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Pseudomonas aeruginosa Strain RW41 Mineralizes 4-Chlorobenzenesulfonate, the Major Polar By-Product from DDT Manufacturing

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Summary

Pseudomonas aeruginosa RW41 is the first bacterial strain, which could be isolated by virtue of its capability to mineralize 4-chlorobenzenesulfonic acid (4CBSA), the major polar by-product of the chemical synthesis of 1,1,1-trichloro-2,2-bis-(4-chlorophenyl)ethane (DDT). This capability makes the isolate a promising candidate for the development of bioremediation technologies. The bacterial mineralisation of 4CBSA proceeds under oxygenolytic desulfonation and transient accumulation of sulfite which then is oxidized to sulfate. High enzyme activities for the turnover of 4-chlorocatechol were measured. The further catabolism proceeded through 3-chloromuconate and, probably, the instable 4-chloromuconolactone, which is directly hydrolyzed to maleylacetate. Detectable levels of maleylacetate reductase were only present when cells were grown with 4CBSA. When the ordinary catechol pathway was induced during growth on benzenesulfonate, catechol was *ortho*-cleaved to *cis,cis*-muconate and a partially purified muconate cycloisomerase transformed it to muconolactone *in vitro*. The same enzyme transformed 3-chloro-*cis,cis*-muconate into *cis*-dienelactone (76%) and the antibiotically active protoanemonin (24%). These observations are indicative for a not yet highly evolved catabolism for halogenated substrates by bacterial isolates from environmental samples which, on the other hand, are able to productively recycle sulfur and chloride ions from synthetic haloorganosulfonates.

Introduction

The chemical synthesis of DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane), which is again and increasingly being used as a vector controlling insecticide after having banned in the 1980s, furnishes the isomers *para,para*-DDT and *ortho,para*-DDT from the condensation of chlorobenzene and chloralhydrate, in the presence of sulfuric acid. Additionally, isomeric chlorobenzenesulfonic acids as well as sulfonated DDTs and other unwanted by-products are formed (Fig. 1). The lipophilic *para,para*-DDT and *ortho,para*-DDT are extracted by a suitable solvent such as diethyl

ether, and the aqueous phase containing the sulfonated haloaromatics was usually dumped into special landfills or, probably, directly into wastewater streams. The dominating residual compound, 4-chlorobenzene sulfonic acid (4CBSA), consequently, is being found in sewage treatment plants, groundwater, marine estuaries (Alonso *et al*., 1999, 2002; Kendall, 1989; Loos *et al*., 2000; Suter *et al.* 1999), and in the leachates of chemical waste deposits; in one case accounting for up to 69% of the total organic carbon of the effluent (Brown *et al*., 1989; Kim *et al*., 1990). 4CBSA itself has some commercial use; it is being found in applications of stains and its structure can be found in the acaricide chlorfenson (4-chlorophenyl 4-chlorobenzenesulfonate, Ovex®) from which it is being released through hydrolysis of the sulfate ester bond, and in anthelmintic and anti-HIV drug preparations (thenium closylate). The amide of 4CBSA, known as Neomagnol® (ICN), has been used as a surface disinfectant.

The bacterial catabolism of sulfonated aromatic compounds has been studied in detail on a number of bacterial isolates. Sulfoaromatics are generally degraded by bacteria upon attack of a mono- or dioxygenase, which releases the xenobiotic sulfonic acid group as benign sulfite which, in turn, is oxidized to sulfate, thus contributing to the global sulfur cycle. On the other hand, several sulfoaromatics are transformed to sulfocatechol and this compound represents a substrate of a sulfocatechol dioxygenase or a specialized protocatechuate dioxygenase (Contzen *et al*., 2001; Cook *et al.*, 1999). The bacterial mineralization of benzenesulfonic acid had been shown to be initiated by a dioxygenolytic removal of the sulfonic acid group as sulfite (Cain and Farr, 1968; Endo *et al*., 1977). Reports on the (bio-) degradation of 4CBSA, however, are very scarce. The recalcitrant compound was assumed to be removed by biological degradation processes from groundwater (Leenheer *et al*., 2001). It was also shown to be depleted within 15 days from a mixed, acclimated continuous culture capable of degrading seven differently substituted benzenesulfonates, but the organism responsible for its degradation could not be isolated from the 5-species-consortium

(Thurnheer *et al*., 1988) and one has to assume the depletion of the compound through mutualistic catabolism. More recently, the use of 4CBSA as a potential sulfur source of the surfactant degrading *Rhodococcus opacus* strain ISO-5 was reported, but the authors didn't provide any further evidence for the fate of the toxic halophenolic carbon backbone (Schleheck *et al*., 2003).

Aromatic compounds are mainly degraded via catechol, protocatechuate or gentisate, and the catabolic sequence of catechol catabolism proceeds through muconate, muconolactone, enol-lactone and 3-oxoadipate, which is then cleaved to form acetate and succinate to enter the Krebs cycle. The breakdown of (poly-) chlorinated aromatics requires specialized enzymes and their catabolism proceeds mostly through chlorinated catechols and muconates, to yield dienelactones as the products of a first dehalogenation; and chloromaleylacetate is finally reduced to 3-oxoadipate by more specialized enzymes. Misrouting of monochlorocatechols into an ordinary catechol pathway may lead to the formation of the antibiotically active compound protoanemonin, which represents a toxic dead-end product (Blasco *et al*., 1995, 1997). In a recent publication convincing evidence was provided that bacterial strains isolated from the environment, which do not possess the halocatechol pathway for productive mineralization of chlorocatechols, may circumvent the formation of protoanemonin or even detoxify it by the action of, probably overexpressed, enzymes of a (methyl-) catechol pathway (Nikodem *et al*., 2003). On the other hand, the corresponding products from chlorocatechol gene clusters of 2,4-dichlorophenoxyacetate degrading *Sphingomonas* strains have proved to function in crude cell extracts but were found to be unstable, eluding chromatographic purification to homogeneity (Thiel *et al*., 2005).

Sulfonated aromatic chemicals like 4CBSA are highly polar compounds and represent severe problems in waterworks during the cleanup of drinking water because of lacking technical systems for their removal: their extremely hydrophilic nature prevents them from being removed by

adsorptive treatment, i.e. with activated charcoal or other absorbants, or by air-stripping. Further, they are extremely recalcitrant and strongly resist microbial breakdown in wastewater treatment systems since they are being found in the effluents as aforementioned. Here we show that 4CBSA can be mineralized by a *Pseudomonas aeruginosa* strain, which was isolated from aerobic River Elbe sediment and water samples taken downstream of Hamburg, Germany, with this compound as its sole source of carbon, sulfur and energy. Strain RW41 currently represents the only known bacterium that exhibits such catabolic capabilities. The isolation of some key intermediates from crude extracts as well as the measured enzyme activities and results from experiments performed with partially purified (chloro)-catechol 1,2 dioxygenase and (chloro)-muconate cycloisomerase allows the proposal of the metabolic pathway involved in the degradation of this chemical. Although the taxon has been considered a potentially pathogenic one in the past, its application in environmental processes seems feasible with regard to the removal of the environmentally important target compound and the microbial recycling of sulfur, chlorine and carbon.

Results and discussion

Isolation, identification and growth of strain RW41 on 4CBSA

Enrichment experiments with 4CBSA as the target carbon source resulted after about four months in a mixed culture utilizing the compound as the only source of carbon and energy. From this culture, strain RW41 was isolated and characterized by standard laboratory procedures. The strain was Gram-negative, oxidase- and catalase-positive, and motile. Its exact taxonomic position was determined by sequencing of its 16S rDNA gene (1488 positions). Cluster analysis showed that the gene sequence grouped with those of bacteria of the γ-subclass of *Proteobacteria* and showed 99.7 % of identity with that of the *Pseudomonas aeruginosa* type strain (Moore *et al.*, 1996; Stackebrandt

et al., 1988), DSM 50071. Further identification tests performed with the API20 NE test system and fatty acid analysis confirmed the taxonomic position of the isolate.

The strain grew relatively slowly on 6.5 mM 4CBSA as sole source of carbon and energy (Fig. 2) with a doubling time of about one day. Almost stoichiometric amounts of chloride and sulfate were released whilst the accumulation of the precursor, sulfite, was transient. Addition of 50 mg L^{-1} yeast extract as a vitamin source to the mineral salts medium was necessary to obtain a reasonable growth rate. An identical generation time of RW41 was obtained when growth studies on 4CBSA were performed in sulphur-free mineral salts medium, an observation which is indicative for the possible utilization of the released sulfonic acid group as a sulphur source. Strain RW41 grew also on benzenesulfonic acid and benzoic acid in liquid medium, and with 4-chlorocatechol on gradient plates, which were used to avoid toxic concentrations of this halogenated phenolic compound. The following, structurally related compounds were not utilized as carbon sources: 3-chloro- and 4-chlorobenzoic acid, all isomeric methylsalicylates and methylbenzoates, 3-chloro- and 4-sulfocatechol, benzene-1,3-disulfonic acid, 2-sulfo-, 3-sulfo- and 4-sulfobenzoic acid, 3-sulfo- and 4-sulfophthalic acid, 4-hydroxy- and 4-aminobenzenesulfonic acid, 5-sulfosalicylic acid, and 4-sulfotoluene.

Characterization of catabolites and enzyme activities of 4CBSA degradation

In order to elucidate the catabolic sequence of the degradative pathway, whole cells of RW41 grown on 4CBSA, BSA, and on acetate as a control, were analyzed for their potential to oxidize potential catabolites to be expected in analogy to the catabolism of BSA from the literature. The data shown in Table 1 provided clear evidence that the catabolic pathways are inducible and are also stable, since they have not got lost during growth with a non-selective carbon source and thus are not located on a mobile element. The oxidation of 4-sulfocatechol as a potential catabolite was always

negligible. On the other hand, higher oxidation rates for chlorocatechols were detected when the strain was pregrown with 4CBSA. The assumption of an additionally induced chlorocatechol pathway was underpinned by the observation of an identical oxidation rate for catechol and 3,5-dichlorocatechol by cell-free extracts of 4CBSA-grown RW41 cells; those cells pregrown with BSA showed only about 1 - 2 % of activity compared to catechol oxidation (data not shown). To confirm the above observations, whole cells and crude cell extracts, both incubated in the presence of 5 and 1 mM of 4CBSA, respectively, were analyzed by HPLC. Despite some depletion of substrate (between 23 and 28%) no potential metabolites were detected, even when NAD(P)H was added to cell extracts in order to stimulate for the accumulation of products to be expected from the initial dioxygenase reaction like 4-chlorocatechol, or the maleylacetate reductase reaction.

The initial catabolic reaction

Because of the capability of strain RW41 to grow with benzoate, it was not possible to generate a stable *cis*-1,2-dihydrodiol from benzoate as a structural analogue of benzenesulfonate in order to prove the mechanism of the initial reaction. This was due to the induced activity of cyclohexa-3,5-diene-1,2-dihydroxy-1-carboxylate (dihydrodiol) dehydrogenase when RW41 was grown with the sulfonate analogue. The same applied to the co-oxidation of the derivative halogenated in the *para* position because also in this case the dihydrodiol dehydrogenase was induced. On the other hand, with crude extracts of 4CBSA-grown cells some low but significant oxygen depletion could be correlated with the presence of this compound and the oxidation rate was also correlated with the amount of enzyme present in the assay. The addition of NADH, after compensation for its oxidation by background activities, resulted in a slightly increased activity for the oxidation of BSA and 4CBSA. Attempts to inhibit the further degradation of assumed catechol or 4-chlorocatechol, however, were not possible because of the high transformation rate for halocatechols due to the induced chlorocatechol 1,2-dioxygenase. Several halocatechols, however, were shown to exhibit

promising inhibitor constants for the conversion of catechol by catechol 1,2-dioxygeanse (Dorn and Knackmuss, 1978). In crude extracts from BSA-grown cells of strain RW41, finally catechol was identified by its specific retention time and UV spectrum upon transformation of BSA when its further oxidation was inhibited by 3,5-dichlorocatechol.

Lower pathway enzymes and activities

Addition of NADH as cofactor did not lead to an increase of specific oxidation rates when assaying the same potential substrates shown in Table 1 with crude extracts by using the oxygen electrode (data not shown). However, when sodium EDTA, a known inhibitor of (chloro-) muconate cycloisomerase requiring divalent metal ions (manganese, Meagher *et al.*, 1990) was added at a 2 mM concentration to extracts incubated in the presence of 1 mM 4-chlorocatechol, HPLC analysis revealed the accumulation of 3-chloro-*cis*,*cis*-muconic acid and, interestingly because of the inhibitory features, of *cis*-dienelactone and of maleylacetic acid.

These observations in conjunction with data presented in Table 1 strongly suggest that 4-chlorocatechol should be an intermediate of the catabolism of 4CBSA. By contrast, from the data presented in Table 1, 4-sulfocatechol can be excluded as a potential intermediate. Neither protoanemonin nor *cis*-acetylacrylate representing its potential product of hydrolysis was oxidized (not shown). Neither sulfocatechol nor protocatechuate 3,4-dioxygenase activity could be detected in cell-free extracts.

Although already during growth on benzenesulfonate a catabolic catechol pathway was found to be active, data of enzyme activities determined in crude cell extracts of 4CBSA-grown RW41 (Table 2) provided clear evidence that an additional chlorocatechol sequence was co-induced. Enzyme activities for the efficient turnover of chlorocatechols and 3-chloromuconate were present, but only

minute amounts of *trans*-dienelactone hydrolase activity could be detected, probably due to its instability in crude cell extracts and especially, in fruitless trials of enzyme purification. Additionally, the activity found in crude extract only for the *trans*-isomer appears to contradict the clear identification of *cis*-dienelactone as a product of chloromuconate cycloisomerase. Monitoring the turnover of 1 mM of 4-chlorocatechol by crude cell extracts by HPLC from cells grown with 4CBSA did not result in the detection of neither dienelactones nor protoanemonin, only maleylacetate accumulated.

In order to elucidate some more specific properties of the 4-chlorocatechol catabolism of strain RW41, the crude extracts obtained from 4CBSA-grown cells were fractionated by anion exchange chromatography. However, repeated analysis of these fractions and also of newly prepared cell material expected to contain (chloro-) catechol 1,2-dioxygenase, (chloromuconate-) cycloisomerase and the dienelactone hydrolase, always resulted in two little resolved peaks for each of the first two enzymes (Fig. 3A, B), irrespective of whether the cells were grown with BSA or 4CBSA. Isoenzymes were not detected and no activity could be found for the dienelactone hydrolase, which we nearly failed before to detect in crude extracts from RW41, probably due to its high instability. Therefore, similarly obtained preparations from *P. knackmussii* B13 grown on 3-chlorobenzoate were used to validate the chromatographic separation of these enzymes. They revealed a clear separation of catechol and chlorocatechol 1,2-dioxygenases with catechol and 3-chlorocatechol as the substrates, respectively, and of both muconate and chloromuconate cycloisomerase, as well as the detection of dienelactone hydrolase activities in the first fractions obtained from separations on the monoQ column (Fig. 3C).

The above observation of a single peak for the (chloro-) catechol 1,2-dioxygenase (C12O) and for the (chloro-) muconate cycloisomerase (MCI) of RW41 can be interpreted in the way that both types

of enzymes are in fact very closely related, probably differing from each other by only very few amino acids, and not yet as differentiated as in B13 whose extract yielded the clearly distinguishable peaks of enzymes of both the catechol and the more specialized chlorocatechol catabolism. Further evidence for this assumption is based in the only moderate activity for 3-chlorocatechol (around 17 % of relative activity compared to catechol) and 4-chlorocatechol but high activity for 3,5-dichlorocatechol when assayed with the oxygen electrode; the B13 enzyme (homology group II) exhibited nearly similar specific activities for the non-chlorinated and the chlorinated substrates (Tab. 2), whilst the chlorocatechol dioxygenase of the homology group III enzymes, which are much more specific for the efficient breakdown of the highly halogenated catechols, showed nearly threefold specific activity with 3-chlorocatechol, compared to catechol as was demonstrated by Potrawfke *et al*. (2001). Comparison of obtained kinetic data from experiments performed with crude cell extracts (Tab. 2) provided some evidence that the additionally induced enzymes of RW41 during growth on the chlorinated substrate can be classified in between the catechol dioxygenases and the chlorocatechol dioxygenases of the homology group II for the conversion of non-halogenated catechol and low- to mid-halogenated catechols (Potrawfke *et al.*, 2001; Reineke, 1998) and may explain that the enzymes are not yet fully evolved ones for the efficient turnover of these halogenated intermediates.

Interestingly, the activity of the dienelactone hydrolase in all experiments we performed was minute and unstable and showed only activity for the *trans* isomer although the *cis* isomer is being formed from an intermediary 4-chloromuconolactone in the catabolic 4-chlorocatechol pathway sequence (Blasco *et al*., 1995, 1997). Activity for only the *trans* isomer was found in 4CBSA-grown RW41 but the analysis of the overall kinetic data suggests a catabolic gene sequence for catechol catabolism to be induced during growth on BSA. A similar observation was made when *Pseudomonas* sp. strain MT1 was grown on chlorosalicylate: also here additional activities for the

trans-DLH and MAR were induced (Nikodem *et al*., 2003). This observation raises the question by which trigger signal the additional DLH and MAR activities of strain MT1 were induced when compared to activities detected during growth on non-chlorinated salicylate.

The only lactone hydrolase activity detected in the cell-free extracts from cells of RW41 grown on 4CBSA was a *trans*-DLH (-like) activity. Since no *trans*-dienelactone was formed in the reaction catalyzed by the partially purified MCI, we assumed that the productive substrate of the hydrolase could only be 4-chloromuconolactone, which has been postulated as the unstable halohemiacetal formed by cycloisomerization of 3-chloro-*cis,cis*-muconate (Blasco *et al.*, 1995). On the other hand, the chlorine in the postulated lactone could be enzymatically replaced by OH^-, and this hemiacetal decay spontaneously and also furnish 3-hydroxymuconate (maleylacetate). This postulate was underpinned by the observation that when using cell-free extracts (where the lactone hydrolase activity was still present), 4-chlorocatechol and 3-chloro-*cis,cis*-muconate were quantitatively transformed into maleylacetate as was judged from HPLC analysis of this conversion (data not shown). By contrast, the partially purified MCI of RW41 transformed 3-chloromuconate into *cis*-dienelactone (90%) and protoanemonin (10%), both of them dead-end products. These observations open new questions related to the evolutionary relationship of MCIs, because some of them have been described to catalyze the conversion of 3-chloromuconate into protoanemonin (Blasco *et al.*, 1995), others into *cis*-dienelactone (Reineke and Knackmuss, 1988) whereas some others form a mixture of both *cis*-dienelactone and protoanemonin, depending on the enzyme of the respective bacterial strain. Here we report that the MCI from RW41 forms mainly *cis*-dienelactone and minute amounts of protoanemonin, whereas the proportion is almost the opposite in strain MT1 (Nicodem *et al.*, 2003). Nevertheless, both strains share in common the direct formation of maleylacetate from 3-chloromuconate in the coupled reaction sequence.

In conclusion, *Pseudomonas aeruginosa* strain RW41, which is capable of growth with 4CBSA as an aromatic compound showing two different substituents and which is being mineralized into benign inorganic ions, induces a chlorocatechol pathway, as proposed in Fig. 4. Formally, the xenobiotic chlorine is being replaced by the physiological OH group before the intermediates can be channelled into the Krebs cycle. The additionally induced catabolic enzymes, however, are seemingly not yet highly evolved with regard to the efficient mineralization of halogenated intermediates. Further genetic (cloning of the catabolic sequences) and biochemical analysis of the corresponding gene products is needed to elucidate this hypothesis in more detail.

Experimental procedures

Bacterial strains, culture conditions, preparation of cell extracts and transformation experiments

Strain RW41 was isolated from aerobic sediments of the River Elbe downstream of Hamburg harbour, which received some input via its tributaries from decommissioned production and waste sites. *Pseudomonas knackmussii* strain B13 (DSM 6978) (Dorn *et al.*, 1974; Stolz *et al.*, 2007) was used as a reference organism in studies on enzyme kinetics for the validation of protocols. Strain RW41 has been identified upon its 16S rDNA sequence comparison, by using standard laboratory procedures, and the API 20 NE test system and fatty acid analysis. It has been deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ) strain collection under accession number DSM 8924. Culture media and growth conditions, the preparation of cell-free extracts for the determination of enzyme activities, and the principle setup of biotransformation experiments were as previously described (Blasco *et al.*, 1995). The mineral salts medium almost free from added sulfur compounds contained ammonium chloride instead of ammonium sulfate.

Enzyme purification and activity assays

Catechol 1,2-dioxygenase and muconate cycloisomerase (MCI) were purified using an FPLC system (Fast protein liquid chromatography, Pharmacia-Amersham Biosciences, Uppsala, Sweden) by anion exchange chromatography and their activities, including that of DLH, were determined as described before (Blasco *et al.*, 1995). The catechol 2,3-dioxygenase was determined according to a published method (Nozaki, 1970), and the sulfocatechol dioxygenase assay was performed as described previously for protocatechuate dioxygenase (Feigel and Knackmuss, 1993). The activity of the maleylacetate reductase was determined as described previously (Kaschabek and Reineke, 1993). Activities of (chloro-)benzenesulfonate 1,2-dioxygenase were determined with the oxygen electrode in 1 ml of 25 mM phosphate buffer (pH 7.5) with varying amounts of crude cell extract, 1 mM of BSA or 4CBSA and 1 mM of NADH. The concentration of the catechol 1,2-dioxygenase inhibitors 3,5-dichloro- or tetrachlorocatechol was 0.1 mM. Soluble protein in the assays was determined as previously described (Bradford, 1976). Protein of whole cell suspensions was determined by the same procedure after heating of the cell suspension at 95°C for 15 min. in the presence of 0.3 M NaOH. Bovine serum albumin served for calibration of these assays.

Analytical methods

Compounds and metabolites were analyzed by HPLC (high-performance liquid chromatography), either on a Class LC10 system (Shimadzu, Kyoto, Japan) equipped with a diode array UV-VIS detector, monitoring routinely the effluent at 210 and 270 nm, or on a similarly equipped HP Series 1050 system (Agilent (Hewlett Packard), Waldbronn, Germany). Spectra were recorded between 200 and 600 nm and stored on the system. Separations were performed on a 150 by 4 mm column filled with RP18 LiChrospher 100, 5 µm (Bischoff, Leonberg, Germany), or on a 150 by 3.9 mm

Waters Nova-Pak column (C-8, 4 µm, Waters Cromatografía, Barcelona, Spain). The solvent was water-methanol, acidified with 0,1% (vol/vol) of *ortho*-phosphoric acid, and the flow rate 1 ml per min. Elution volumes and UV maxima (nm) of separated metabolites, on the Shimadzu system, were as follows at 18% methanol: 4-sulfocatechol, 1.8 ml (233/282); 4-sulfophenol, 2.1 ml, (229/268); maleylacetic acid, 2.6 ml (190); *trans*-dienelactone, 3.6 ml (274); protoanemonin, 5.5 ml (260); 4CBSA, 6.2 ml (190/225/265); *cis*-dienelactone, 6.7 ml (276); 3-chloromuconic acid, 12.1 ml (206/255); catechol, 14.3 ml (280); 4-chlorocatechol, 24 ml (285); 4-chlorophenol, 27.4 ml (226/283). Oxygen uptake rates were determined with a Clark-type oxygen electrode (model DW-1, Hansatech Instruments, Kings Lynn, UK) with whole cell suspensions and crude extracts. Chloride ion concentrations were determined with a flow-injection system (FIA) developed by the Fraunhofer-Institut für Grenzflächen- und Bioverfahrenstechnik, Stuttgart, Germany. Sulfate was measured by the method of Bertolaccini and Barney (1957) and sulfite ion concentrations were determined by the method of Johnston *et al*. (1975). Spectrophotometric determinations were performed at 25°C on a model UV 2100 or UV 2401 PC recording spectrophotometer (Shimadzu Corp., Kyoto, Japan).

Chemicals

4-Sulfocatechol was synthesized as previously described (Quilico, 1927). Protoanemonin and *trans*-dienelactone were prepared biologically and chemically according to previously described methods (Blasco *et al.*, 1995, Shaw, 1946); *cis*-dienelactone was obtained from Stefan Kaschabeck, Bergische Universität-Gesamthochschule Wuppertal, Wuppertal, and later-on from Michael Schlömann, Technische Universität Freiberg, respectively. Maleylacetate was obtained upon transformation of *cis*-dienelactone with crude cell extracts. *cis*-acetylacrylate was prepared from the commercial *trans* isomer (Lancaster Synthesis, Morecambe, UK) by UV irradiation (Seltzer and

Stevens, 1968). 3-chloro- and 4-chlorocatechol were obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany, together with 3,5-dichlorocatechol. A sample of tetrachlorocatechol (Sigma-Aldrich) was obtained from Yoon-Seok Chang, Pohang University of Science and Technology, Pohang, Republic of Korea. 3-chloro-*cis,cis*-muconate was always freshly prepared from 4-chlorocatechol by partially purified chlorocatechol 1,2-dioxygenase from *Pseudomonas knackmussii* strain B13. 4-chlorobenzenesulfonic acid (90% technical grade) was obtained from Sigma-Aldrich (Steinheim, Germany), a 1 M saturated aqueous solution extracted twice with ethylacetate to remove contaminating 1,1-bis(4-chlorobenzene)sulfone (as identified by GC/MS) and further purified by threefold crystallization from boiling water/methanol. A commercial sample of the sodium salt of 4CBSA (TCI, Japan) was obtained from Toru Matsui, COMB, University of the Ryukyus, Okinawa, Japan. All other chemicals were of analytical grade (S < 30 ppm).

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Figure legends

Fig. 1. Chemical production of DDT and by-products. o*rtho,para*-DDT, about 20 % yield of organic solvent-soluble fraction, is always produced as an unwanted by-product without insecticidal activity (insecticidal *para,para*-DDT [boxed] is about 80%). The aqueous reaction broth contains mainly 4-CBSA (boxed structure), its isomers and (di-)sulfonated DDTs. Partially compiled from tentative structural data suggested by Kim *et al*. (1990).

Fig. 2. Growth of *Pseudomonas aeruginosa* strain RW41 with 4-chlorobenzenesulfonate. Growth was monitored by measuring Optical Density (▼), depletion of 4-CBSA (■), release of chloride (●) and of sulfite (□), which oxidized in the medium to sulfate (▲).

Fig. 3. Separation of catabolic enzymes from *P. aeruginosa* strain RW41 crude cell extracts by anion exchange chromatography. A: Elution of (chloro-) catechol 1,2-dioxygenase (C12O, ▲) and (chloro-) muconate cycloisomerase (MCI, Δ) activity of 4-CBSA-grown RW41. Similar activities were determined in fractions from crude extract of RW41 cells grown with benzenesulfonate (B). As a reference for the separation of enzymes specific for the catabolism of catechol and chlorocatechols, the crude extract of 3-chlorobenzoate-grown *Pseudomonas knackmussii* B13 was chromatographed under identical conditions (C): fractions containing additionally (chloro-) dienelactone hydrolase activity (DLH, ●) are displayed, which was not detected in RW41 crude extracts and fractions. Arrows indicate catabolic enzyme activities of the catechol 1,2-dioxygenase ▲ and muconate cycloisomerase Δ (termed Type I), and of the co-induced chlorocatechol 1,2-dioxygenase ▲ and chloromuconate cycloisomerase Δ (termed Type II). Note that B13 isoenzymes elute clearly separated, in contrast to RW41 enzymes.

Fig. 4. Catabolic pathway for 4-chlorobenzenesulfonate by *Pseudomonas aeruginosa* RW41. Structures are given in their physiological form at neutral pH. Reaction 1: putative 4CBSA dioxygenase, sulfite will be released spontaneously. Reaction 2: chlorocatechol 1,2-dioxygenase. Reaction 3: (chloro-) muconate cycloisomerase. This enzyme catalyzes the conversion of 3-chloro-*cis,cis*-muconate into *cis*-dienelactone and minute amounts of protoanemonin. Reaction 4: Enelactone hydrolase activity. Reaction 5: maleylacetate reductase. Maleylacetate and 3-oxoadipate are depicted in their predominant physiological enol form, which dominate at neutral pH.

Table 1. Specific oxygen uptake rates of whole cell suspensions of *Pseudomonas aeruginosa* strain RW41

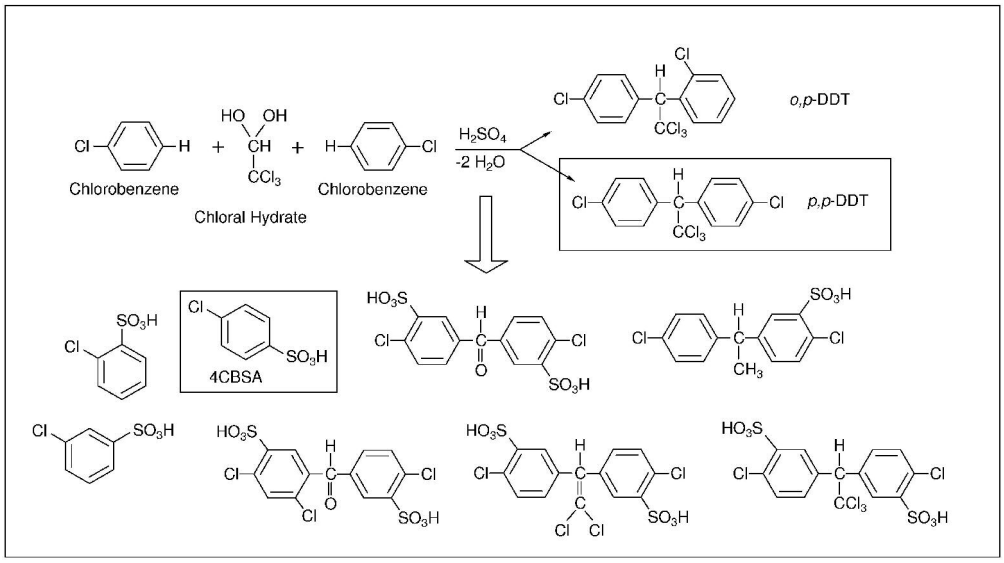
	Specific oxidation rates after growth with		
Substrate	4CBSA	BSA	Acetate
4CBSA	15	17	<2
Benzenesulfonate	17	22	<2
Catechol	143	123	4
3-Chlorocatechol	35	8	<2
4-Chlorocatechol	99	14	<2
3,5-Dichlorocatechol	141	<2	<2
4-Sulfocatechol	6	5	<2

Specific rates (nmoles per min per mg of protein) were assayed with washed cell suspensions pregrown on the indicated substrates and corrected for endogenous respiration. Data represent means of at least two independently performed experiments; the standard deviation was always below 14 %.

Table 2. Specific enzyme activities of crude extracts of *P. aeruginosa* RW41 grown on non-halogenated and halogenated substrate

Enzyme	Substrate	Activity after growth on BSA	Activity after growth on 4CBSA
Catechol 1,2-dioxygenase	Catechol	165	246
	3-Chlorocatechol	3	41
	4-Chlorocatechol	20	137
	4-Sulfocatechol	<1	<1
Muconate cycloisomerase	*cis,cis*-Muconate	28	35
	3-Chloromuconate	3	17
Dienelactone hydrolase	*trans*-Dienelactone	<1	20
	cis-Dienelactone	<1	<1
	Protoanemonin	<1	<1
Maleylacetate reductase	Maleylacetate	<1	3.4

Specific enzyme activities (nmoles x min^{-1} x mg^{-1}) were determined with crude cell extracts prepared from washed cells pregrown on the indicated substrates. Data represent means of at least two independently performed experiments; SD was always below 18% with a confidence interval of 90% at least. Activities of a catechol 2,3-dioxygenase were not found in RW41. ND, not determined.



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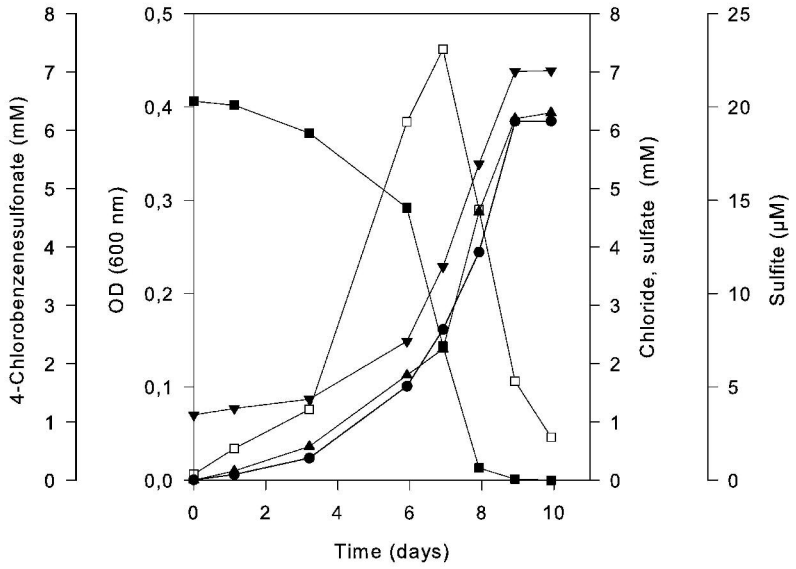

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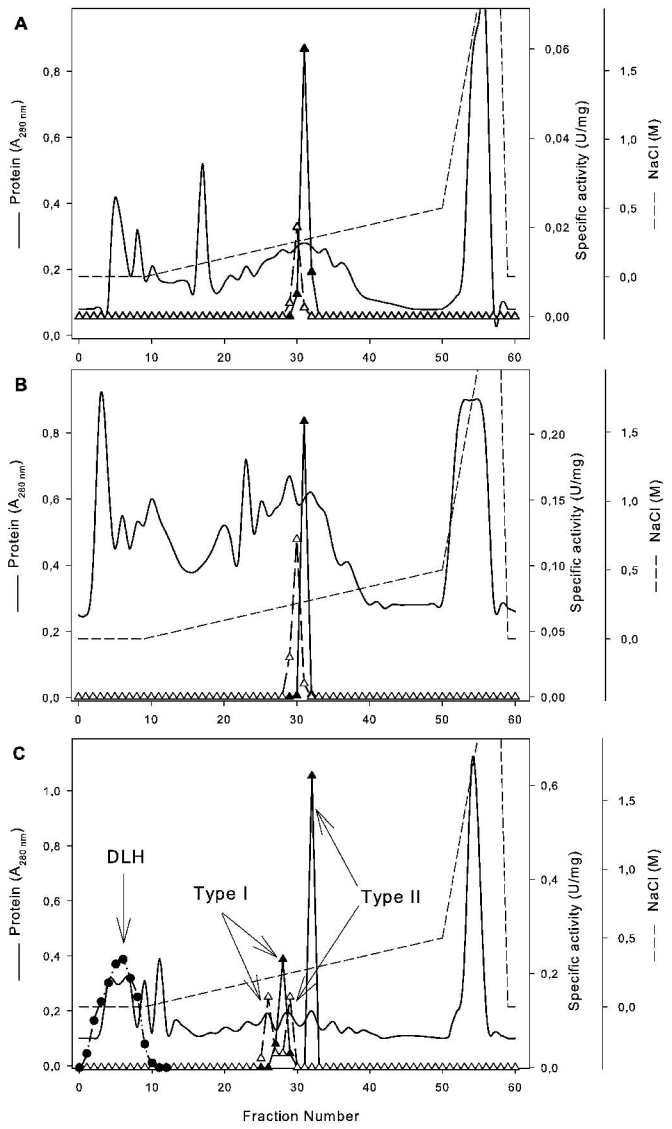

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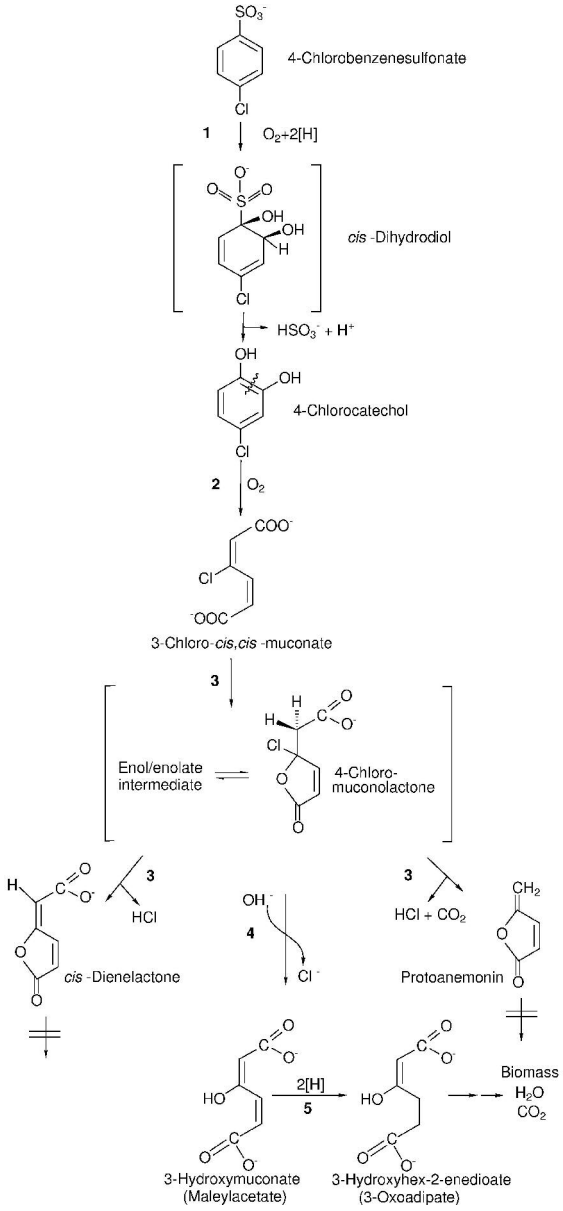

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