Transposition of the TOL catabolic genes (Tn4651) into the degradative plasmid pSAH of Alcaligenes sp. O-1 ensures simultaneous mineralization of sulpho- and methyl-substituted aromatics

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Mixtures of 2-aminobenzenesulphonate (trivial name orthanilic acid, OA) and 3-methylbenzoic acid (3-MB), which are degraded by enzymes of plasmid-encoded pathways, can exert inhibition of growth and respiration in Alcaligenes sp. O-1 and Pseudomonas putida mt-2 depending on the ratio of their concentrations. The pronounced inhibition of Alcaligenes sp. O-1 growing on OA by the addition of equimolar amounts of 3-MB is characterized by a rapid inactivation of the OA-converting desulphonation activity. The exconjugant Alcaligenes sp. O/T was selected for simultaneous breakdown of OA and 3-MB by assembling the catabolic pathways from the plasmids pSAH (OA) and pWW0 (3-MB) of the above strains. The transpositional insertion of the TOL catabolic genes (Tn4651) from pWW0 into the recombinant plasmid of the exconjugant O/T was detected by Southern blot hybridization using the TOL plasmid as a probe. The exconjugant showed a rapid inactivation of OA desulphonation activity similar to the parent strain. However, following induction of the TOL catabolic genes and mineralization of 3-MB, the exconjugant O/T recovered and displayed high desulphonation activity, thus allowing sequential breakdown of both substrates. Our results clearly extend the expression range of the TOL catabolic genes, but not the replication ability of the plasmid, to the genus Alcaligenes.

Introduction

Although there are numerous reports on the bacterial biodegradation of aromatic compounds, the area of mixed substrate interactions has been grossly neglected. The environmental aspects of microbial utilization of mixed substrates were discussed by Weide (1983). In a natural environment, it is unlikely that micro-organisms will be faced with single aromatic compounds; in polluted sites mixtures are more likely to occur. Studies with a growth-supporting primary substrate and a non-growth-supporting secondary ( xenobiotic) substrate have established that certain mixtures are degraded more rapidly than xenobiotic compounds presented individually (Smith, 1990). There are only a few reports of interactions between biodegradable aromatic compounds when both are presented as growth-supporting substrates. Stimulating and antagonistic effects have been observed (Bauer & Capone, 1988; Arvin et al., 1989).

It is well established that most bacteria able to catabolize aromatic compounds cannot simultaneously degrade chloro- and methyl-substituted substrates because the pathways involved are incompatible, i.e. chlorocatechols generated via ortho-ring fission inactivate the 2,3-dioxygenase required for meta-cleavage of methyl-substituted aromatics. So far, defined mixed cultures (Schmidt et al., 1983) and construction of genetically engineered strains (Rojo et al., 1987) have been the approaches to avoid these restrictions on simultaneous biodegradation.

We observed that methyl-substituted aromatics interfered with the mineralization of sulphonated aromatic compounds (Jahnke, 1990); i.e. the growth of Alcaligenes sp. O-1 on orthanilic acid ceases immediately upon addition of 3-methylbenzoic acid (3-MB) which itself is not mineralized. On the other hand, the presence of...
orthaniilic acid (OA) may inhibit the growth of *Pseudomonas putida* mt-2 on its substrate 3-methylbenzoate. The xenobiotic compounds are readily biodegradable by the respective strains when presented individually.

Since both xenobiotic substrates are degraded by plasmid-encoded pathways (Williams & Murray, 1974; Jahnke et al., 1990), we speculated that the inability to degrade the sulpho- and methyl-substituted aromatics simultaneously could be overcome by a natural gene transfer. Consequently, the objective of this study was a selection for events which would allow the assembly and function of both degradation pathways in either of the two strains involved.

### Methods

**Bacterial strains and plasmids.** The degradative strains *Alcaligenes* sp. O-1, encoding OA-mineralization on plasmid pSAH (Jahnke et al., 1990) and *Pseudomonas putida* mt-2 harbouring the archetypal TOL plasmid pWW0 (Williams & Murray, 1974) and their derivatives were used (Table 1).

**Media, culture conditions and analysis of substrate utilization.** The procedures were as described by Jahnke et al. (1990). In the shake-flask experiments the substrates were added simultaneously in the range of concentrations indicated in Fig. 1.

Respiration of 3 ml cell suspensions was measured in the presence of 3 mM substrate with a Clark-type oxygen electrode and an oxygen analyser (model 53, Yellow Springs Instrument Co.) in a thermostatically (27 °C) controlled vessel as described previously (Siegmund & Diekmann, 1989). Cultures induced by growth (3 d) on the substrate were diluted in growth medium and adjusted to an OD₆₀₀ of 0.4 for strains O-1 and O/T with OA, 0.3 for strain O/T with 3-MB, and 0.15 for strain mt-2. The primary respiration rate was monitored for 10 min. Then 0.1 ml of the second substrate was injected to the final concentrations indicated in the text and the new respiration rate was monitored for a further 10 min.

**Preparation of cell extracts and enzyme assay.** The methods of Jahnke et al. (1990) were followed except for two modifications: (a) succinate-grown cultures were diluted 1:10 in 6 mM-OA-mineral medium, incubated overnight in 100 ml shake-flasks for use as 10% (v/v) inocula in the 10 litre bioreactor; (b) the second substrate, 3 mM-3-MB, was subsequently added to mid-exponential phase cells grown on OA as indicated in the text.

Desulphonation activity in cell extracts was determined by the method of Thurnheer et al. (1986). In contrast, release of sulphite from bacterial colonies was assayed on mineral agar according to Johnston et al. (1975). OA dioxygenase was assayed by O₂ uptake at 30 °C as described above in 3 ml cell extracts containing 2 mg protein ml⁻¹ and 0.15 mM-NADH. Crude extract was centrifuged for 90 min at 15000 g and at 4 °C and the supernatant diluted with 75 mM-Tris/HCl, pH 7.3, to adjust the protein concentration. Protein was determined according to Bradford (1976). The reaction was started by injection of OA to a final concentration of 3 mM. The endogenous O₂ consumption rate, recorded in the absence of substrate, was subtracted from the overall reaction rate. For certain experiments, Sephadex G-25 columns (PD-10, Pharmacia) were used to eliminate interfering low molecular mass compounds from the crude extracts prior to enzyme assays. The proteins were eluted with 50 mM-Tris/HCl, pH 7.3.
Expression of TOL genes in Alcaligenes

Bacterial conjugation. The mating experiments were carried out as described previously (Jahnke et al., 1990), except that additional markers were used. Ta3 was introduced into Alcaligenes sp. O-1 for counterselection with kanamycin the chromosomal location of this insertion being confirmed by further matings with the plasmid-free recipient P. putida PaW130 (Jahnke, 1990). Only the two plasmids of the donor Alcaligenes sp. O-1::Ta3 were transferred. Secondly, the archetypal TOL plasmid pWW0 was transferred by conjugation into P. putida PaW130. This strain has a chromosomally encoded resistance towards rifampicin (Keil et al., 1985) and was subsequently named P. putida PaW130TOL (Table 1).

Exconjugants of Alcaligenes sp. O-1 from the mating experiments were (i) confirmed by a high titre (1:1024) using a specific antiserum against Alcaligenes sp. O-1 (Jahnke et al., 1990) and (ii) chemotaxonomically differentiated by their polyamine pattern from any P. putida PaW130TOL donor clones that may have acquired a kanamycin resistance spontaneously. Exconjugants of Alcaligenes sp. O-1 contain 2-hydroxyputrescine as a beta-subclass-specific marker (Busse & Auling, 1988); this is not present in authentic pseudomonads (Auling, 1989). Exconjugants of Alcaligenes sp. O-1 were previously described by Jahnke et al. (1990).

Plasmid isolation, digestion and hybridization. The methods employed were previously described by Jahnke et al. (1990).

Results

Growth inhibition of Alcaligenes sp. O-1 and P. putida mt-2 by mixed aromatic substrates

A previous study on plasmid-encoded breakdown of OA by Alcaligenes sp. O-1 (Jahnke et al., 1990) demonstrated that this property was encoded by a large conjugative plasmid, pSAH. Plasmid pSAH shows no homology to the archetypal TOL plasmid pWW0 which confers the ability to catabolize toluene or 3-MB (as the soluble substrate) on P. putida mt-2. Each strain is unable to degrade the aromatic substrate of the other. In the presence of both aromatic compounds the degradation of either OA by Alcaligenes sp. O-1 or of 3-MB by Pseudomonas putida mt-2 was increasingly inhibited depending on the ratio and the concentrations provided (Fig. 1). The inhibitory effect of 3-MB on the degradation of OA in Alcaligenes sp. O-1 was more pronounced than that exerted by OA on 3-MB degradation in P. putida mt-2. Some relief of inhibition occurred by increasing the concentrations of the primary substrate of both strains. Nevertheless, it appeared that the inhibition of OA mineralization in Alcaligenes sp. O-1 could not be overcome above a threshold concentration of 2 mM-3-MB.

These concentration-dependent inhibition phenomena were further studied by additional respiration measurements with cells induced by either aromatic compound. The respiration of Alcaligenes sp. O-1 with OA was increasingly inhibited by raising the concentration of 3-MB (Fig. 2a). In analogous experiments, the respiration of P. putida mt-2 with 3-MB did not appear to be inhibited by raising the OA concentration. There was a distinct sensitivity of Alcaligenes sp. O-1 towards higher concentrations of 3-MB. Using the same assay and conditions as described for Fig. 2(a), the respiration of cells of strain O-1 was inhibited by addition of 9 mM-3-MB to various degrees, depending on the substrate used for previous growth. Thus there was only slight inhibition with succinate and benzoate (20 or 32% reduction only), whereas the inhibition was more pronounced with 4-toluenesulphonic acid, OA and benzenesulphonic acid (48, 53 and 55% reduction).

Because desulphonation is an essential step in the biodegradation of sulphonated aromatics by Alcaligenes sp. O-1 (Thurnheer et al., 1986), the inhibitory effect of 3-MB on this enzymic reaction was investigated. Using OA as substrate, the desulphonation activity of homogenates prepared from cells of strain O-1 grown on OA was progressively inhibited by increasing concentrations
of 3-MB (Fig. 2b). However, this enzymic reaction appeared to be less susceptible to inhibition by 3-MB than did the respiration.

In order to examine whether 3-MB was causing a reversible inhibition or an irreversible inactivation, the time-course of this inhibition was investigated at the enzymic level during growth in the following modified experiment. *Alcaligenes* sp. O-1 was induced on 6 mM-OA in a 10 litre bioreactor until the culture reached the mid-exponential phase of growth. Subsequently, 3-MB was added to a final concentration of 3 mM, i.e. equimolar to the residual OA present in the supernatant (Fig. 3). Upon addition of 3-MB, both growth and utilization of OA ceased immediately. The desulphonation activity of the fully induced culture fell from 20 μkat (kg protein)^{-1} to undetectable levels within 4 h. Neither recovery of growth on OA nor of desulphonation activity was observed with *Alcaligenes* sp. O-1.

Conjugation experiments

In order to combine the properties to degrade both sulpho- and methyl-substituted aromatics in one microorganism, matings were made between the two biodegra-
Expression of TOL genes in Alcaligenes

Fig. 5. (a) Inhibition and inactivation of the desulphonation activity (□) of the exconjugant Alcaligenes sp. O/T growing on 6 mm-OA (◇) in a 10 litre bioreactor by addition of 3 mm-3-MB (●) at an OD_{578} (■) of 0.65, and recovery by simultaneous growth on both compounds. (b) The time-course of induction of polypeptide patterns specific for either of the compounds was followed by SDS-PAGE of bacterial homogenates. The lanes contained (from left to right): pre-inoculum cells grown with succinate (A); cells grown with OA for 16 h (B); the same cells immediately (C) after addition of 3-MB, 6 h (D), 9 h (E) and 29 h (F) after addition of 3-MB, and (as a control) cells grown for 50 h with OA (G). The filled arrowhead (•) indicates the position of a polypeptide at 32 kDa which appeared concomitantly with the desulphonation activity and disappeared upon induction of 3-MB degradation. The open arrowheads (<) mark the two polypeptides induced during growth on 3-MB. The Figure comprises data of two cultivations.

Degradative strains Alcaligenes sp. O-1 and P. putida mt-2 after the introduction of additional markers for selection of exconjugants. Using Alcaligenes sp. O-1::Tn5 as donor and P. putida PaW130TOL as recipient, no exconjugants were obtained. However, exconjugants containing both degradative pathways were obtained in filter matings using P. putida PaW130TOL as donor and Alcaligenes sp. O-1::Tn5 as recipient. On mineral agar supplemented with kanamycin and both aromatic substrates (OA and 3-MB), the frequency of conjugation was typically 3 × 10^{-7} per donor. The frequency of spontaneous mutation of the donor P. putida PaW130TOL to Km' (typically 10^{-9}) necessitated additional characterization of the exconjugants. They were all serologically and chemotaxonomically (Fig. 4) confirmed as derivatives of Alcaligenes sp. O-1.

Degradative capabilities of the exconjugant Alcaligenes sp. O/T

The exconjugant Alcaligenes sp. O/T grew well overnight on minimal agar containing both OA and 3-MB. However, the colonies did not release sulphite during the first 12 h of rapid growth. This indicated preferred growth on 3-MB and delayed breakdown of OA. When added as mixed substrates in various ratios to shake-flask cultures, both OA and 3-MB were broken down, as evident by HPLC analysis of culture supernatants. When
The inhibition of respiration by 3-MB of the exconjugant O/T, grown on OA, was also investigated. Remarkably, the cells displayed the same rapid inhibition of respiration and even the same concentration dependence (Fig. 2a) as previously observed with the parental strain *Alcaligenes* sp. O-1.

The exconjugant O/T (Fig. 5) was grown in a 10 litre bioreactor with 6 mM-OA and 3-MB was added after the onset of growth (Fig. 3). However, contrary to the parental strain (cf. Fig. 3), the exconjugant O/T recovered from inhibition by 3-MB and resumed growth at a reduced growth rate \( (\mu_{\text{max}} \approx 0.05 \text{ h}^{-1}) \). During the 10 h inhibition period the concentration of both substrates, OA and 3-MB, remained constant. In the initial phase of growth on OA as the sole source of carbon and energy, the exconjugant O/T clearly displayed the characteristic protein pattern (Fig. 5) known from growth of the parental strain *Alcaligenes* sp. O-1 with OA (Jahnke *et al.*, 1990). Of the four OA-specific polypeptide bands (65, 43, 40, and 32 kDa), only the 32 kDa protein disappeared upon addition of 3-MB. Other additional polypeptide bands (63 and 60 kDa) were obviously induced by this second substrate. Enzyme assays revealed that the desulphonation activity of the exconjugant O/T, fully induced by growth on OA, fell to undetectable levels within 4 h of 3-MB addition (Fig. 5). A high OA dioxygenase activity (333 \( \mu \text{kat kg}^{-1} \)) was found immediately prior to addition of 3-MB; 4 h later, OA dioxygenase activity was no longer detectable. Eluates from PD-10 columns had a specific OA dioxygenase activity of 193 \( \mu \text{kat (kg protein)}^{-1} \) when prepared prior to the addition of 3-MB, but again no residual activity was found 4 h later. However, after recovery from inhibition, a high desulphonation activity (43 \( \mu \text{kat kg}^{-1} \)) was found in the late exponential phase of the second growth period, which was more or less characterized by simultaneous degradation of both substrates.

**Plasmid analysis of the exconjugant Alcaligenes sp. O/T**

Two plasmids were detected in the exconjugant O/T. One was identical in size with the small cryptic plasmid pME1702 of *Alcaligenes* sp. O-1 (Jahnke *et al.*, 1990). The other was larger than either pWW0 (donor) or pSAH (recipient). *Eco*RI restriction endonuclease digestion patterns showed its size to be about 193 kb (not shown). Digestion of the parent degradative plasmids
gave approximately 172 kb for pSAH and approximately 115 kb for pWW0 as expected. Comparison of the digestion patterns revealed fragments of both pWW0 and pSAH in the plasmid of the exconjugant (not shown). Southern blots of EcoR1 digests were hybridized against digoxigenin-dUTP-labelled DNA of the archetypal TOL plasmid pWW0 as probe. All fragments of pWW0 (Lehrbach et al., 1983) were present in the control, even when they were undetectable in the agarose gel. Strong hybridization signals were detected in the digest of the exconjugant plasmid (Fig. 6). Within the detection limit of the method used, there was no hybridization signal against chromosomal DNA when both chromosomal and plasmid DNA were separated following Eckhardt-lysis as described by Jahnke et al. (1990). The sum of the pWW0-specific fragments in the enlarged plasmid was calculated as 56 kb. The hybridization fragments were homologous to that region of pWW0 which harbours the TOL catabolic genes.

**Discussion**

The rapidity and severity of inhibition by 3-MB of respiration (Fig. 2) and of catabolism of sulphonated aromatics, observed both in the parent strain *Alcaligenes* sp. O-1 (Fig. 3) and the exconjugant O/T (Fig. 5), might indicate that an essential cofactor is inactivated. The apparent irreversible inactivation of the OA-metabolizing enzymes in both strains could also be explained by breakdown of only one protein component (the 32 kDa polypeptide) while the characteristic OA-induced protein pattern was evident in the SDS-gel. Since the exconjugant O/T recovered from inhibition by 3-MB (Fig. 5), further studies will have to show whether the distinct desulphonation activities found in the two growth phases are identical. It would also be of interest to find out whether a preparation of the recently identified two-component (43 and 17 kDa) desulphonating enzyme of *Alcaligenes* sp. O-1 (Thurnheer et al., 1990; A. M. Cook, unpublished results) shows a similar progressive inhibition of the desulphonation reaction in the presence of increasing concentrations of 3-MB as indicated by our data in Fig. 2(b).

This work shows the conjugal transfer of the TOL catabolic genes (3-MB degradation; Assinder & Williams, 1990) to, and expression in, *Alcaligenes* sp. O-1::Tn5. This catabolic activity was reduced fourfold in the new host, when calculated from the extended lag phase and prolonged generation time. Moreover, the exconjugant strain O/T did not lose the ability to degrade OA that is encoded by the degradative plasmid pSAH. Incompatibility of pWW0 and pSAH might explain our inability to demonstrate the (opposite) conjugal transfer to and expression of pSAH in *P. putida* harbouring plasmid pWW0, since both the transfer of pSAH and the expression in a plasmid-free strain of *P. putida* have been previously shown (Jahnke et al., 1990). The ability of pWW0 to replicate in *Alcaligenes* sp. O-1 is not yet known, because incompatibility with the cryptic plasmid pME1702 might also preclude its establishment in this host. Hitherto, the archetypal TOL plasmid pWW0 has been referred to as having a broad host range (Benson & Shapiro, 1978), with an expression range of the catabolic genes limited to *Pseudomonas* spp. (Burlage et al., 1989). An uncommon TOL-like plasmid has been described by Hughes et al. (1984) for strain 345 of *Alcaligenes eutrophus*, which is not a member of the genus *Alcaligenes* as defined phylogenetically (Busse & Auling, 1992). Since pWW0 was not found in the exconjugant O/T, which breaks down both xenobiotic substrates and expressed new polypeptides upon growth on 3-MB (Fig. 3), transposition of TOL catabolic genes (Tn4651) remains as the only explanation. This was confirmed by specific hybridization with pWW0. Obviously, the transposition of Tn4651 into pSAH was accompanied by secondary events because the size of the enlarged plasmid in the exconjugant differed from that expected for a simple transposition event. Adding the inserted fragments to pSAH would result in a plasmid of 228 kb. However, the plasmid found had a size of only 193 kb, indicating that 35 kb had been deleted from pSAH in favour of the new insertion.

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M. Jahnke and others


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