast, the enzyme for the next metabolic step of the modified *ortho* pathway, i.e., chloromuconate cycloisomerase, was expressed at a low level, the activity during growth on benzoate and 2,4-D being reduced to about 10% in comparison with *R. eutropha* JMP134 growing on 2,4-D. Catechol 2,3-dioxygenase activity initiating the *meta* cleavage pathway was not detected in strain EF43 (Table 2).

The stability of pJP4 was regularly verified by plasmid preparation during all chemostat cultivation experiments. The plasmid was shown to be stably maintained by strain EF43 in both the presence or the absence of 2,4-D or 3-chlorobenzoate.

Discussion

Taxonomic status

Based on its 16S rRNA sequence and additional taxonomic characteristics, strain EF43 was assigned to the genus *Halomonas*. This identification is supported by the presence of the 19 16S rRNA signature features characteristic of the genus *Halomonas* (Dobson and Franzmann 1996) and by the composition of the major fatty acids 18:1 plus 19:0 cyclo (53.1%), 16:1 plus 17:0 cyclo (15.5%), and 16:0 (12.5%), which is consistent with the major fatty acid composition of the genus *Halomonas* (Dobson and Franzmann 1996). This identification is further confirmed by physiological characteristics (sodium ion requirement, salt tolerance up to 20% NaCl and growth optimum at 0.6 M sodium ion concentration, nitrate reduction) and by the substrate utilization pattern. Strain EF43 grows on acetate, succinate, β-hydroxybutyrate, DL-lactate, and L-α-alanine, which were described as characteristic substrates of the genus *Deleya* (Balows et al. 1992), a genus which was unified with the genus *Halomonas* by Dobson and Franzmann (1996).

As indicated by the phylogenetic analysis, the closest relatives of strain EF43 are soda lake alkaliphiles (Duckworth et al. 1996) such as Crater Lake isolate 12C1 (Acc. No. X92135; 97% similarity) and Lake Elmenteita isolate WE5 (Acc. No. X92140; 97% similarity), which should also be assigned to the genus *Halomonas*, and the Mono Lake isolate *Halomonas* sp. ML-185 (Acc. No. AF140007; 97% similarity). 2,4-D degradation under alkaline conditions by members of the *Halomonadaceae* has been described previously (Maltseva et al. 1996). From these findings and our results, we conclude that the *Halomonadaceae* are potential degraders of chloroaromatic compounds in alkaline and saline environments, as was recently confirmed by conjugation experiments with a *gfp*-tagged pJP4 and soda lake isolates as recipients (Kleinsteuber et al. 2000).

Expression of the 2,4-D degradative pathway

The broad host range plasmid pJP4 encoding the 2,4-D degradative pathway of the neutrophilic bacterium *Ralstonia eutropha* JMP134 was successfully transferred into bacteria isolated from an alkaline environment and was shown to be stably maintained in the majority of transconjugants, indicating that the host range of this IncP1 plasmid also includes alkaliphiles. The degradative genes were expressed in at least some of the transconjugants, enabling them to degrade 2,4-D and 3-chlorobenzoate under alkaline conditions, as demonstrated with isolate EF43. However, the significant degradation of 2,4-D or 3-chlorobenzoate was observed only if an additional carbon source was provided. A similar

effect was previously observed with other pJP4 transconjugants (Feng et al. 1994).

2,4-D and 3-chlorobenzoate degradation was observed during growth on benzoate, pyruvate, or α-ketoglutarate, or in R2A medium. Only slight or no degradation occurred during growth on succinate or acetate. Remarkably, the degradation of 3-chlorobenzoate was completely inhibited during growth on succinate. This effect might be due to transcriptional repression of the chlorocatechol catabolic operon, since succinate has been described as a catabolite repressor specific to the modified *ortho* cleavage pathway encoded by the *clcABD* operon in *Pseudomonas putida* (McFall et al. 1997a).

The plasmid pJP4 encodes the entire degradative pathway down to β-ketoadipate (Streber et al. 1987; Perkins et al. 1990; Matrubutham and Harker 1994; Kasberg et al. 1995; Pérez-Pantoja et al. 2000; Laemmli et al. 2000) including a 2,4-D transport protein (Leveau et al. 1998) and the two identical transcriptional activators TfdR and TfdS (Matrubutham and Harker 1994; You and Ghosal 1995; Leveau and van der Meer 1996). The *tfd* genes are expressed in concert due to the TfdR/S-regulated promoters (Leveau et al. 1999; Laemmli et al. 2000) which are induced by the metabolites 2,4-dichloromuconate (Filer and Harker 1997) or 2-chloromuconate (McFall et al. 1997b). Consequently, the complete degradation of 2,4-D and 3-chlorobenzoate and the funneling of metabolites into the tricarboxylic acid cycle should be possible in bacteria possessing the β-ketoadipate pathway (Harwood and Parales 1996), provided that the *tfd* genes generally are expressed. Since strain EF43 degrades benzoate via the *ortho* cleavage pathway, the lack of β-ketoadipate coenzyme A transferase and/or β-ketoadipyl coenzyme A thiolase cannot be the reason for the inability to grow on 2,4-D or 3-chlorobenzoate. Although during growth on benzoate the enzymes of the β-ketoadipate pathway are induced, benzoate shows no enhancing effect on the 2,4-D degradation rate in comparison with nonaromatic growth substrates such as pyruvate or α-ketoglutarate. However, the presence of an additional carbon source enables strain EF43 to significantly degrade 2,4-D or 3-chlorobenzoate, whereas these chloroaromatic compounds are not sufficient as sole sources of carbon and energy for the strain, indicating a biochemical bottleneck.

In the case of 2,4-D, this bottleneck may result from the fact that the initial step of assimilating 2,4-D is catalyzed by an α-ketoglutarate-dependent dioxygenase. α-Ketoglutarate must be supplied and — because it is oxidatively decarboxylated to succinate in this step — regenerated in order to initiate and maintain the cleavage reaction; otherwise 2,4-D will not be utilized despite the presence of 2,4-D/α-ketoglutarate-dioxygenase (Müller and Babel 2000). This supply requirement might result from the fact that, in contrast to the activity of the initial reaction, the activity of consecutive enzymatic steps in 2,4-D degradation, and finally the formation of acetylCoA and succinate from the phenolic intermediate, is rather low in strain EF43. The 2,4-D/α-ketoglutarate dioxygenase might catalyze a futile reaction under these conditions by wasting carbon from the pool of metabolites as CO_2, which would result in a shortage of carbon and energy and thus prevent the balancing of assimilatory and dissimilatory routes.

This explanation is not as obvious in the case of 3-chlorobenzoate. The enzymatic profile is similar in that it is characterized by a high rate of activity in the initial step followed by strongly reduced rates in consecutive parts of the pathway. However, the requirement of two substrates is not the reason for the bottleneck in this case. The metabolism of 3-chlorobenzoate into tricarboxylic acid cycle intermediates is balanced, including the expenditure and gain of reduction equivalents in the initial metabolic steps. However, whether the availability of reduction equivalents in stoichiometric proportion is guaranteed is questionable, bearing in mind that conversion of substrate via this sequence is the only source of energy to satisfy maintenance requirements. The role of reduction equivalents is suggested by the results of batch experiments, which showed that the degradation of 3-chlorobenzoate proceeded much better when NADH emerged early from the auxiliary substrates, such as was the case for pyruvate, α-ketoglutarate, and, to a lesser extent, benzoate.

The rate-limiting step in 2,4-D degradation by strain EF43 appears to be chlorocatechol conversion, since the activity of at least the chloromuconate cycloisomerase is very low compared with that of the natural host of pJP4, and the accumulation of 2,4-dichloro-*cis,cis*-muconate during 2,4-D degradation is likely. Activities of (chloro)dienlactone hydrolase (TfdE) and (chloro)maleylacetate reductase (TfdF) were not determined, and the possible accumulation of the metabolites chlorodienlactone or chloromaleylacetate has not yet been examined. As a consequence, it cannot be excluded that one of the further chlorocatechol converting enzymes (TfdE or TfdF) causes the bottleneck in 2,4-D degradation by strain EF43. The reasons for this imbalance must be further elucidated by mRNA and protein analyses. The genes *tfdC*, *tfdD*, *tfdE*, *tfdF,* and *tfdB* are organized in one operon as well as $tfdD_{II}$, $tfdC_{II}$, $tfdE_{II}$, $tfdF_{II}$, and $tfdB_{II}$ (Leveau et al. 1999; Laemmli et al. 2000). Although both modules I (*tfdCDEFB*) and II ($tfdD_{II}C_{II}E_{II}F_{II}B_{II}$) are expressed and are required for efficient degradation (Laemmli et al. 2000), the activity of TfdD and TfdE is primarily supplied by module I, whereas the TfdF activity is likely to be supplied by module II (Pérez-Pantoja et al. 2000). As demonstrated by Pérez-Pantoja et al. (2000), *Ralstonia eutropha* JMP222 derivatives containing cloned modules I or II grew on 3-chlorobenzoate, whereas in the case of *Pseudomonas putida* KT2440, only the derivative containing module I was able to grow on 3-chlorobenzoate. These results suggest a different transcriptional efficiency of the TfdR-regulated promoters of modules I and II, leading to an insufficient activity of the chlorocatechol-converting enzymes in those host strains not efficiently transcribing both modules, and consequently to the accumulation of metabolites during the degradation of 2,4-D or 3-chlorobenzoate.

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