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Microbiology and Pollution: the Biodegradation of Natural and Synthetic Organic Compounds [and Discussion]

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Microbiology and pollution: the biodegradation of natural and synthetic organic compounds

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Natural products are normally biodegradable, and problems arise mainly when excessive amounts accumulate as wastes. Recent research has suggested some new ways of recycling the organic materials in domestic refuse and sewage, in the organic by-products of industry and in agricultural wastes. If processes can be made economical, these materials could provide sources of energy and of animal foodstuffs, and environmental pollution could be decreased.

The chemical residues from herbicides and pesticides present potential biological hazards to the operatives and to the environment. Many chemicals used in industry would be hazardous if allowed to remain in industrial effluents. Some of these materials can be rendered innocuous by microbial action, and it may be possible to develop strains with improved metabolic activities enabling them to deal more effectively with novel chemical compounds. Methods of constructing strains and the use of their enzymes for degrading synthetic chemicals are discussed.

Introduction

Biological recycling

The turnover, or recycling, of biological materials depends, in the long run, on the activities of microorganisms. They have been engaged in biological recycling since life began and have continued to evolve new metabolic activities in order to cope with the compounds produced by the synthetic operations of plants and animals. If the attack by microorganisms is faster than we would wish, it is termed biodeterioration, and action is taken to prevent it. If the process is too slow for the material presented, we call it pollution. There are many different ways of defining pollution and many different ways of solving pollution problems. I am going to take a very narrow view and consider the problems presented to microorganisms in breaking down organic molecules into products that we would consider harmless. I shall not spend time considering ways of reducing the amounts of domestic refuse, or the waste materials of industry, although in some cases the best approach to the problem might be to reduce the amount of material discarded. I shall refer only briefly to the recovery of nutrients from waste material, although microbial processes are involved. The publication of *War on waste* (H.M.S.O. 1974) and conferences such as *Food from waste* (Birch *et al.* 1976) lead to the hope that a more responsible attitude may be reached towards conserving more of the resources of society.

There are many paradoxes in the methods used to dispose of normal plant and animal residues. Horse manure is considered to be so valuable that, as well as being used by farmers, it is sold to gardeners as a good organic fertilizer. Waste from intensive farming units, on the other hand, often presents pollution problems. These residues are potential nutrient sources, and processes are now in operation for treating poultry waste and incorporating it into animal feedstuff (Rolfe 1976). Other, more complex, systems have been developed in which the waste

product of one species is introduced into the food chain of other organisms. Paper and plant material may be added at some stages of these cycles and microbial fermentation steps may be used to provide energy for part of the operations. These systems represent deliberate and organized attempts to reproduce the economical advantages of natural food chains with the discarded residues of a complex industrial society (Hughes 1975; Bull *et al.* 1979).

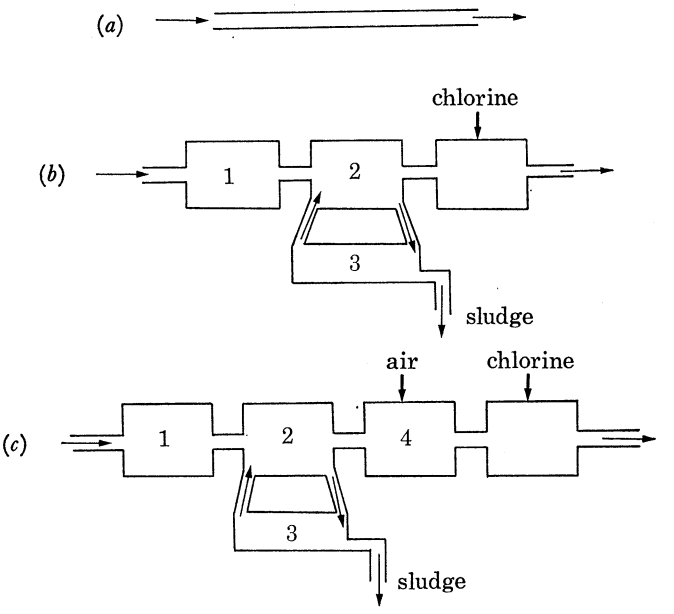

FIGURE 1. Sewage may be discharged into the sea, estuaries or rivers. (*a*) No treatment. (*b*) In primary treatment plants the raw sewage enters tank 1 and debris is removed by skimming or sieving. In tank 2 the solid material settles and goes into the sludge tank 3. The liquid passes out and may be treated with chlorine before discharge. (*c*) Secondary treatment plants follow the same initial procedure as (*b*), but, in activated sludge plants, the liquid passes into tank 4, where it is vigorously aerated and organic material is broken down by microbial action.

Microorganisms and sewage

The waste products of human metabolism from most of the dwellings in this country disappear into sewage systems and are joined by wastes from factories, shops, markets and surface drains. The simplest method of disposal is to release new sewage into the sea or a river (figure 1*a*). The expectation is that harmless microorganisms will oxidize all the organic matter and that pathogens will not survive long in these conditions. This system works quite well up to a point and may be adequate for small, isolated communities, but microorganisms have limitations, and, with large towns and cities, the amount of sewage becomes too great to be dealt with in this way. The system has the advantage of being cheap. There are no residues left behind, but, equally, nothing useful is recovered.

In many sewage plants the sewage is run through a series of tanks. First, debris is removed by sieving and skimming and the sewage then passes into settling tanks, where much of the solid organic material settles as a sludge. The effluent now has a much lower content of organic matter. The sludge remains and has to be disposed of in one way or another (figure 1*b*).

Secondary sewage treatment includes a stage in which the organic matter is treated by microbial action. In activated sludge systems (figure 1*c*) the effluent from the settling tank is led into a series of tanks, in which it is stirred and aerated. The inoculum for the process is derived

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from the sewage sludge itself and consists of a community of microorganisms that work together to oxidize much of the organic matter. It is possible to isolate and identify known bacterial species from activated sludge, but some of the organisms are difficult to fit into the tidy slots of microbial taxonomists, while others are difficult, if not impossible, to propagate in pure culture. The activated sludge process is capable of removing many organic chemicals from industrial wastes and has long been highly regarded by microbial biochemists as a source of bacterial strains for metabolic studies. The microbiology of a successful activated sludge system

TABLE 1. ORGANISMS OCCURRING IN SEWAGE PLANTS

(Samples from the outlet channel of the aeration tank from ten plants were plated on tryptone–glucose extract agar enriched with vitamins (t.g.e.v.a.). After 10 days at 20 °C, all colonies were transferred to master plates (t.g.e.v.a.), incubated for 3 days, and replica plated on test media. Tests were: growth on glutamate, in seawater, on acetate and on glycerol; gelatinase activity; anaerobic growth at 20 °C; Kovacs cytochrome oxidase; penicillin resistance. Of 1387 isolates, 1069 were assigned to 15 separate groups according to phenotype. Results from four sewage plants are shown below.)

plant	main input	predominant groups of bacteria
C	industrial	1, 2†, 4
D	industrial	1, 2, 4†, 7, 15
G	brewery	11, 14†
K	domestic	2, 4, 7, 11, 13†

† Most abundant (Banks *et al.* 1976).

is still largely unknown, but there is evidence to suggest that the population is adapted to suit the demands of its own particular sewage input. Davies and colleagues at York University examined the bacterial flora of ten different sewage plants which differed in the types of catchment areas and to some extent in methods of operation (Banks *et al.* 1976). They assigned their isolates to 15 groups on the basis of 8 tests for metabolic properties and found that there was a different microbial profile for each of the sewage plants. Table 1 shows the predominant groups of bacteria in four sewage plants, two with the same industrial catchment area, but slightly different in operation, one with mainly domestic input and one serving a large brewery. For plant G, with the domestic brewery discharge, they found that over one-third of the isolates belonged to a single bacterial group.

The aerated activated sludge treatment removes most of the organic material but leaves inorganic ions in solution. The presence of excessive amounts of phosphates and nitrates in the effluent is undesirable, particularly if it is to be discharged into a river from which water is withdrawn at sites downstream. Several microbiological methods may be used to remove nitrate. In one process, the liquid waste after oxidation is passed into an anaerobic tank in which denitrifying bacteria convert nitrate to nitrogen gas which is released into the atmosphere. When secondary treatment is based on trickle filtration, the conditions in the filter beds may allow aerobic oxidation at the surface to be followed by anaerobic reduction of nitrate to nitrogen in the lower levels.

In other systems, the removal of inorganic ions is accomplished by sand or soil filtration, and this can be associated with growing crop plants to take up the salts from the soil. Several schemes in the United States incorporate large land treatment areas for this purpose, and the filtered water may be passed into rivers or recovered for other use. The artificial Santee Lakes in the San Diego region of California are fed by water recovered by soil filtration of the effluent from the town sewage plant, and include boating lakes and a swimming pool. Water recovery

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for recreational use is important in this area of low rainfall. Both anaerobiosis and soil filtration are combined in the Flushing Meadows project in Phoenix, Arizona, where the sewage effluent is loaded onto the soil filtration beds so heavily that anaerobic conditions are established. After denitrification has occurred the beds are dried out and reloaded (Stevens 1974).

The sludge remaining after sewage treatment is, in some cases, disposed of by dumping at sea, but other, and more conservative, methods are also in use. Composting of sewage sludge is a relatively recent development (Finstein & Morris 1979), but various forms of processed sludge have been used as fertilizers for many years. Many British sewage plants carry out anaerobic digestion of the sludge, and this not only reduces the volume, but produces methane as a by-product. With current fuel shortages, the production of methane by anaerobic digestion of this unwanted material takes on increasing importance. Other organic residues can be subjected to digestion by methanogenic bacteria, and the design of small, efficient anaerobic fermenters offers the opportunity of dealing economically with organic disposal from small units (Hawkes *et al.* 1978).

Toxic effects

The microorganisms of the sewage plants are natural communities that have become adapted to these very specialized ecological niches. There is sufficient flexibility to accommodate wide fluctuations in the input of organic chemicals, but the operations can be brought to a halt if substances reaching the sewage stream kill off members of the microbial community or produce physical conditions that hamper their operations. Seeding with particular bacterial strains has been attempted on some occasions but has not been adopted as a general practice. The use of specialized strains of microbes for transforming potentially toxic chemicals into harmless products is probably best carried out at source.

Biodegradation of oil

Oil at sea

A current worldwide pollution problem is the presence of oil in the seas and estuaries. Yet oil is a natural product and can be degraded by microbial action. There are natural oil seeps on land which are removed by microorganisms and the same processes occur with natural oil seeps in the ocean. Wilson *et al.* (1974) calculated that oil seepage into the sea from deep underwater fissures reaches about 600 000 t per annum. Examination of one of the areas of high seepage, north of Santa Barbara, did not indicate any deleterious effect on marine life, and surface oil swept onto the shore was degraded without accumulation (Wardley-Smith 1976). This indicates that naturally occurring microbial strains have the potential for dealing with oil pollution resulting from commercial oil handling operations.

Many conferences have been held to discuss ways in which oil pollution could be reduced, what international regulations should be drawn up and how they could be enforced. The most dramatic and best known occasions of major oil pollution have been those of major disasters to large oil tankers, as with the Torrey Canyon in 1967 and the Amoco Cadiz in 1978. These presented special problems and led to the development of equipment for rapid dispersal of large slicks of crude oil. The aim was to get the spilled oil into a suitable physical state for microbial attack. This is particularly difficult when tarry lumps are formed from the oil or when agitation sets up stable emulsions, the so-called chocolate mousse effect. Although estimates vary widely (Wardley-Smith 1976; Hughes & McKenzie 1975), authorities agree that

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pollution from accidental spills is quantitatively less significant than loss during normal tanker operations or from shore installations. The amount of oil pollution originating from the two latter sources can be reduced by microbial treatment.

TABLE 2. CATABOLIC PLASMIDS OF *Pseudomonas*

plasmid	10^{-6} × relative molecular mass†	primary growth substrate	reference
CAM	160	camphor	Rheinwald *et al.* (1973)
OCT	27	octane	Chakrabarty *et al.* (1973)
SAL	51	salicylate	Chakrabarty (1976)
NAH	49	naphthalene	Dunn & Gunsalus (1973)
TOL	78	toluene	Williams & Murray (1974)

† Bukhari *et al.* (1977). The OCT plasmid occurs with a separate transfer factor K. Other isolates may carry plasmids of different size, e.g. a range of TOL plasmids of relative molecular masses 60–170 × 10^6.

Microbial activities

The metabolic pathways for the degradation of the organic compounds found in crude oil are fairly well understood. In nature, a mixture of microorganisms is responsible for the biodegradation of oil, but many well known species are able to remove one or more of the components. For these organisms, the hydrocarbons represent a few of many different potential growth substrates. In nature, such compounds come and go in the microenvironment, and bacteria have evolved regulatory systems that ensure that the synthesis of enzymes for the initial attack on these compounds is induced only when required. Thus, for an organism with the genetic information for utilizing benzene as a carbon source, the enzymes for degrading benzene are induced when benzene reaches the bacterial environment. Some of these organisms have evolved an additional and highly effective system for responding to the appearance of a variety of potential growth substrates. The essential genes of bacteria are carried on a single chromosome, but genes specifying enzymes required for the catabolism of some of these more unusual growth substrates may be carried on small extrachromosomal pieces of DNA that are known as plasmids (table 2).

Plasmids have been described carrying genes for the catabolism of octane (Chakrabarty *et al.* 1973), of naphthalene (Dunn & Gunsalus 1973), of camphor (Rheinwald *et al.* 1973) and of toluene (Williams & Murray 1974) (see table 2), as well as of a number of other compounds (Chakrabarty 1976). Some plasmids can be transferred very readily to other bacteria, and this enables useful genetic information to be spread very rapidly through a population. A few years ago, Chakrabarty constructed a strain carrying NAH, SAL and a hybrid CAM-OCT plasmid (Friello *et al.* 1976), and showed that it was much more effective in degrading crude oil than a mixture of the strains carrying a single plasmid. He suggested that this constructed strain would be useful for spraying on oil slicks at sea to speed up the rate of biodegradation. Few people think that this would be effective, since the multiplasmid strain would be in competition with the marine bacteria in their own habitat. Further, faster growth with oil as a carbon source would need an additional supply of nitrogen to support bacterial growth. However, although strains constructed in this way are not likely to be effective in the open sea, they could be very useful for the degradation of hydrocarbon residues in closed systems. (This is a very promising general method for constructing strains for treating industrial effluents.) Refinery wastes may also be treated by systems similar to those used in sewage plants. After oil–water separation,

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the oil layer is returned to the refinery and the oily water is treated microbiologically. A source of nitrogen is added to promote bacterial growth, and the oily components are removed by the aerated activated sludge method or by other microbial aeration treatments (McKinney 1963).

Tankers

After oil has been discharged from tankers at their terminals, the empty tanks are filled with sea water as ballast. This mixes with the residual oil in the tanks, and at a later time the oily water is discharged and the tanks washed out with fresh sea water before returning to the loading port. The amount of oil released this way can be substantially reduced by successive transfers involving oil–water separation on the tanker. Some of the oil residue is still left in the tanker when the new load is taken on. This is the load-on-top method. The oily ballast water can also be transferred to separator tanks on shore for treatment, and that is essentially the same problem as dealing with refinery effluents.

A more radical approach is to treat the oily ballast during the voyage of the tanker. An experiment on these lines gave promising results (Gutnick & Rosenberg 1977). The experimental vessel was an oil tanker with one of the slop tanks carrying 107 m^3 of oily ballast water over a layer of thick oily sludge. This was supplemented with urea (7.6 mM) and K_2HPO_4 (0.57 mM) to assist bacterial growth, and the tank was aerated. After 4 days the thick layer of sludge had disappeared completely from the experimental tank and was unchanged in the control tank. Gutnick & Rosenberg (1977) suggest that this could be developed into an operational method without difficulty.

Normal tanker voyages vary in the distances travelled in ballast and in the ambient temperatures encountered and it might be possible to construct a number of different oil-degrading strains adapted for particular conditions.

Biodegradation of synthetic chemicals

Herbicides and pesticides

Organic compounds used as pesticides and herbicides include many synthetic chemicals that do not occur naturally but are nevertheless removed from soil by biological transformations. Some, like the herbicide 2,4-D (2,4-dichlorophenoxyacetate), are degraded fairly rapidly with half lives of a few days or weeks, while DDT (4,4-dichlorodiphenyltrichloroethane) is an example of a very persistent compound (table 3). Several microbial genera, particularly species of *Arthrobacter*, *Bacillus*, *Nocardia*, and *Pseudomonas*, can utilize a number of synthetic herbicides and pesticides. However, in nature, complete biodegradation may be due to the activities of a mixed population rather than a single strain. Also, there will be several different potential growth substrates available for the soil microbes, and these may include other xenobiotics as well as a mixture of organic compounds of biological origin. Studies on the rates of disappearance of particular pesticides often show a lag period before any significant changes occur, and this may be due either to chemical changes or to adaptation of the original microbial population. Some recent studies have indicated ways in which strains with new metabolic capabilities might evolve.

The commonly used herbicide 2,4-D is known to be degraded by a pathway that has evolved to catabolize aromatic compounds that are normally found as intermediates in the breakdown

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of natural products. In some organisms, the genes are located on a plasmid (Pemberton & Fisher 1977).

Dalapon (2,2-dichloropropionate) is utilized by several microbial species acting alone (Goring *et al.* 1975). Senior *et al.* (1976) reported a stable community of seven different micro-organisms growing on Dalapon in chemostat culture. Of these, three could grow on Dalapon as sole carbon source and were classed as primary utilizers, while the secondary utilizers relied on metabolites released by members of the first group. The mixed population grew faster on Dalapon than any of its individual members by themselves. After some time, a new primary utilizer appeared in the chemostat culture and the authors were able to conclude that it had arisen by mutation from one of the secondary utilizers in the original population. Most studies

TABLE 3. PERSISTENCE OF PESTICIDES IN SOILS

pesticide group	example	estimated time for 50 % to disappear/month
non-persistent	2,4-D (2,4-dichlorophenoxyacetate)	< 0.5
slightly persistent	Dalapon (2,2-dichloropropionate)	0.5–1.5
moderately persistent	Dichlobenil (2,6-dichlorobenzonitrile)	1.5–6
persistent	DDT (4,4-dichlorodiphenyltrichloroethane)	> 6

After Goring *et al.* (1975).

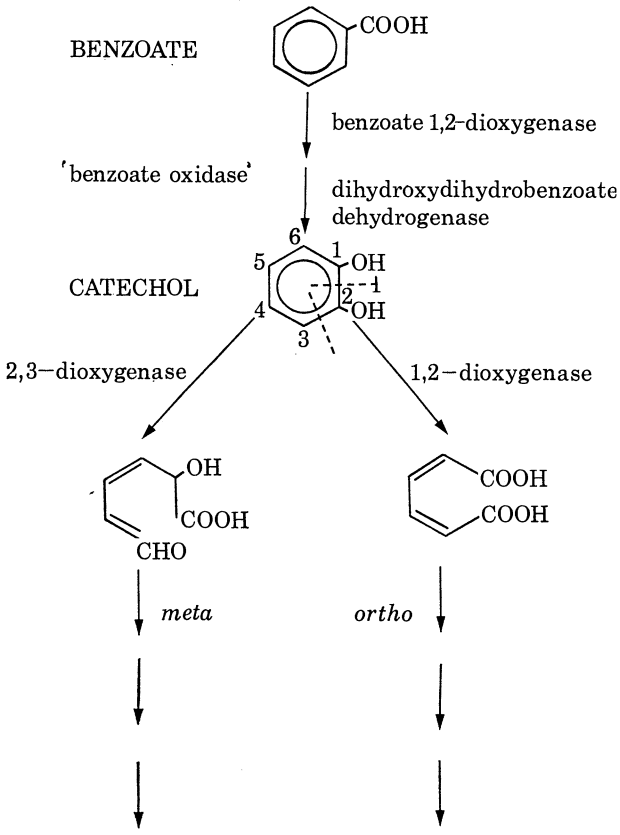

FIGURE 2. Benzoate, and substituted benzoates, may be metabolized by conversion to catechols, followed by aromatic ring cleavage. Catechol 1,2-dioxygenase, *ortho* cleavage, produces *cis*, *cis*-muconate from catechol, which is metabolized further by the enzymes of the β-ketoadipate pathway. Catechol 2,3-dioxygenase, *meta* cleavage, produces 2-hydroxymuconate semialdehyde, which is metabolized further by enzymes of the α-keto acid pathway (Dagley 1971; Clarke & Ornston 1975).

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on microbial adaptation are carried out with pure cultures, but this laboratory system showed how a mixed population of bacteria could adapt to the challenge of a novel chemical compound under conditions that are not too far removed from those operating in soil in the open ground.

Dichlorobenil (2,6-dichlorobenzonitrile) is an example of a fairly persistent herbicide, and the biodegradation of chlorobenzoates illustrates the way in which more complex adaptation to xenobiotics may occur in nature. The first step in the biodegradation of the benzoates is oxidation to a catechol by the action of a benzoate oxygenase and a dehydrogenase (Dagley 1971). The aromatic ring of a catechol may be cleaved between the hydroxyl groups by a 1,2-dioxygenase (*ortho* cleavage) or adjacent to the hydroxyl group by a 2,3-dioxygenase (*meta* cleavage) (Clarke & Ornston 1975) (see figure 2). Whether or not a particular microbial strain can metabolize a particular substituted benzoate will depend on the specificity of both the benzoate oxidase and the catechol oxygenase systems. Since these are normally inducible enzymes, the specificity of the regulatory systems for their synthesis is equally important. For complete biodegradation of substituted benzoates and catechols, it is essential that metabolites later in the pathways should be accepted as substrates by the appropriate bacterial enzymes. For the halogenated compounds to be degraded, it is necessary that the halogen should be eliminated as a halide ion at some step of the pathway. Microbial degradation of synthetic organic molecules was discussed by Dagley (1972, 1975).

Utilization of chlorobenzoates

Some chlorobenzoates are used as herbicides, while others arise as metabolites of the partial breakdown of industrial products such as the polychlorinated biphenyls. Few bacteria have the ability to utilize chlorobenzoates for growth, but Knackmuss and colleagues have followed the emergence of strains that acquired this ability after a period of adaptation. They obtained strains of bacteria that could utilize 3-chlorobenzoate, 4-chlorobenzoate and 3,5-dichlorobenzoate. *Pseudomonas* strain B13 was isolated by enrichment culture with 3-chlorobenzoate. It oxidizes 3-chlorobenzoate to 3-chlorocatechol and employs the *ortho* cleavage pathway for subsequent breakdown. It is unable to utilize 4-chlorobenzoate, since the benzoate oxidase produced by B13 has a very narrow specificity and will not accept 4-chlorobenzoate as a substrate. Although it cannot oxidize 4-chlorobenzoate, strain B13 can oxidize 4-chlorocatechol. It produces two catechol 1,2-dioxygenases, and the combined activities of these isoenzymes is sufficient for oxidation of 4-chlorocatechol (Dorn & Knackmuss 1978*a*, *b*) (figure 3).

Pseudomonas putida strain mt-2 can utilize benzoate and the methyl-substituted benzoates, *m*- and *p*-toluate, for growth. Growth on *m*- and *p*-toluate depends on the presence of the TOL plasmid (Williams & Murray 1974). The benzoate 1,2-dioxygenase determined by the TOL plasmid has a broader specificity than the B13 enzyme and it can accept 4-chlorobenzoate as a substrate, as well as benzoate and the methylbenzoates. The methylcatechols are catabolized by *meta*-pathway enzymes, but the cleavage of 4-chlorocatechol by TOL catechol 2,3-dioxygenase yields dead end products (figure 4).

Bacteria able to utilize 4-chlorobenzoate and 3,5-dichlorobenzoate for growth were selected from chemostat culture after a prolonged adaptation period (Hartmann *et al.* 1979). The chemostat was inoculated with a mixed microbial population in soil samples from the Gottingen area, supplemented with cultures of the two defined *Pseudomonas* strains, B13 and mt-2.

At the initial stage the compounds supplied as a carbon source for growth were 3-chlorobenzoate, which could be used by B13, and 4-methylbenzoate, which could be used by strain

mt-2. After one month, 4-chlorobenzoate was added as an additional carbon source. After a further month of operation with the three substituted benzoates, the chemostat culture could be kept going with 4-chlorobenzoate alone. At this point, 3,5-dichlorobenzoate was added in gradually increasing amounts, and, after 6 months, a strain was obtained that grew well on agar plates, with 3,5-dichlorobenzoate as the sole carbon source (Hartmann *et al.* 1979).

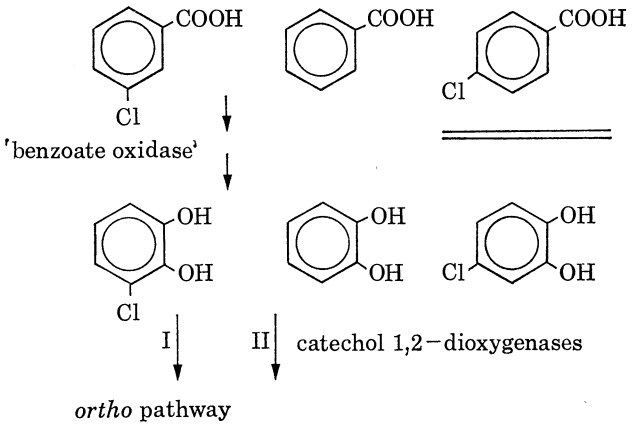

FIGURE 3. Metabolism of benzoate and chlorobenzoates by *Pseudomonas* strain B13. The benzoate 1,2-dioxygenase of B13 acts on 3-chlorobenzoate but not 4-chlorobenzoate. Benzoate induces catechol 1,2-dioxygenase I and 3-chlorobenzoate induces both dioxygenases I and II. Catechol 1,2-dioxygenase II has higher relative activities for 3-chlorocatechol and 4-chlorocatechol (Dorn & Knackmuss 1978*a*, *b*).

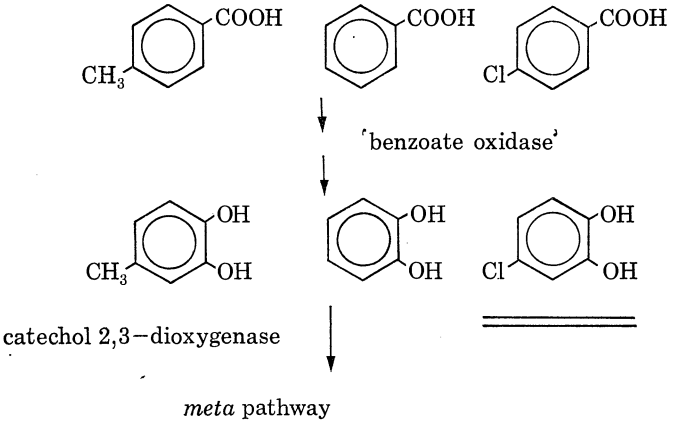

FIGURE 4. *Pseudomonas putida* (*arvilla*) strain mt-2 utilizes benzoate and *m*- and *p*-toluate. The benzoate 1,2-dioxygenase coded by the TOL plasmid also acts on 4-chlorobenzoate. Ring cleavage by catechol 2,3-dioxygenase produces dead end products (Hartmann *et al.* 1979).

The characteristics of strain WR912 suggested that it possessed a benzoate 1,2-dioxygenase with the broad specificity of the enzyme determined by the TOL plasmid of strain mt-2. Cultures of WR912, grown on benzoate or the chlorobenzoates, had high *ortho* cleavage activities for 3-chloro-, 4-chloro- and 3,5-dichlorocatechol and in this respect resemble strain B13. The novel organism WR912 has therefore the necessary enzyme specificity and the necessary inducer specificity for utilizing 3-chlorobenzoate, 4-chlorobenzoate and 3,5-dichlorobenzoate as growth substrates. It was reasonable to conclude that exchange of genetic information had occurred during the long selection periods and that this had been accompanied by one or more

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mutational events. In this experiment they were following the stepwise evolution of new catabolic activities.

Genetic transfer and mutation

The possibility of genetic exchange between *Pseudomonas* strains mt-2 and B13 could be tested directly (Reineke & Knackmuss 1979). The TOL plasmid of *P. putida* mt-2 can be transferred to other *Pseudomonas* species by conjugation and, if there are no barriers to gene expression, the recipient gains the capacity to grow on *m*- and *p*-toluate. Exconjugants from mating mixtures of strains mt-2 and B13 were found to have some of the characters of each of the parent bacteria (table 4). WR211 was obtained by plating a mixture of a streptomycin-resistant mutant of B13 with the streptomycin-sensitive mt-2 on agar plates containing *p*-toluate and streptomycin. This exconjugant grew on 3-chlorobenzoate, but not on 4-chlorobenzoate, and carried the TOL plasmid.

TABLE 4. CONSTRUCTION OF NOVEL STRAINS OF *PSEUDOMONAS* UTILIZING HALOBENZOATES

		benzoates used as growth substrates			
strain	derivation	3-chloro-	4-chloro-	3,5-dichloro-	4-methyl-
B13	wild type	+	−	−	−
P. putida (TOL)mt-2	wild type	−	−	−	+
WR211	conjugation between mt-2 and B13 selection on *p*-toluate	+	−	−	+
WR216	spontaneous mutation from WR211 selection on 4-chlorobenzoate	+	+	−	−
WR241	conjugation between mt-2 and B13 selection on 4-chlorobenzoate	+	+	−	−
WR941	spontaneous mutation from WR241 selection on 3,5-dichlorobenzoate	+	+	+	−

Data from Reineke & Knackmuss (1979) and P. A. Williams (personal communication).

Strain WR216 was derived from WR211 by spontaneous mutation on plates containing 4-chlorobenzoate. Strain WR241 was an exconjugant from mt-2 and B13 plated directly on 4-chlorobenzoate, but the frequency indicated that both plasmid transfer and mutation had taken place. Strain WR241 gave rise, by a further mutation, to a strain, WR941, able to utilize 3,5-dichlorobenzoate. These experiments showed that novel catabolic activities could be acquired by plasmid exchange and mutation.

Transfer of the TOL plasmid from *P. putida* mt-2 to *Pseudomonas* sp. B13 was not sufficient to produce a strain able to utilize 4-chlorobenzoate for growth, even though this compound can be oxidized by the TOL-coded benzoate 1,2-dioxygenase. Both the 4-chlorobenzoate-utilizing strains, WR216 and WR241, had lost the ability to grow on 4-methylbenzoate and P. A. Williams (personal communication) suggests that loss of some of the *meta*-pathway enzymes may be a prerequisite for growth on 4-chlorobenzoate. The sequence of events leading up to strain WR941 involved (*a*) transfer of the TOL plasmid to B13, (*b*) a mutation to allow 4-chlorobenzoate to be utilized, and (*c*) a further mutation to allow 3,5-dichlorobenzoate to be utilized. The initial attack on 4-chlorobenzoate and 3,5-dichlorobenzoate is carried out by the TOL-coded benzoate 1,2-dioxygenase, and the subsequent metabolism depends on the *ortho*-pathway enzymes originating from strain B13. There are close similarities between WR941

derived by genetic experiments and strain WR912 obtained by selection in continuous culture over a long period, indicating that similar genetic events occur in the natural environment.

Old and new microbes

We have seen that natural communities of microorganisms can be employed very effectively for recycling plant and animal wastes, and many of the residues of our industrial society. Synthetic chemicals need not present insoluble problems, since microbial populations can acquire new metabolic activities by mutation and rearrangement of genetic information. This adaptation is more likely to occur in nature if the new synthetic chemical bears some resemblance to a natural product for which metabolic pathways have already evolved. This limitation to microbial infallibility should be borne in mind when designing new herbicides and pesticides that will be released into the environment.

Experiments in microbial evolution in the laboratory have shown that enzyme specificity can be altered by successive mutational steps and that mutations in regulatory genes can lead to very high levels of certain enzymes (Clarke 1978). Transfer of genetic information between bacteria by the processes of conjugation, transduction and transformation can increase the genetic potential. Plasmid transfer by conjugation offers many possibilities for strain construction, particularly since some plasmid genes are carried as short DNA segments (known as transposons) that can pass from one plasmid to another or from a plasmid to a chromosome (Bukhari *et al.* 1977). *In vitro* methods of DNA manipulation can also be used for strain construction (Murray 1979). Although laboratory-made strains may not be very effective in open situations, they may be of very great value in treating industrial effluents. For some toxic compounds it might be preferable to use a microbial enzyme rather than the microorganism itself. The organophosphate insecticide parathion can be metabolized by a stable community of bacteria in continuous culture. One of the organisms produces an enzyme that hydrolyses parathion to *p*-nitrophenol and diethylphosphate, thus reducing the toxicity. Munnecke (1976, 1977) has shown that soluble or immobilized preparations of parathion hydrolase are very effective in detoxifying residues left in containers or on clothing, and suggests that this could be an effective method for destruction of dangerous pollutants.

Biological methods of waste treatment using enzyme preparations, constructed strains or mixed populations of microorganisms offer many advantages. The Japanese project *Microbiology for environmental cleaning* started in 1974 and brought together many microbiologists and other experts, with the aim of developing new techniques for dealing with pollution. They report (Arima 1978) that, although the problems are very complex, they can see many possible industrial applications in the future and can point to some processes that are already practicable. Slater & Somerville (1979) predict that biological treatment will play an increasing role in effluent treatment, and point out that it may be more economical than chemical methods for dealing with industrial wastes.

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Discussion

J. R. Postgate, F.R.S. (*A.R.C. Unit of Nitrogen Fixation, University of Sussex, Brighton BN1 9RQ, U.K.*). What are the prospects of using microorganisms that are derepressed for enzymes that can catabolize exotic substrates to deal with chemical spillages? For example, this approach could have been used in dealing with a recent spillage of fluoroacetate; however, a suitable organism was not available sufficiently quickly to be of use. Should we not have a national culture collection of organisms suitable for dealing with such spillages? Who would finance this collection?

P. H. Clarke. Yes, this is a good idea. For example, microorganisms capable of carrying out dehalogenations are common and it is easy to evolve improved strains.

C. J. Duggleby (*Department of Molecular Biology, University of Edinburgh, Kings Buildings, Edinburgh EH9 3JR, U.K.*). Not all catabolic plasmids are as readily transmissible as TOL; does Professor Clarke feel that transfer of these other plasmids occurs in nature?

P. H. Clarke. Yes.

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