Mineralization of orthanilic acid is a plasmid-associated trait in *Alcaligenes* sp. O-1

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A bacterial isolate (O-1) which degrades different substituted benzenesulphonates was identified as a strain of *Alcaligenes* sp. using chemotaxonomic, DNA:DNA hybridization and serological methods. Its ability to mineralize orthanilic acid (2-aminobenzenesulphonate) is correlated with the presence of a 117 MDa plasmid, designated pSAH. Selective curing of pSAH resulted in the abolition of the orthanilic acid mineralization phenotype, which could be fully restored by re-establishment of pSAH. A second plasmid of 6.8 MDa, designated pME1702, with no detectable functions, is also present in *Alcaligenes* sp. O-1. Upon conjugation with *Alcaligenes* sp. O-1 as donor, the plasmid-free recipient *Pseudomonas putida* PaW130 acquired the ability to utilize orthanilic acid. Exconjugants of *P. putida* PaW130 harboured both plasmids (pSAH and pME1702) originating from *Alcaligenes* sp. O-1. Growth characteristics, the inducibility of enzyme activity (desulphonation), and expression of characteristic proteins during mineralization of orthanilic acid indicate that a plasmid-encoded cluster of genes is involved in the biodegradation of orthanilic acid. Southern blot hybridizations did not reveal any relationship between pSAH and the archetypal TOL plasmid pWW0, which also mediates degradation of substituted aromatic compounds.

Introduction

Sulphonated aromatic compounds are building blocks of detergents and azo dyestuffs. Introduction of sulphonic substituents improves the water solubility of aromatic compounds, but enhances their recalcitrance (Bretscher, 1981; Cook *et al.*, 1986), so that aromatic sulphonates may accumulate in the environment (Leisinger, 1983). Although the biodegradability of these compounds has been reported by several laboratories, pure cultures which efficiently attack benzenesulphonates (Thurnheer *et al.*, 1986; Zürrer *et al.*, 1987; Locher *et al.*, 1989) or naphthalenesulphonates (Brilon *et al.*, 1981; Ohe & Watanabe, 1988; Wittich *et al.*, 1988) have only recently been isolated, using specific enrichment techniques.

Among a collection of 17 isolates which degraded benzenesulphonates, strain O-1, tentatively identified as a *Pseudomonas* sp. by a few criteria, was found to be the most versatile, degrading three different analogues

(Thurnheer *et al.*, 1986). A large plasmid has been detected in this strain (Auling, 1986, Report on EMBO Fellowship no. ASTF 5111) and a plasmid-mediated biodegradative capacity was therefore suspected. Analogous examples of plasmid-mediated degradation of a diversity of aromatic pollutants are well documented (for a review see Frantz & Chakrabarty, 1986).

We now report on the identification of isolate O-1, and the contribution of a 117 MDa plasmid to the biodegradation of orthanilic acid by this organism. Intra- and interspecies transmissibility of this plasmid was examined as was its relationship to the archetypal TOL plasmid, pWW0 (Williams & Murray, 1974).

Methods

Bacterial strains and plasmids. These are listed in Table 1.

Media, culture conditions and analysis of substrate utilization. The mineral medium described by Thurnheer *et al.* (1986) was supplemented with either 6 mM-orthanilic acid (MO) or 10 mM-succinic acid (MS). The biodegradative strains were maintained under selective conditions (*Alcaligenes* sp. O-1 on MO agar; *Pseudomonas putida* PaW1 on 5 mM-*m*-toluate agar as described by Williams & Murray, 1974). The complex medium (PYE) used in some experiments has been described by Busse & Auling (1988). Antibiotics were added as indicated. Cultures were incubated at 30 °C unless otherwise stated.

Abbreviations: % D, degree of binding; PYE, peptone yeast extract; SSC, standard saline citrate; TBE, Tris/borate/EDTA; TE, Tris/EDTA.

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Table 1. *Bacterial strains and plasmids*

Strain	Relevant characteristics		Source or reference
<i>Alcaligenes faecalis</i>	DSM 30030 ^T		K. H. Hinz Kerstens & De Ley (1984)
<i>piechaudii</i>	NCTC 11970 ^T		P. Kämpfer Kiredjian <i>et al.</i> (1986)
<i>xylosoxidans</i> subsp. <i>denitrificans</i>	NCTC 8582 ^T	Formerly <i>Alcaligenes denitrificans</i>	K. H. Hinz Kerstens & De Ley (1984)
<i>xylosoxidans</i> subsp. <i>xylosoxidans</i>	KM 534 ^T	Formerly <i>Achromobacter xylosoxidans</i>	K. H. Hinz Kiredjian <i>et al.</i> (1986)
sp.	O-1	pSAH, pME1702; degradation of benzenesulphonates	Thurnheer <i>et al.</i> (1986)
sp.	Cur30	pSAH cured; pME1702; Cm ^R , Km ^R ; degradation-negative derivative of strain O-1	This work
' <i>Alcaligenes paradoxus</i>	DSM 66 ^T		DSM Kerstens & De Ley (1984)
<i>eutrophus</i>	DSM 531 ^T		DSM Kerstens & De Ley (1984)
<i>Comamonas acidovorans</i>	DSM 39 ^T	Formerly <i>Pseudomonas acidovorans</i>	DSM Tamaoka <i>et al.</i> (1987)
<i>Pseudomonas putida</i>	mt-2	Obtained as PaW1; pWW0; degradation of <i>m</i> -toluate	P. A. Williams Williams & Murray (1974)
<i>putida</i>	PaW130	plasmid-free derivative of <i>P. putida</i> PaW1; Rif ^R	P. A. Williams Keil <i>et al.</i> (1985)
<i>putida</i>	Ex5, Ex11	pSAH, pME1702; exconjugants of <i>P. putida</i> PaW130 following matings with <i>Alcaligenes</i> sp. O-1	This work
<i>Escherichia coli</i>	PM191	pWW0-3004, 2.25 kb <i>Xho</i> I fragment carrying <i>xylE</i> from the TOL plasmid pWW0	P. A. Williams Chatfield & Williams (1986)

Abbreviations: Cm, chloramphenicol; Km, kanamycin; Rif, rifampicin; R, resistance; T, type strain; Strains denoted K. H. Hinz were kindly provided by Dr. Hinz, Tierärztliche Hochschule, Hannover, FRG., those denoted P. A. Williams were from Dr. P. A. Williams, University College of North Wales, Bangor, Gwynedd, UK., and the strain denoted P. Kämpfer was from Dr. P. Kämpfer, Technische Universität Berlin, FRG. DSM, German Collection of Microorganisms and Cell Cultures Ltd., Braunschweig, FRG; NCTC, National Collection of Type Cultures (Central Public Health Laboratory), London, UK.

High-performance-liquid-chromatography (HPLC) of orthonilic acid was done with a Beckman apparatus (model 332) and an UV absorbance detector (model 160) according to Thurnheer *et al.* (1986). Absorbance was measured at 254 nm.

Enzymes and chemicals. Orthonilic acid (97%) was purchased from Fluka, as was formamide; restriction endonucleases were from BRL or Boehringer and were used in accordance with the manufacturer's specifications; agarose was from Pharmacia. All other chemicals were obtained from Merck and were of reagent grade or better.

Preparation of cell extracts and enzyme assay. In order to detect inducible proteins specific for degradation of orthonilic acid, cells grown in MS were used as a 10% (v/v) inoculum for growth in MO. The inoculum was washed in saline at room temperature prior to resuspension in prewarmed MO. Biodegradation was carried out in a 10 litre fermenter at 30 °C, 300 r.p.m. and at an aeration rate of 3 litres min⁻¹. Samples of biomass were harvested (10000 g, 10 min, 4 °C) at different growth phases for determination of desulphonation activity (Thurnheer *et al.*, 1986) and polyacrylamide gel electrophoresis (PAGE) of cellular proteins (Auling *et al.*, 1986). For SDS-PAGE, 20 µl samples of cell extracts were supplemented with 1 µl 10% (w/v) SDS and 1 µl 2-mercaptoethanol prior to heat denaturation (100 °C, 5 min). Samples were analysed on an 8–15% (w/v) polyacrylamide

gradient gel. Reference molecular mass standards were purchased from Pharmacia.

Taxonomic methods. Biochemical features, identification and probability calculation (API 20NE) of isolate O-1 were examined according to the instructions of the manufacturer (api bioMérieux GmbH, Nürtingen, FRG). Polyamines were prepared and analysed according to Busse & Auling (1988). Chromatography of quinones, isolation of DNA, determination of the G + C content and DNA:DNA hybridization followed procedures previously described (Auling *et al.*, 1986). Prior to renaturation experiments at 61 °C using a buffer containing 20% (v/v) formamide (puriss. p.a.), the DNA concentration was determined according to Richards (1974). Preparation of anti-*Alcaligenes* sp. O-1 serum raised in rabbits and agglutination tests were carried out essentially as described by Auling *et al.* (1978). For double immunodiffusion assays (Ouchterlony, 1949) small petri dishes (5 cm in diameter) containing a 5 ml layer of 1% (w/v) agarose were incubated at 37 °C in a humidity chamber. The precipitation lines were stained and visualized as described by Chase (1967).

Plasmid isolation, digestion and hybridization. For plasmid analysis, a modification of the method of Eckhardt (1978) was followed. Colonies grown on PYE were suspended in 20 µl TBE buffer (Maniatis *et al.*, 1982) containing 20% (w/v) sucrose and 7% (w/v) Ficoll 400. A solution

(10 µl) of lysozyme (1 mg ml⁻¹) and RNAase (10 µg ml⁻¹) was added immediately before loading 10 µl of this mixture into the wells of a horizontal 0.5% agarose gel, containing 0.5% SDS. Complete lysis was achieved by adding 10 µl TBE containing 30% (v/v) glycerine, 7% (w/v) SDS and 0.05% bromophenol blue. Electrophoresis was carried out at 20 V for 30 min, then at 80 V for 3 h.

For preparative isolation of plasmid DNA, the alkaline SDS lysis procedure of Kado & Liu (1981), modified by Nies *et al.* (1987), was used routinely.

To prepare plasmid pSAH free of either contaminating chromosomal or pME1702 DNA the procedure of Wheatcroft & Williams (1981) was basically followed. Cleared lysates obtained from 50 ml freshly harvested cells (Nies *et al.*, 1987) were subsequently ethanol-precipitated at 4 °C overnight (Maniatis *et al.*, 1982), resuspended in TE buffer (10 mM-Tris/HCl, 10 mM-EDTA; pH 8), and finally concentrated (Centricon C10, Amicon) to approximately 500 µl. This plasmid solution was layered onto a preformed 16 ml sucrose gradient (Baxter-Gabbard, 1972) followed by centrifugation at 100000 *g* and 20 °C for 4 h. Fractions (0.5 ml) were collected and analysed by agarose gel electrophoresis. Fractions containing pure pSAH DNA were combined and concentrated by ethanol precipitation at -20 °C.

Restriction fragments were either purified with a glassmilk solution (Geneclean Kit, Bio101 Inc.) or directly random primed-labelled with digoxigenine-dUTP (Boehringer) before use as probes in hybridizations (Southern, 1975) on nitrocellulose filters. Because of the high sensitivity of the technique only 1 µg of digested DNA was submitted to electrophoresis (0.8%, w/v, agarose, 20 V, 16 h). Hybridizations were done at 68 °C following the manufacturer's protocol with SSC (150 mM-NaCl and 15 mM-sodium citrate, pH 7.0) as the standard buffer. Hybridization stringency was calculated according to Meinkoth & Wahl (1985); 0.1 × SSC was used for high-, and 0.6 × SSC for low-stringency conditions.

Plasmid curing and bacterial conjugation. For plasmid curing experiments, cells were repeatedly subcultured in PYE at either 27, 37, or 42 °C according to Trevors (1986). Cultures were incubated at the corresponding temperature until the late exponential phase of growth, diluted tenfold and repeatedly re-incubated until this phase of growth was reached again. Samples were first spread on PYE agar followed by replica plating on MO agar for selection of mutants incapable of degrading orthonilic acid. Clones were purified and loss of mineralization capability was confirmed by HPLC of supernatants from liquid cultures.

For mating experiments, overnight cultures were mixed, spotted onto nitrocellulose filters (0.2 µm pore size; Sartorius) and finally placed on PYE agar. After an appropriate incubation period the cells were resuspended from the filters and plated onto selective medium.

Exconjugants from matings (24 h) with *Alcaligenes* sp. Cur30 as recipient were selected on MO supplemented with chloramphenicol and kanamycin (each 100 µg ml⁻¹). Exconjugants from matings (72 h) with *P. putida* PaW130 as recipient strain were selected on MO supplemented with 300 µg rifampicin ml⁻¹.

Results

Identification of isolate O-1

A two-step procedure for identification of xenobiotic-degrading Gram-negative aerobic bacteria has been proposed (Busse *et al.*, 1989). This involves rapid identification at the genus level by chemotaxonomic methods and subsequent allocation to a species by DNA:DNA hybridization and other methods.

When the quinone system of strain O-1 was analysed, only a ubiquinone with eight isoprenoid units in its side chain (Q-8) was detected. This finding excludes O-1 from species of the fluorescent pseudomonads (Oyaizu & Komagata, 1983; Auling *et al.*, 1986) and from the genus *Acinetobacter*, which both possess an ubiquinone Q-9 and are also well known for their biodegradation potential (Dagley, 1986). The presence of 2-hydroxyputrescine and putrescine as dominant polyamines in strain O-1 suggests its affiliation to the beta subclass of the Proteobacteria (Busse & Auling, 1988).

The multiple-test system API 20NE identified strain O-1 as *Alcaligenes denitrificans* with a probability of 69.9%. However, the taxonomic status of the genus *Alcaligenes* is controversial (De Ley *et al.*, 1986; Busse & Auling, 1991). In order to allocate strain O-1 either to species within the genus *Alcaligenes* as defined at present (Kiredjian *et al.*, 1986) or to those which are phylogenetically distant even from the family *Alcaligenaceae* (De Ley *et al.*, 1986), an antiserum was raised against this isolate. The antiserum obtained had a high titre (>1:1024) in the homologous reaction. In the heterologous reactions with type strains of the genus *Alcaligenes*, the following titres were obtained: *Alcaligenes xylosoxidans* subsp. *xylosoxidans* KM 534 (1:512), *Alcaligenes xylosoxidans* subsp. *denitrificans* NCTC 8582 (1:64), *Alcaligenes faecalis* DSM 30030 (1:8), *Alcaligenes piechaudii* NCTC 11970 (<1:8). In Ouchterlony double diffusion assays (Fig. 1a, b) strain O-1 and *Alcaligenes xylosoxidans* subsp. *xylosoxidans* KM 534 displayed the highest cross-reactivity (both three to five precipitation lines). With *Alcaligenes xylosoxidans* subsp. *denitrificans* NCTC 8582, two precipitation lines were visible. Partial serological relatedness, indicated by spur formations, was observed to *Alcaligenes faecalis* DSM 30030, '*Alcaligenes*' *eutrophus* DSM 531 and *Comamonas acidovorans* DSM 39.

Similarity of strain O-1 with both reference strains of *Alcaligenes xylosoxidans* was also observed by PAGE of cellular proteins (data not shown). The G + C content of 66.1 mol%, obtained by six determinations, is not contradictory to an allocation of strain O-1 to either of the two subspecies. DNA homology measurements using the initial renaturation rate method of De Ley *et al.* (1970) confirmed a relatively close relatedness of O-1 to both type strains, *A. xylosoxidans* subsp. *xylosoxidans* KM 534 and *A. xylosoxidans* subsp. *denitrificans* NCTC 8582. The values obtained (51% *D* to NCTC 8582 and 40% *D* to KM 534) did not allow the inclusion of strain O-1 in either of the two subspecies.

Plasmid analysis

Two plasmids were detected in *Alcaligenes* sp. O-1. The molecular masses of the plasmids were estimated from

Fig. 1. Cross-reactivity of anti-*Alcaligenes* sp. O-1 serum with bacterial homogenates from related (a) and phylogenetically less related (b) species from the beta-subclass of Proteobacteria, and from *P. putida* PaW130 exconjugants (c). The higher number of precipitation lines visible in (c) is due to a more intensive staining procedure for detection of even weak cross-reactivity. Approximately 30 µg protein of bacterial homogenates, prepared by ultrasonic treatment (30 000 g supernatants) were challenged with 30 µl of anti-*Alcaligenes* sp. O-1 serum (central well). Abbreviations: A.x, *Alcaligenes xylosoxidans* subsp. *xylosoxidans* KM 534^T; A.d, *Alcaligenes xylosoxidans* subsp. *denitrificans* NCTC 8582^T, identical with DSM 30026; A.e, '*Alcaligenes*' *eutrophus* DSM 531^T; A.f, *Alcaligenes faecalis* DSM 30030^T; A.p, '*Alcaligenes*' *paradoxus* DSM 66^T; P.a, *Comamonas acidovorans* (formerly *Pseudomonas acidovorans*) DSM 39^T; PaW, recipient *Pseudomonas putida* PaW130; Ex₅, Ex₁₁, exconjugants *P. putida* Ex5 and *P. putida* Ex11; O-1, *Alcaligenes* sp. O-1, control for the homologous reaction (a superscript T denotes the type strain of the genus or species).

Fig. 2. Analysis of the plasmid content of *Alcaligenes* sp. O-1 and various 'cur' mutants. Following DNA gel electrophoresis (a), hybridization of the corresponding Southern blots (b) with digoxigenine-dUTP-labelled pSAH DNA (150 ng ml⁻¹) was carried out in 0.1 × SSC. Tracks contained: A, *P. putida* PaW130; B, N, *P. putida* mt-2; C, M, *Alcaligenes* sp. O-1 (positive control); D to L, different 'cur' mutants. chr, chromosomal DNA.

three independent electrophoresis runs with reference plasmids (Rochelle *et al.*, 1985) as 117 and 6.8 MDa, respectively (data not shown). Restriction endonuclease digestion patterns (*Eco*RI) confirmed these estimations (see Fig. 4). The larger plasmid was designated pSAH (sulphoaromatics, high molecular mass) and the smaller, at present under investigation in the Microbiology Institute of the ETH Zürich, Switzerland, designated pME1702 (T. Leisinger, personal communication).

Instability and conjugative transfer of the degradative activity

Alcaligenes sp. O-1 grows well at 30 °C (optimum temperature) with orthonilic acid as sole source of carbon and energy (Thurnheer *et al.*, 1986). However, when cultures were incubated at 42 °C the ability to degrade orthonilic acid was completely lost within 20 generations. Clones which failed to degrade orthonilic

Fig. 3. Induction of specific polypeptides and desulphonation activity during growth of *P. putida* Ex5 with orthanilic acid as sole carbon and energy source. (a) Protein patterns (SDS-PAGE) of the following cultures: A, MS inoculum culture, late exponential phase; B–F, growth with orthanilic acid after 12 h (B), 25 h (C), 30 h (D), 40 h (E) and 50 h (F); G, *Alcaligenes* sp. O-1 after 50 h (control). The empty arrowheads (◁) mark the four polypeptides which are distinctly induced during growth of *P. putida* Ex5 on orthanilic acid. The two of these which appeared concomitantly with the desulphonation activity are marked with full arrowheads (◄). (b) Growth (●), desulphonation activity (▲) and orthanilic acid concentration (■) during the above experiment.

acid were isolated and designated 'cur' mutants. At either 27 °C or 37 °C, no loss of the biodegradative capability was observed within the equivalent experimentation period. However, upon prolonged cultivation (more than 50 generations) on rich medium (PYE) at 37 °C spontaneous loss of the ability to grow on MO also occurred at a low (0.1%) rate (data not shown).

All of the 'cur' mutants analysed retained the small plasmid pME1702, but plasmid pSAH, present in the wild-type, was not detectable in any of them (Fig. 2). To exclude the possibility of integration of pSAH into the bacterial chromosome as a result of the curing treatment

(Sinclair *et al.*, 1986) Southern hybridization analysis with pSAH as probe was carried out. Sequence homology to pSAH could not be detected within either chromosomal or pME1702 DNA in any of the 'cur' mutants (Fig. 2) although less than 0.05 pg of homologous pSAH DNA was clearly detectable in control experiments following the specifications of the manufacturer.

The capability of *Alcaligenes* sp. O-1 to mineralize orthanilic acid is therefore strongly correlated with the presence of the large plasmid pSAH.

Intraspecies conjugation could be demonstrated between the donor *Alcaligenes* sp. O-1 and the recipient

Fig. 4. Comparison of plasmids resident in *Alcaligenes* sp. O-1 with the archetypal TOL plasmid pWW0. (a) Agarose gel electrophoresis of restriction fragments (*Eco*RI) of plasmids pSAH and pME1702 from *Alcaligenes* sp. O-1 (A) and plasmid pWW0 from *P. putida* mt-2 (B). Fragments corresponding to pME1702 are arrowed (►). Fragment sizes are given in kb. Hybridizations were done either with fragments of plasmid pWW0, under stringent conditions (b), or with the *Pvu*II restriction fragment of *xylE* (catechol 2,3-dioxygenase) from plasmid pWW0, under less stringent conditions (c). Digoxigenine-dUTP-labelled DNA (100 ng ml⁻¹ of pWW0 or 50 ng ml⁻¹ of the *xylE* fragment) was used as probe.

Alcaligenes sp. Cur30. This 'cur' mutant regained the ability to grow on orthonilic acid concomitantly with the restoration of plasmid pSAH (not shown). No spontaneous mutation processes interfered with the observed frequency of conjugation (typically 1×10^{-6} per donor). A coupled interspecies transfer of pSAH and pME1702 was observed between *Alcaligenes* sp. O-1 as donor and *P. putida* PaW130 as recipient (not shown). *P. putida* PaW130 exconjugants acquired the ability to grow on orthonilic acid with a typical conjugational frequency of 1×10^{-6} per donor. If the donor strain *Alcaligenes* sp. O-1 was omitted from these matings, no biodegradative clones could be obtained.

Neither *P. putida* PaW130 nor the corresponding exconjugants cross-reacted with anti-*Alcaligenes* sp. O-1 serum, either in agglutination ($<1:8$) or in Ouchterlony assays (Fig. 1c). The exconjugants had a polyamine pattern representative of the fluorescent pseudomonads (Busse & Auling, 1988). Upon spraying with catechol the exconjugants, grown on orthonilic acid, formed a yellow compound representing 2-hydroxymuconic semialdehyde (Inouye *et al.*, 1983). This test indicated that they had acquired a new catechol 2,3-dioxygenase activity upon conjugation because the plasmid-free recipient does not possess an equivalent enzyme activity (Keil *et al.*, 1985). Polypeptides of identical molecular masses were specifically induced during growth on orthonilic acid in both the exconjugants and the donor *Alcaligenes* sp. O-1 (Fig. 3), namely two polypeptides of 61 and 45 kDa in the early exponential and two more of 43 and 28 kDa in the mid-exponential phase of growth. A fifth polypeptide of 34 kDa, present in strain O-1, was only weakly induced in the exconjugant *P. putida* Ex5. The exconjugant displayed the highest activity of desulphonation in the late exponential phase of growth, as was observed for the donor *Alcaligenes* sp. O-1 by Thurnheer *et al.* (1986). The decline of desulphonation activity during the stationary phase of growth explains the extremely long lag phase observed with cultures of both donor and exconjugant after reinoculation from induced, but stationary-phase cells (Fig. 3).

Comparison of pSAH with the archetypal TOL plasmid pWW0

The archetypal TOL plasmid pWW0 is the most extensively studied among the related (Lehrbach *et al.*, 1983) degradative plasmids which mediate mineralization of substituted aromatic compounds (Burlage *et al.*, 1989). In order to examine the possibility that pSAH has evolved from pWW0, the molecular relationship of the two plasmids was studied. With restriction fragments of total pWW0 as a probe no homology could be detected (Fig. 4b, A) under conditions of either high or low stringency in Southern blot hybridizations of plasmid DNA from *Alcaligenes* sp. O-1. Hybridization with the internal *Pvu*II restriction fragment of pWW0-3004 (Chatfield & Williams, 1986) which carries the TOL-specific *xylE* gene (catechol 2,3-dioxygenase), showed no homology either (Fig. 4c, A). However, a clear signal was found at the 7.8 kb position (fragment E, Burlage *et al.*, 1989) of *Eco*RI-digested pWW0-DNA as expected (Fig. 4c, B). Hybridization of total DNA of *Alcaligenes* sp. O-1 with the same probe also revealed no signal (data not shown), indicating that no catechol 2,3-dioxygenase related to *xylE* is present in this strain.

Discussion

The taxonomic methods applied placed isolate O-1 in the neighbourhood of either *Alcaligenes xylosoxidans* subsp. *denitrificans* NCTC 8582 (formerly *Alcaligenes denitrificans*) or *Alcaligenes xylosoxidans* subsp. *xylosoxidans* KM 534 (formerly *Achromobacter xylosoxidans*). This has been confirmed by serological techniques and PAGE, both recommended as reliable taxonomic markers for Proteobacteria (Murray *et al.*, 1990). According to the definition of Johnson (1984) the values obtained for DNA:DNA homology with the reference strains of the two subspecies (51% and 40%) are too low to allow incorporation of isolate O-1 in either of these taxa. Further studies including more strains would be necessary to establish a new species of *Alcaligenes* to accommodate isolate O-1. As a consequence of this, and as the nomenclatural status of the genus *Alcaligenes* is still under dispute (Kerstens and De Ley, 1984; De Ley *et al.*, 1986; Kiredjian *et al.*, 1986; Busse & Auling, 1991), formal taxonomic proposals for a species designation for isolate O-1 are avoided at present.

The large plasmid pSAH (117 MDa) present in *Alcaligenes* sp. O-1 is required for mineralization of orthanilic acid whereas the small plasmid pME1702 (6.8 MDa) remains cryptic. This was concluded from the observations that (i) curing of pSAH coincided with loss of the capacity to degrade orthanilic acid, and (ii) subsequent re-establishment of the original phenotype was achieved upon introduction of pSAH into the 'cur' mutant *Alcaligenes* sp. Cur30. Hybridization of total bacterial DNA with purified plasmid pSAH as a probe verified physical loss of plasmid pSAH in all 'cur' mutants derived from *Alcaligenes* sp. O-1. This observation excluded any integration of pSAH into the bacterial chromosome, a result often observed in curing experiments with other metabolic plasmids (Jeenes & Williams, 1982; Meulien & Broda, 1982; Eaton & Timmis, 1986; Sinclair *et al.*, 1986). The small plasmid pME1702 seemed to be rather stable, as no treatment (heat, ethidium bromide and acridine orange) of the 'cur' mutants resulted in plasmid-free derivatives, which was concluded from colony hybridizations of more than 2000 clones, using pME1702 as a probe (M. Jahnke, unpublished results).

With regard to the conjugative transfer of plasmids pSAH and pME1702 into *P. putida* PaW130 we assume that only the large plasmid pSAH is self-transmissible. Plasmid pME1702 may be mobilized passively because it seems to be too small to encode all the gene functions necessary for its own conjugative transfer. Plasmid pSAH possibly has a rather broad host range as it mediates gene transfer between *Alcaligenes* sp. and *Pseudomonas putida*, which belong to the beta and

gamma subclasses, respectively, of the Proteobacteria (Stackebrandt *et al.*, 1988).

Upon conjugative transfer of pSAH from *Alcaligenes* sp. O-1 into a foreign genetic background (*P. putida*) no difference between donor and recipient was detected with regard to the expression of the orthanilic acid degradation phenotype. For example, growth rate, growth yield, retarded induction of both growth and specific polypeptides were identical (this paper; M. Jahnke, unpublished results). This indicates that a complete set of genes (both regulatory and structural) for transport and mineralization of orthanilic acid may be clustered on this large degradative plasmid. Strains free of plasmid pSAH neither grew on nor attacked orthanilic acid.

In order to estimate the number of degradative gene functions mediated by pSAH, Tn5 mutagenesis of *Alcaligenes* sp. O-1 is currently being done in our laboratory. Thus far it cannot be excluded that pSAH either mediates just the initial attack on the sulphonic substituents or provides only regulatory factors necessary for efficient degradation of orthanilic acid. Therefore it would be interesting to study expression of the orthanilic acid degradation phenotype upon conjugative transfer of pSAH into a recipient completely unable to mineralize aromatic compounds.

On the other hand, horizontal gene transfer has been discussed by Thurnheer *et al.* (1988) to explain results from experiments with a defined co-culture established for improved biodegradation of seven different sulphonated aromatic compounds. As *Alcaligenes* sp. O-1 was a member of this co-culture, we suspect that conjugative transfer of pSAH was involved in such events which resulted in strains of improved biodegradation capability. The calculated transfer frequencies for intra- and interspecies conjugation of pSAH were in the range reported for other catabolic plasmids, e.g. SAL (Chakrabarty, 1972), TOL (Williams & Murray, 1974) and NAH (Dunn & Gunsalus, 1973; Ferrer *et al.*, 1986). These degradative plasmids of *Pseudomonas* spp. encoding the genes for the catechol *meta*-cleavage pathway (Frantz & Chakrabarty, 1986) were all shown to be appreciably homologous with one another (Farrell & Chakrabarty, 1979; Lehrbach *et al.*, 1983). An evolutionary inter-relationship of TOL (pWW0) with the new degradative plasmid pSAH of *Alcaligenes* sp. O-1 was originally suspected. However, our results indicate no significant homology between these two plasmids (assuming a sufficient sensitivity of the non-radioactive technique applied). The heterogeneity of the genes encoding catechol 2,3-dioxygenase in pWW0 and pSAH indicates the presence of a new enzyme in *Alcaligenes* sp. O-1 because the *meta*-cleavage pathway is involved in the degradation of orthanilic acid (A. Cook, personal

communication). This was concluded from the inhibition of ring cleavage by 3-chlorocatechol (Thurnheer *et al.*, 1990). Recent results (Keil, 1990; Saint *et al.*, 1990) imply that the structural gene for catechol 2,3-dioxygenase may have been evolved independently several times.

Although the degradative plasmids pSAH and pWW0 display no homology they may interact in a common host as both are conjugative. However, our preliminary data show that pSAH and pWW0 cannot coexist in *Alcaligenes* sp. O-1, indicating that plasmid pSAH might belong to the IncP-9 group (Lehrbach *et al.*, 1983). The recombination of catabolic genes resulting from conjugative interference of pSAH and pWW0 is under investigation in our laboratory, using both conventional and microcosm experiments.

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