the chromosomal \( \beta \)-oxoadipate pathway. This combination of metabolic possibilities is available to neither the wild type nor the cured strains.

The transmissibility of many plasmids obviously increases the opportunities for genetic rearrangements as outlined above, including recombination with other plasmids or with chromosomal material, and is one way strains may arise from mixed populations with new metabolic capabilities. There are at least two barriers to the spread of an evolving metabolic ability or its advantageous association with a pre-existing ability, both within and between species and genera of bacteria. The first is the transfer of genes between organisms, in which it is clear that plasmids could play a vital role. The second is the lack of genetic homology within the recipient organism that prevents recombination and thus the utilization of the transferred genes. The incorporation of genes in a plasmid could also facilitate the overcoming of this barrier.

Results such as those of Audus (1960) show three features characteristic of many studies on the degradation of xenobiotics: (a) a long lag phase of up to several months before any disappearance of the compound can be detected; (b) the difficulty of isolating pure cultures capable of the complete breakdown even after the onset of disappearance; (c) the inherent instability of the degradative ability in pure strains of competent bacteria, once isolated, when removed from selective media. One possible explanation is that the lag phase represents a period during which processes of plasmid exchange and recombination result in the selection of one or more organisms that finally have a battery of enzymes capable of the complete degradation. The instability of the competent isolates might therefore be a result of the responsible genes being located on an unstable and hence easily lost plasmid.

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**Evolution of Catabolic Pathways**

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Catabolic pathways are complex units of function mediated by specific sets of enzymes and, in some cases, transport systems. The pathways normally are integrated into cellular processes by control mechanisms that regulate the enzymes or transport systems by modulating the synthesis or the activity of the proteins. Thus the evolution of a physiologically effective catabolic pathway requires the acquisition of a full set of structural and
regulatory genes. A satisfactory theory accounting for the evolutionary acquisition of a catabolic pathway should provide a description of the following.

1. The genetic background of the organism before it acquired the pathway.

2. The mutations that led to the expression of the new pathway. These may include:
   (a) Modification mutations (base changes in DNA, for example) that altered the structure of genes and gene products.
   (b) Deletions, transpositions and inversions that led to the loss of gene expression and thus caused the redirection of metabolic flow.
   (c) Deletions, transpositions and inversions that led to the loss of gene expression and thus caused the redirection of metabolic flow.
   (d) Transpositions changing the orientation of functional genes.

3. Processes of gene transfer that may have led to new combinations of genes.

4. Selective pressures that favoured the growth of organisms with new sets of genes.

It is not surprising that the most significant advances in the study of catabolic evolution have been made with micro-organisms. The homogeneity of pure microbial cultures makes their biochemistry relatively simple to explore, mutant and recombinant strains frequently can be identified by the appropriate choice of growth medium, gene transfer among micro-organisms can be quite rapid, and selective conditions favouring the growth of strains with new combinations of genes can be clearly defined.

Novel catabolic activities can appear in cells grown under selective laboratory conditions, and in some instances have been shown to result from only one or a few mutations. We shall use the term ‘mechanistic studies’ to describe the analysis of catabolic acquisition by organisms in the laboratory. The obstacles to growth that are potentially removed by mutation have been summarized by Clarke (1974): ‘A compound is not utilized if it is unable to enter a cell, if there are no enzymes that convert it to suitable metabolic intermediates, if the enzymes are not induced by the compound, if the enzymes which can attack the compound do so at a rate too low to be effective, or if the compound is an inhibitor of an essential cellular activity’. When no more than a few mutations are required to overcome these barriers, the process of metabolic acquisition may be studied in the laboratory. Analyses of this kind give an indication of what may happen as catabolic pathways evolve. Detailed accounts of mechanistic studies can be found in several excellent reviews (Clarke, 1974; Hartley, 1974; Hegeman & Rosenberg, 1970).

Mechanistic studies have shown that, under laboratory conditions, organisms frequently acquire the ability to utilize a new compound as a result of a mutation in a regulatory element that controls the biosynthesis of either enzymes (Brown & Clarke, 1970; Canovas & Johnson, 1968; Charnetzky & Mortlock, 1974a; Cocks et al., 1974; Mortlock et al., 1965; Tanaka et al., 1967) or transport systems (Wu et al., 1968) that act on the compound. A gene duplication leading to the production of large amounts of a protein with an essential activity has been observed as an initial response to selection in a chemostat on limiting concentrations of growth substrate with yeast (Adams & Hansche, 1974) and bacteria (Horiuchi et al., 1962; Rigby et al., 1974). Once the gene for a protein is expressed, mutations may alter the protein’s specificity so that it can accommodate a novel substrate in catalysis. In some cases, relatively few mutations may be required to change the substrate specificity of an enzyme (Francis & Hansche, 1972; Oliver & Mortlock, 1971). As part of an analysis of the acquisition of altered amidases in Pseudomonas aeruginosa, Brown & Clarke (1972) showed that a single amino acid substitution altered the specificity of the wild-type acetamidase so that it acted upon acetanilide. Several structural gene mutations were required to produce a mutant amidase that acted upon some of the amides tested by Clarke and her associates (Brown et al., 1969; Clarke, 1974). Hartley’s group found that multiple mutagenesis with nitrosoguanidine was required to alter the ribitol dehydrogenase of Klebsiella aerogenes so that the enzyme possessed an increased affinity for xylitol (Burleigh et al., 1974).

Metabolic acquisition may result from regulatory mutations with the consequence of either a loss of inductive control leading to the constitutive production of an enzyme under all growth conditions or a change in the specificity of induction so that a novel growth substrate may elicit the synthesis of an enzyme that acts upon it. Mutations that lead to constitutivity may be caused by the inactivation of a repressor protein, and
therefore may occur much more often than mutations that alter the structure of regulatory proteins so that novel metabolites may elicit enzyme synthesis. Many constitutive mutant strains grow well under the conditions in which they were selected and appear to be stable during storage on non-selective growth media. This raises the possibility that at least two genetic steps are required for the evolution of new regulatory genes. The first mutation inactivates a regulatory gene and results in the constitutive production of an enzyme; the second leads to the reassertion of inductive control by the acquisition of a new regulatory gene. The extent to which such mutational sequences may contribute to the evolution of new regulatory genes is unknown. Since almost all of the catabolic enzymes and transport systems produced by wild-type bacteria are under strict inductive control, it appears that mutations causing the constitutive production of proteins are counter-selected effectively in the natural environment. Mutations that alter the specificity of induction have been identified (Brammar et al., 1967; Canovas et al., 1968; LeBlanc & Mortlock, 1971); organisms bearing these mutations maintain a rigorous control over induced enzyme synthesis under most growth conditions. Mutant strains in this class may occur so infrequently that they escape detection in some mechanistic experiments, but they may make a significant contribution to the evolution of new regulatory genes in the harsh and fluctuating conditions of Nature.

The strength of mechanistic studies lies in their direct and well-defined approach to the unit processes of mutation and selection in the acquisition of new metabolic capabilities. These investigations have provided a conceptual basis for analysing the evolution of regulatory genes and information about the evolutionary routes open to existing enzymes, but natural evolutionary events cannot be reconstructed on the basis of mechanistic studies alone. Indeed the fundamental design of most mechanistic studies is to shift the phenotype of organisms away from the phenotype that evolved in the natural environment. Therefore essential complements to mechanistic analyses are descriptive studies that attempt to perceive what did happen as catabolic pathways evolved.

By comparing the properties of presently existing genes and gene products, descriptive studies demonstrate evolutionary relationships indirectly rather than directly as in mechanistic studies. In attempts to elucidate the evolution of enzymes and induction mechanisms that participate in catabolic pathway, descriptive studies seek information about the following questions. (1) Within a single organism, what is the evolutionary relationship between isofunctional proteins? (2) What is the evolutionary relationship between enzymes catalysing analogous reactions within a cell? (3) Sometimes divergent metabolic sequences are employed to achieve a single physiological function in members of different biological groups. Did the different biochemical sequences evolve independently? (4) Distinctive induction mechanisms are used to govern the expression of some catabolic enzymes in different microbial genera. Do the differences in regulation reflect the independent evolution of the pathway from separate genetic origins in each biological group? (5) Have the particular patterns of induction found within well circumscribed taxa been selected to permit organisms to adapt to specific niches in the natural environment?

By addressing these questions, descriptive studies can contribute insights into the mechanisms of evolution, evolutionary changes that are tolerated within a single protein, and the selective pressures that may have operated in the evolution of induction mechanisms and in the divergence of protein structure. Evidence for or against the evolutionary affinities of proteins may be adduced from examination of the following characteristics: (1) oligomeric structure (Lebherz & Rutter, 1973; London, 1974); (2) protein–protein interaction as revealed by the hybridization of subunits from heterologous enzymes (Queener & Gunsalus, 1970; N. W. Patel et al., 1973; Crawford, 1975); (3) serological cross-reactivity (Stanier et al., 1970; London & Kline, 1973); (4) amino acid composition [Metzger et al. (1968), but see also Prager & Wilson (1971)]; (5) amino acid sequences (Ambler & Wynn, 1973; Ambler, 1974; Hartley, 1974; Crawford, 1975); (6) similar binding domains as revealed by X-ray-diffraction analysis (Rossman & Liljas, 1974). It is clear that a minimum requirement for comparative work is that at least one of the proteins should be obtained in pure form and

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characterized so that it may be used as the basis for comparison. It also is evident that the significance of the comparison increases as the characterization moves through the laborious course to chemical definition.

(a) Some of the methods described above have been applied to proteins that catalyse identical or similar catabolic reactions within an organism. Representatives of some bacterial genera elaborate two or more catabolic enzymes with identical biochemical function (Canovas & Stanier, 1967; Charnetzky & Mortlock, 1974b; Koo & Adams, 1974; Patel et al., 1975; Wilson & Mortlock, 1973), and it is possible that the structural genes for these enzymes were derived from a single ancestral gene. The multiple forms of the enzymes increase the physiological flexibility of the cell by allowing each enzyme to be regulated independently. Most catabolic enzymes are not subject to allosteric regulation at the level of their activity; control is exercised by governing their synthesis at the level of transcription. Therefore these enzymes, if they have a common evolutionary origin, have not been subjected to selective pressures for structural divergence in order to bind chemically distinct substrates or allosteric effectors. On these grounds, the enzymes might be expected to have closely similar structures if additional selective forces did not contribute to their evolution. Recent evidence suggests enzymes that catalyse identical biochemical reactions within bacteria have diverged structurally (Koo & Adams, 1974; Patel et al., 1975). The physiological basis for the structural divergence has not been established. Selective forces that favour divergence of homologous structural genes within an organism may operate at the level of the genes. Genetic recombination is favoured at regions with homologous DNA sequences. Therefore it might be expected that intrachromosomal (or intraplasmid) recombinational events might decrease the genetic stability of homologous genes within an organism. The available evidence indicates that duplicated structural genes are unstable within bacteria and are lost with a frequency as high as $10^{-2}$ per cell plated (Folk & Berg, 1971; Jackson & Yanofsky, 1973; Hartley, 1974). Mutations that introduce variations into the nucleotide sequence of DNA coding for homologous genetic regions would increase their stability and, if the base substitutions did not interfere with the activity of the gene products, might be selectively advantageous.

(b) Enzymes that catalyse chemically analogous reactions within an organism may share a common evolutionary origin. Comparison of the oligomeric structures of enzymes that catalyse similar reactions in the $\beta$-oxoadipate pathway of Pseudomonas putida has suggested that the enzymes may be homologous (Meagher & Ornston, 1973; R. N. Patel et al., 1973; Parke et al., 1973), but more detailed analysis indicates substantial differences in primary structure between putatively homologous enzymes (Parke, 1975). Possible selective factors that could act as evolutionary determinants in the divergence of homologous structural genes within an organism may operate at the level of the genes, as postulated above for isofunctional enzymes, or at the level of proteins. The evolution of substrate specificity may have required a correlated divergence of subunit binding regions. Many catabolic enzymes are oligomeric, and the self-association of their subunits appears to be accurate, even when putatively homologous subunits are undergoing self-assembly in the same cell. The regions of primary sequence that determine the accurate self-assembly of the different oligomers presumably are the products of divergent evolution.

(c) Fungi and bacteria employ different biochemical mechanisms to dissimilate some aromatic substances to $\beta$-oxoadipate (Cain et al., 1968; Stanier & Ornston, 1973; Cook & Cain, 1974). The most plausible interpretation of the biochemically distinct pathways is that they evolved independently in each biological group, and this inference is supported by the observation of major differences between the oligomeric structures of the carboxymuconate lactonizing enzymes (cycloisomerases) from fungi (Thatcher & Cain, 1974) and bacteria (R. N. Patel et al., 1973).

(d) Bacteria employ a single mechanism to dissimilate aromatic growth substrates via the $\beta$-oxoadipate pathway, but differences in the evolutionary history of the organisms are reflected in variations in the induction mechanisms used to govern the synthesis of enzymes of the pathway. The closely related fluorescent pseudomonads, Ps. aeruginosa.
and *Ps. putida*, use similar mechanisms to govern induction of enzymes of the β-oxo-
adipate pathway (Ornston, 1966; Kemp & Hegeman, 1968). Isofunctional enzymes for the pathway in the two species share a number of serological determinants (Stanier et al., 1970), and the organization of genes for the pathway appears to be roughly the same in the two species (Kemp & Hegeman, 1968; Wheelis, 1975).

Synthesis of the enzymes of the β-oxo-adipate pathway is governed by different mechanisms in *Pseudomonas* and *Acinetobacter* (Canovas & Stanier, 1967). Representatives of these two genera differ morphologically, and the G+C content of their DNA differs by about 20%. The differences in inductive control are reflected in the extent of co-ordinate induction, in the metabolites that act as inducers, and in the presence or absence of iso-
functional enzymes. The enzymes of the β-oxo-adipate pathway from the two genera do not cross react serologically (Stanier et al., 1970) as would be expected if the structural genes for the enzymes were evolutionarily remote. A different impression suggestive of close evolutionary homology emerges from the limited amino acid-sequence information that is available. The N-terminal amino acid sequence of one enzyme of the β-
oxo-adipate pathway, muconolactone isomerase, has been compared. Only one clear difference exists in the first 14 residues of the *Ps. putida* and *A. calcoaceticus* mucono-
lactone isomerases (Patel et al., 1974) and thus the proteins appear to be homologous. Therefore the different induction mechanisms used by the representatives of each species cannot be attributed to the independent evolution of the pathway from separate genetic origins as has been suggested (Canovas et al., 1967). Moreover, the presence of homolo-
gous enzymes in two genera, which by other taxonomic criteria would appear to be dis-
tant, suggests that gene transfer may have played a role in the evolution of the β-oxo-
adipate pathway of the two genera.

(e) The evolution of distinctive induction mechanisms in different microbial species may be influenced by the nutritional opportunities available to the representatives of each species in the natural microniches. Evidence that ecological factors may have influenced the evolution of induction mechanisms has come from the analysis of transport systems (Meagher et al., 1972; Ornston & Parke, 1976). β-Oxoadipate does not act as an inducer in *A. calcoaceticus* and the induction mechanisms of this species preclude the use of this compound as a growth substrate by wild-type cells. In contrast, β-oxoadipate has a major inductive role in fluorescent pseudomonads. That β-oxoadipate acts as a growth sub-
strate for the latter bacteria in their natural environment is indicated by the observation that representatives of *Ps. putida* elaborate an uptake system that is induced by and ap-
pears to act upon β-oxoadipate.

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Factors in Haemolymph of the Mussel, *Mytilus edulis* L., of Possible Significance as Defence Mechanisms

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A system comparable with the highly specific immune response of vertebrates has never been described in any invertebrate group. However, many invertebrates contain constitutive factors of apparently broad specificity which are postulated to have a defensive role. Such lytic and agglutinating factors occurring in mussel haemolymph have been examined. Mammalian erythrocytes provide a well-characterized model system for the study of those factors interacting with membranes.

Body fluid (haemolymph) was collected by centrifugation of the mussels after removal of the shell and about 2 ml of fluid was obtained from 4 g of tissue (one animal). This was similar in protein (5 mg/ml) and ionic concentrations to haemolymph collected directly from the heart (0.1 ml/animal).

Lysozyme (EC 3.2.1.17) activity was detected in every mussel examined, by using *Micrococcus luteus* as substrate (Litwack, 1955) and hen’s-egg-white lysozyme as a standard; 20 individuals contained a mean of 8.15 (s.d. ±2.28) μg of lysozyme/ml of