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Obligately Anaerobic Bacteria in Biotechnology

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ABSTRACT

New obligately anaerobic bacteria are being discovered at an accelerating rate and it is becoming very evident that the diversity of anoxic biotransformations has been greatly underestimated. Furthermore, among contemporary anaerobes there are many that thrive in extreme environments including, for example, an impressive array of both archaebacterial and eubacterial hyperthermophiles. Free energy for growth and reproduction may be conserved not only via fermentations but also by anoxygenic photophosphorylation and other modes of creating transmembrane proton potential. Thus forms of anaerobic respiration in which various inorganic oxidants (or indeed carbon dioxide) serve as terminal electron acceptors have greatly extended the natural habitats in which such organisms may predominate. Anaerobic bacteria are, however, often found in nature as members of close microbial communities (consortia) that, although sustained by syntrophic and other relations between component species, are liable to alter their composition and character in response to environmental changes, e.g., availability of terminal oxidants. It follows that the biotechnological exploitation of obligately anaerobic bacteria must be informed by knowledge both of their biochemical capacities and of their normal environmental roles. It is against this background that illustrative examples of the activities of anaerobic bacteria are considered under three heads:

1. *Biodegradation/Bioremediation,* with special reference to the anaerobic breakdown of aromatic and/or halogenated organic substances;
2. *Biosynthesis/Bioproduction,* encompassing normal and modified fermentations; and

3. *Biotransformations,* accomplished by whole or semipermeabilized organisms or by enzymes derived therefrom, with particular interest attaching to the production of chiral compounds by a number of procedures, including electromicrobial reduction.

Index Entries: Anaerobes; biodegradation; dehalogenations; fermentations; biotransformations; chiral bioreductions.

INTRODUCTION

Humans were exploiting anaerobic microbial processes long before their nature was comprehended, e.g., yeast ethanol fermentations, the lactic bacterial fermentations of food preservation, or the anaerobic digestion of cellulose by rumen microbes. This makes it the more remarkable that until relatively recently the scope and potential of anaerobic biotransformations were grossly underestimated. In part this may have been one legacy of the designation by Priestley and Lavoisier of oxygen as "the vital force," but, more likely, it came about because initially the isolation, growth, and manipulation of obligately anaerobic bacteria called for specialist skills and equipment not possessed by the majority of microbiologists, let alone biochemists. The problems were exacerbated by the fact that many of the more interesting anaerobic bacteria are naturally quite slow growing. The ready availability of anaerobic cabinets, and of "room temperature catalysts" capable of stripping the last remnants of dioxygen from gaseous mixtures has now removed all such obstacles to their cultivation and study. Consequently, over the last 20 years or so there has been an almost explosive increase in the number and diversity of newly identified genera and species of obligately anaerobic bacteria.

Many of these bacteria have been shown to accomplish metabolic feats that were previously deemed implausible, and in some instances reactions occur anaerobically that cannot proceed under aerobic conditions. One is therefore no longer able to suppose that if a transformation cannot be accomplished aerobically it will certainly not proceed anaerobically. However, that an ingenious repertoire of anoxic biochemistry has been disclosed should surprise no one, for anaerobes are presumed to have enjoyed a long period of evolutionary experimentation before obligate aerobes put in an appearance. Thus there is now scarcely a naturally occurring organic compound that cannot be fermented by some bacterium or other, whereas anoxygenic phototrophy and forms of anaerobic respiration using electron sinks as different as nitrate, Fe(III) or Mn(IV), sulfate, or carbon dioxide have extended their natural habitats.

Growing as they do in the absence of dioxygen, obligately anaerobic bacteria sustain low redox potentials in their immediate vicinities, and many are notable for their potent reducing properties. Furthermore, any oxygen atom incorporated into their substance or into their metabolic

intermediates and products must have originated in some substrate other than dioxygen. The emergence of dioxygen-producing photosynthetic organisms and thereafter of heterotrophic, aerobically respiring organisms was of key importance in making possible the rapid evolution of metazoa and hence of larger, highly differentiated organisms. Yet, so far as bacteria were concerned, the ability to use dioxygen as a terminal electron acceptor in respiration and as a substrate in biochemical reactions, in the main afforded only alternative routes to ends that could already be achieved by anaerobic means. Indeed, even in contemporary aerobes the majority of mainstream metabolic processes are wholly anaerobic in character, notable exceptions being the dioxygen-dependent biosynthesis of sterols, and the oxygenative breakdown of lignins.

The establishment of a global aerobic atmosphere brought in its train the challenge of sensitivity to oxygen toxicity, and it is tempting to speculate that the tendency of many obligate anaerobes to exist in nature as components of mutualistically cooperative microbial communities has, at least in part, been driven by their need to inhabit niches wherein they are protected from exposure to potentially lethal levels of dioxygen. In the mixed consortium this is assured by the dioxygen-scavenging activities of facultative and obligate aerobes that, in their respiration, consume reduced organic compounds generated by the anaerobes.

Contemporary anaerobes are, of course, the outcome of several routes of continuing evolution, and present species include many that have specialized in their activities and/or have succeeded in establishing themselves in an extreme environment that is hostile to competitors. Thus, among obligately anaerobic bacteria we find acidophiles, alkalophiles, and halophiles, and a particularly impressive range of thermophiles and hyperthermophiles (*1*). In many situations the pattern of community living extends to consortia that are stabilized by a variety of syntrophic relationships. One classic form of anaerobic syntrophism is based on hydrogen gas exchange in which one type of bacterium (the hydrogenogen) can profitably utilize its normal organic substrate only if the hydrogen that is generated thereby is immediately scavenged by a hydrogen-consuming companion organism (the hydrogenotroph) for which the hydrogen serves as a prime electron donor.

Among consortia based on such hydrogen transfer are those that sustain methanogenic or rumen-based anaerobic fermentations. Other relationships are more subtle. For example, from polluted river sediment was isolated a two-membered bacterial association that accomplished complete anoxic breakdown of *para*-cresol under nitrate-reducing conditions. One species anaerobically converted the *para*-cresol to *para*-hydroxybenzoate but could not proceed to degrade this product. However, the second bacterial species, though unable to attack *para*-cresol, could utilize the *para*-hydroxybenzoate, thereby supplying to its partner products of hydroxybenzoate breakdown that the latter could utilize for its growth (2). Again, the strictly anaerobic bacterium *Pelobacter acidigallici* (*3*) can ferment several

trihydroxybenzenes but not syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid). Yet when it was grown in coculture with the homoacetogenic anaerobe *Acetobacterium woodii* the mixed culture fully mineralized added syringate. The *Acetobacterium* initiated the attack by *O*-demethylation of the syringate, so supplying to the companion *Pelobacter* a fermentable substrate, i.e., gallate. Addition to the community of a methanogen, for example, *Methanosarcina barkeri* or *Methanothrix soehngenii*, enabled methoxylated gallic acid derivatives to be fully converted into methane and carbon dioxide. Substituting the sulfate-reducing hydrogenotroph *Desulfobacter postgatei* for the methanogen, ensured that 3,4,5-trimethoxybenzoate was completely degraded, via acetate, to carbon dioxide (*4*).

The species composition of an anaerobic community, plus the ease/extent to which it can expand its capacities during a period of acclimation (by adaptation and/or the recruitment of new species or additional genetic information), will evidently determine the range of its biodegradative and biosynthetic abilities, though these will also be influenced by what terminal electron sinks are available. Generally speaking, the most versatile anaerobic respirers will choose preferentially to utilize that oxidant whose redox couple has the highest potential. Thus, nitrate would tend to be utilized before Mn(IV) and Fe(III), which in turn would tend to be consumed in advance of sulfate, which would itself be used before methanogenesis could prevail. There are circumstances therefore wherein anaerobic environments are stratified or zoned according to which anaerobic oxidant is predominant. The pattern may evidently change with time as the result of microbial activities, diffusion, and eventual exhaustion of one or more of these electron sinks, and shifts in distinctive anaerobic bacterial communities will reflect this change. It has been suggested that the steady state, dissolved H_2 concentration is diagnostic of these communities since it decreases as the electron-accepting avidity of the predominant terminal sink increases, i.e., in the order methanogenic > sulfate-reducing > Fe(III)-reducing > Mn(IV) reducing > denitrifying (*5*). It is to be expected that some organic compounds might more readily be biotransformed in more reducing environments than in zones of higher redox potential.

One theme that should therefore run like a *leitmotif* through any account of the possibilities that obligately anaerobic bacteria display as agents of biotechnological transformations, is that their exploitation is likely to be the more effective to the extent that it reflects their usual environmental situations and roles. An apt example is afforded in the context of the hydrogen syntrophic consortium. In the anaerobic treatment of organically polluted waste waters by the upflow anaerobic sludge blanket (UASP) procedure, the active organisms associate into granular flocs (*6,7*), which effectively concentrate the working biomass. On the same principle somewhat more compact syntrophic biomethanation (SB) granules can be produced from a mixture of syntrophic plus methanogenic anaerobes that, as in nature, work concertedly to convert fermentatively produced volatile

fatty acids into methane and carbon dioxide. It is feasible that into such associations there could be introduced specialist anaerobes, e.g., dehalogenators, *O*-demethylators, and so on, to extend the degradative capacities of the biogranules and so produce purification agents that are "precustomised" to tackle specific pollutants (*1,8,9*).

An even more obvious matching of desirable properties with the natural habit of the chosen bioagent is possible when anoxic transformations are to be undertaken at higher than usual temperatures using either whole organisms or thermoduric enzymes. In these circumstances it is evidently sensible to turn to thermophilic anaerobes, both eubacterial and archaebacterial (*1,10*). Many of these have been isolated from microbial communities that inhabit hot volcanic springs, and in recent years the range of hyperthermophilic archaebacteria has been greatly extended by isolations from submarine hydrothermal vents and from terrestrial or marine solfataric muds (*1*). Thus the hyperthermophilic *Methanopyrus kandleri*, which grows at 110°C, was isolated from such a hydrovent (*11,12*), as were obligately anaerobic carboxydotrophic bacteria that produce carbon dioxide from the volcanic gases emerging from such vents (*13*). Sulfidogenic hyperthermophilic archaea (obligate anaerobes including species of *Archaeoglobus, Thermococcus,* and *Pyrococcus*) have been isolated from production fluids drawn from deep oil reservoirs below the bed of the North Sea and the permafrost of Alaska (*14*). A distinctively different bacterium was isolated from Atlantic II Deep Brines in the Red Sea and was named *Flexistipes sinusarabici* (*15*). Not only was this anaerobic organism, during growth at temperatures up to 64°C, tolerant of high salinity (25%) and of potentially toxic levels of heavy metals, but it proved to be the first representative of an entirely new phylum of bacteria. The basis for the abilities of such organisms to grow at unusually high temperatures is probably multifactorial. It has, for example, been suggested that the resistance to thermal denaturation of intracellular proteins of *Methanothermus fervidus* might be due to interactions with the high internal concentration (300 m*M*) of 2,3-diphosphoglycerate in this bacterium (*16*). However, a large number of enzymes isolated from thermoanaerobes have been found to be very thermostable and simultaneously to have unusual stability to organic solvents, detergents, and extremes of pH. For example, *Pyrococcus woesei* and *Pyrococcus furiosus* are species of a genus of hyperthermophilic archaebacteria that were isolated from a submarine, solfataric sandy sediment through which hot seawater and volcanic gases percolated at 100°C. Both organisms grow on starch in a medium wherein elemental sulfur is available for reduction to H_2S and the atmosphere consists of H_2 plus CO_2. In the laboratory, however, cultures can be grown in a nutrient-rich medium without sulfur and under N_2 plus CO_2 to yield substantial crops of cells from which several highly thermoduric enzymes can be purified. Thus the α-amylase of *P. woesei* is active over a temperature range of 40–130°C (*17*), whereas the α-glucosidase of *P. furiosus* has a temperature optimum from 105–115°C and a half-life of 46–48 h at

98°C (*18*). The serine protease of *P. furiosus* similarly has a half-life of 33 h at 98°C (*19*).

Just as the opportunity to sample submarine thermal vent communities threw up a bewildering array of novel obligate anaerobes so, as other special communities are discovered, can we expect to find other obligate anaerobes with unusual but useful features. Thus the microbial reduction of CO_2 to acetate is a major electron sink reaction in the hindgut fermentations of wood-feeding cockroaches and termites and versatile anaerobic acetogens, such as *Acetonema longum* and *Clostridium mayombei*, have been isolated from these locations (*20,21*). *Methanobacterium arbophilicum* and *Propionispira arboris* were obtained from wetwoods of living trees (*22,23*), whereas sediments from hypersaline and/or high pH lakes have yielded, among other organisms, *Desulfohalobium retbaense* (*24*), *Halobacteroides halobius* (*25*), alkalophilic and halophilic methanogens (*26,27*), and halophilic, methylotrophic anaerobes (*28*). *Clostridium paradoxum*, which is capable of growth at pH 11 and at temperatures above 60°C, was isolated from anaerobic sewage digestors in the US (*29*). A thermophilic, strictly anaerobic strain of *Dictyoglomus*, which only utilized xylan (fermenting this to acetate, H_2 and CO_2) was isolated from a paper-pulp cooling tank at a paper-board factory in Finland (*30*). Most intriguingly, an anaerobic dehalogenating microbial community was isolated from marine sediment that housed burrows of the hemichordate *Saccoglossus kowalewskii* which synthesises 2,4-dibromophenol; this compound accumulates in the mucus linings of the burrows where it apparently inhibits growth of aerobic bacteria (*31*). Indeed, the ability of marine anaerobic dehalogenators to degrade anthropogenically derived substances may, in part, be due to activities directed toward naturally occurring analogs formed by a number of marine inhabitants, including macroalgae. When such a xenobiotic substrate is poorly water soluble and/or potentially toxic, it might beneficially be subjected in the laboratory to bacterial degradation in a biphasic aqueous–organic system (*32*).

The picture emerges of an exceedingly rich, obligately anaerobic microbiota that constitutes a reservoir of biodiversity scarcely yet exploited in many areas of biotechnology. There is also hope that progress can be made in "dissecting out" the component organisms of some of the anaerobic syntrophic consortia and of contriving ways to grow them in the absence of their usual companions. Thus, for example, in the presence of the hydrogenation catalyst Pd-$BaSO_4$ *Syntrophus wolfei* effected butyrate oxidation with olefin reduction, i.e., in the absence of any normal interspecies hydrogen transfer (*33*). A partly successful attempt was made to grow *Acidaminobacter hydrogenoformans* using an artificial electron accepting system consisting of semipurified hydrogenase from *Desulfovibrio vulgaris*, propylviologen sulfonate as redox mediator, and 2-anilino-1,4-naphthoquinone dissolved in dibutylphthalate as the terminal electron sink (*34*).

Continued experimentation along these lines could extend the range of reductive processes that may be accomplished by hydrogenogens. On the other hand, the ability of natural hydrogenotrophs to utilize so well gaseous H_2 as primary electron donor can be advantageous in any putative bioreductive agent.

A short article such as this cannot hope to be comprehensive. Therefore in what follows, examples will only be considered that illustrate principles and general opportunities for the exploitation of anaerobes. Fortunately, there are many recently published articles that are broad in scope and detailed in content. They include a compendium of accounts of the biology of anaerobic microorganisms (*35*), two books on the biotechnological attributes of the clostridia (*36,37*), other books on fermentations (*38*) and biocatalysis (*39*), and a substantial report on anaerobic biotransformations (*40*). Schink has written percipiently on the principles and limits of anaerobic degradation (*41,42*) and splendid reviews have appeared on subjects as diverse as the anaerobic degradation of aromatic compounds (*43*), microbial anaerobic respiration (*44*), microbial reductive dehalogenations (*45*), anaerobic utilization of Fe(III) and Mn(IV) (*5,46*), thermophiles (*47,48*) and hyperthermophiles (*10,49*), the physiology and enzymology of anaerobic starch-utilizing thermophiles (*50*), the production of fuels and chemicals from biomass (*51*), solvent production (*52*), methanogenesis (*53,54*), and the biology, ecology, and biotechnological applications of anaerobic bacteria adapted to environmental stresses (*1*). Many articles and reviews continue to appear in journals devoted to the problems of the water and waste-disposal industries, on the subjects of anaerobic purification of organically polluted waters (*55*), and on both dry and wet forms of anaerobic digestion of putrescible matter (*56–59*). From all of these sources it is evident that the biotechnological promise of obligately anaerobic bacteria may best be considered under the three heads:

1. Biodegradation/Bioremediation;
2. Biosynthesis/Bioproduction; and
3. Biotransformations.

BIODEGRADATION/BIOREMEDIATION

Aerobic degradation is likely to be the fate that awaits most of the organic matter that is produced annually, with anaerobic breakdown being a feature of a more restricted range of ecological niches, including aquatic sediments and the gastrointestinal tracts of animals. However, the great range of degradative abilities possessed by obligately anaerobic bacteria, and the hope that they can be put to profitable use in a biotechnological context, are both well illustrated by consideration of anaerobic aromatic degradation and anaerobic dehalogenation.

Aromatic Degradation

Possibly it was because oxygenases had been shown to play such a crucial role in the ring-opening reactions accomplished by aerobic degraders of aromatic organic compounds that for a long period the capacities of anaerobic bacteria to undertake ring fission reactions were grossly underestimated. The same hesitancy was not displayed regarding degradation of heterocyclic compounds, for it had long been recognized that a range of purines and pyrimidines were fermentable by anaerobic bacteria. For example, a number of pyrimidine-fermenting clostridia were known first to reduce the weakly aromatic ring system and then hydrolytically to cleave the product. A more remarkable reaction initiated the anoxic attack on nicotinic acid carried out by *Clostridium barkeri* when the pyridine ring was first hydroxylated via a selenium- and molybdenum-containing, NAD-dependent nicotinate hydroxylase (*60*).

A review article written by W. C. Evans in 1977 (*61*) was particularly influential in focusing attention on the range and role of anaerobic dissimilation of aromatic compounds. Evans had a longstanding interest in animal nutrition and recognized that the forage consumed by ruminants contained natural aromatic compounds that did not emerge unscathed from their encounter with the anaerobic rumen microbiota. He was also aware that Tarvin and Buswell in 1934 (*62*) had reported that benzoate, phenylacetate, phenylpropionate, and cinnamate were completely utilized by a methane-forming sewage sludge sample incubated under strictly anaerobic conditions. Yet it was with the anoxygenic phototroph *Rhodopseudomonas palustris* that he and Dutton (*63*) obtained suggestive evidence of anaerobic reductive metabolism of benzoate via cyclohexane carboxylate followed by dehydrogenation to cyclohex-1-ene carboxylate and a "CoA-mediated β-oxidation" that involved hydration, dehydrogenation, and then ring cleavage of the 2-oxocyclohexane carboxyl-CoA to produce pimelate. Following in the footsteps of others who had demonstrated that denitrifying bacteria could utilize aromatic compounds, Williams and Evans (*64*) went on to show that a strain of *Paracoccus denitrificans* that aerobically decarboxylated benzoate to catechol and then metabolized this via the *ortho* pathway, degraded benzoate quite differently under anaerobic denitrifying conditions, via a reaction sequence similar to that employed by *Rps. palustris*. However, adipate rather than pimelate was produced as an intermediate.

A similar route of benzoate catabolism was followed in methanogenic consortia and it was later convincingly shown that in all of these instances the first step in the anaerobic catabolism of benzoate was its activation to form benzoylCoA, with all of the subsequent intermediates being produced as their CoA esters. BenzoylCoA thus assumes a central role in the anaerobic metabolism of aromatic compounds of various kinds, including phenol, 2-aminobenzoate, phenylacetate, and phthalates (Fig. 1). Recently, an oxygen-sensitive enzyme system has been described that is capable of

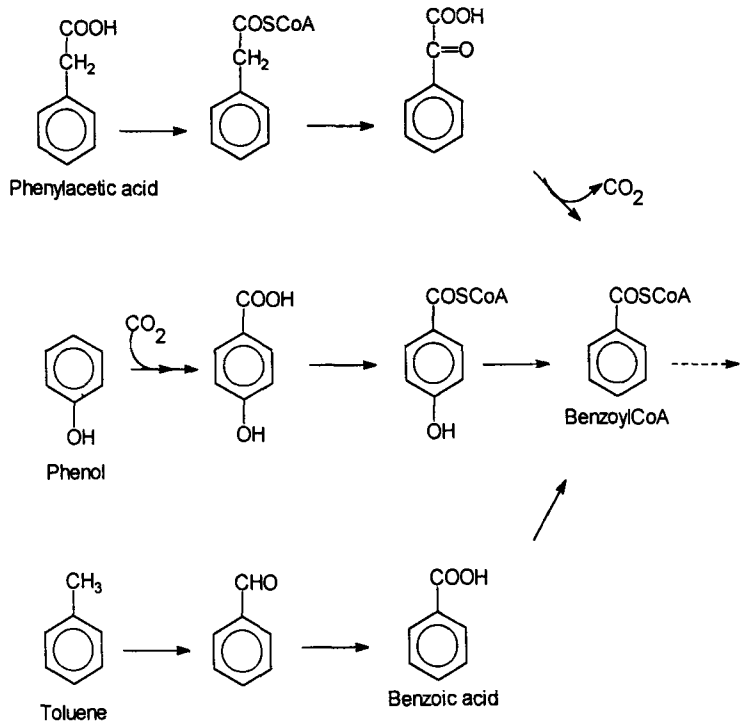

Fig. 1. Proposed central role for benzoyl-CoA in the anaerobic degradation of various aromatic organic compounds (*outline scheme only*).

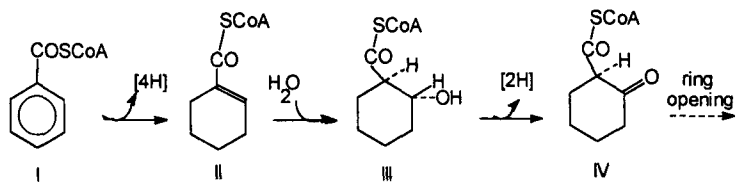

Fig. 2. Course of initial anaerobic degradation of benzoyl-CoA. (I) benzoyl-CoA, (II) cyclohex-1-enecarboxyl-CoA, (III) *trans*-2-hydroxycyclohexanecarboxyl-CoA, (IV) 2-oxocyclohexanecarboxyl-CoA.

reducing benzoylCoA to *trans*-2-hydroxycyclohexane carboxylCoA (*65*) (*see* Fig. 2). The facultative anaerobe *Alcaligenes xylosoxidans* subsp. *denitrificans*, which during anaerobic growth on nitrate can utilize benzoate, carries the genes specifying enzymes of benzoate catabolism on a plasmid. This bacterium can additionally completely mineralize several haloaromatic and methoxyaromatic compounds, including vanillate (*66*).

Phenols and hydroxyphenols follow differing routes of anaerobic degradation depending on the nature and distribution on the aromatic ring of other substituent groups. The anaerobic degradation of catechol (by reductive dehydroxylation) is generally slower than that of resorcinol or phenol, which is the reverse of the usual aerobic trend (*67*). Under

anaerobic conditions *para*- and *meta*-cresols seem to be more easily degraded than *ortho*-cresol. At one time it was thought that this might be because *ortho*-cresol might not induce synthesis of the enzymes necessary for its degradation since in several consortia it could be cometabolized with toluene, which served as the required inducer. However, a denitrifying bacterium has been isolated that can grow anaerobically on *ortho*-cresol as sole source of carbon and energy (*68*). Compounds in which the aromatic ring is activated by two or three *meta* hydroxyl groups are particularly readily metabolized under anaerobic conditions, and it was at first generally believed that to be vulnerable to anoxic degradation the benzene ring had to carry oxygen-containing substituent group(s). Yet it soon became obvious that benzene itself, as well as toluene and xylenes, could be utilized under denitrifying (*69,70*) or methanogenic (*71*) conditions. Hydroxylated intermediates were detected in the methanogenic consortia, suggesting that phenol might be formed from benzene, and *para*-cresol from toluene, though little, as yet, is known of the mechanism of hydroxylation of the benzene ring. Two other routes for anoxic breakdown of toluene were proposed as a consequence of findings made with denitrifying organisms, namely that toluene could be oxidatively condensed with acetylCoA to form phenylpropionylCoA and thence benzoylCoA (*72*), or that toluene is directly oxidized to benzyl alcohol and then to benzoyl CoA (*73–75*). The suggestion that the methyl group of toluene could be directly oxidized acquired legitimacy from the isolation and characterization of a flavoprotein *para*-cresol methyl hydroxylase from an *Achromobacter* (*76*). With toluene as substrate it has now been demonstrated that methanogenic consortia can simultaneously carry out ring oxidation (to *para*-cresol), methyl oxidation (to benzyl alcohol) and, to lesser extents, ring oxidation to *ortho*-cresol, ring reduction to methylcyclohexane and demethylation to benzene (*43*) (*see* Fig. 3). The inherent syntrophism of the methanogenic consortium is well illustrated by the manner in which it anaerobically degrades ferulate, i.e., 4-hydroxy-3-methoxycinnamate (*77*). Normally, this leads to the formation of such intermediates as cinnamate, phenylpropionate, phenylacetate, benzoate, pimelate, and adipate, but when the activity of the H_2-utilizing methanogens is suppressed (by added bromoethane sulfonate) then more reduced products such as toluene, benzene, ethylbenzene, and phenol put in an appearance (*71*).

Nor must the degradative abilities of anaerobic sulfate-reducing bacteria be underestimated. It has been calculated that these bacteria are able to metabolize over 50% of the organic detrital input to coastal marine sediments (*78*). Various of the more recently isolated species can completely mineralize many types of organic reductant and not, as was the case with the early isolates of *Desulfovibrio* species, merely effect their partial oxidation. Among such newly described organisms are species of *Desulfococcus* that, like *Desulfotomaculum sapomandens*, can oxidize benzoate as well as fatty acids (*79*). Various *Desulfobacterium* species metabolize

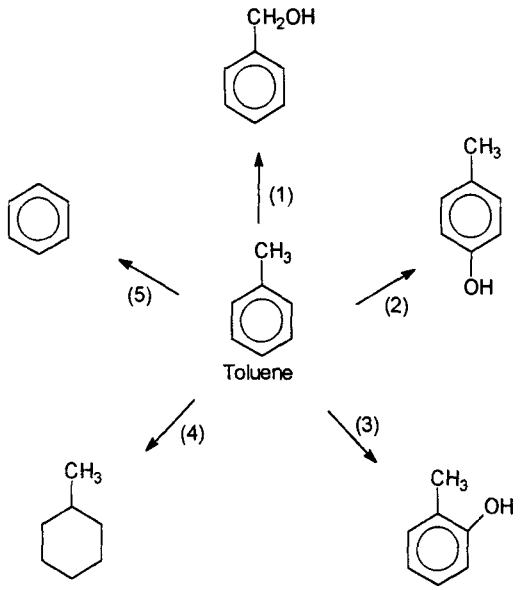

Fig. 3. Possible routes of anaerobic transformation of toluene in mixed methanogenic cultures: (1) methyl oxidation, (2) ring oxidation, (3) ring oxidation, (4) ring reduction, (5) demethylation. Routes (1) and (2) are probably the most important (*43*).

phenolic and indolic compounds and aniline (*80–83*), whereas *Desulfobacula toluolica* completely degrades toluene (*84*). Some of the sulfate reducers are particularly versatile in the range of energy conserving processes that they can exploit. A number are fermentative when sulfate is not available, and species of *Desulfovibrio* and *Desulfobulbus* have been reported to reduce nitrate with specific yields of ATP greater than they could achieve using sulfate as terminal electron acceptor. Some species, including *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris*, can directly reduce Fe(III) to produce siderite, i.e., $FeCO_3$ (*85*). Indeed, with sulfate as an electron acceptor *D. desulfuricans* could not metabolize gaseous H_2 below a partial pressure of 10^{-5} atm., whereas with Fe(III) as terminal respiratory oxidant H_2 was utilized to levels below 10^{-6} atm, leading to suggestions that in some aquatic sediments Fe(III) might be the preferred anaerobic electron acceptor.

Desulfuromonas acetoxidans, which can utilize acetate or alcohols or pyruvate at the expense of Fe(III) or Mn(IV) reduction (*86*) is, on the basis of 16S rRNA sequence homology, closely related to *Geobacter metallireducens*, which was the first obligately anaerobic bacterium enriched for its ability to couple the oxidation of acidic products of other fermentative bacteria, or H_2 gas, to the reduction of Fe(III) or Mn(IV) (*5*). It is therefore interesting to note that *G. metallireducens* in pure culture is capable of oxidizing a variety of aromatic compounds, including toluene, phenol, and *para*-cresol as well as benzoate (*87*). Mixed populations or pure cultures of sulfate-reducers have been found to mineralize 3- and 4-picoline as well

as benzoate (*88*), 3-amino-benzoate (*89*), methoxybenzoate (*90*), catechol (*81,91*), toluene and xylenes (*92*), and furfural (*93*).

Like *Rhodopseudomonas palustris, Rhodocyclus gelatinosus* is able to grow anaerobically (phototrophically) or aerobically (but via a different route) on a wide range of aromatic substrates (*40*). Relatively little attention has been paid to the degradative capacities of other phototrophic anaerobes but doubtless they will prove to possess diverse abilities in this regard. Certainly the growth of purple photosynthetic bacteria contributes to the purification of heavily organically polluted water in sewage lagoons, and purification plants have been constructed to maximize the degradative role of these bacteria (*94*).

Fermentative anaerobes can contribute importantly to the degradation of a wide range of substituted aromatics, though sometimes only after a period of acclimation. For example, the majority of homoacetogenic bacteria can grow at the expense of methoxy-substituted aromatics. Thus most species of *Acetobacterium*, the acetogenic clostridia including *Clostridium thermoaceticum*, and organisms such as *Sporomusa malonica* and *S. termitida* all undertake *O*-demethylation, with the methyl group generally being oxidized to CO_2 by a reversal of the acetylCoA pathway that these bacteria employ for CO_2 reduction. Though some species require to be supplied with additional CO_2 to serve as an ancillary electron acceptor, other species can use the aromatic substrate itself for this purpose. *Clostridium thermoaceticum* is particularly versatile in that it can reduce carboxylate groups, oxidize aldehyde groups, decarboxylate aromatic acids, and hydroxylate the benzene ring (*95*). *Acetobacterium woodii* can be selectively enriched by its ability to grow on methoxylated aromatic acids, including 3,4,5-trimethoxybenzoate (*96*). *Syntrophococcus sucromutans* is unusual in using sugars as electron donors with a variety of electron sinks including methoxyaromatics (*97*). *Eubacterium limosum* is another well-known fermentative anaerobe that will grow on a range of methoxy-substituted aromatics, including 3-methoxysalicylate, which *Acb. woodii* does not utilize (*98*). *Eubacterium oxidoreducens* ferments pyrogallol, gallate, phloroglucinol, and quercetin when H_2 or formate are available as electron donors (*99*), its fermentation of gallate involving both a novel decarboxylase and a transhydroxylase (*100*). Unlike *Eubacterium oxidoreducens, Pelobacter acidigallici* can ferment various trihydroxybenzene and trihydroxybenzoate derivatives in the absence of any externally supplied electron donor, its growth on gallate being particularly rapid (*3*).

There are many instances of H_2 syntrophic cocultures being elected by virtue of the utilization of an aromatic substrate by the hydrogenogen. A particularly interesting example was that of a benzoate fermenting coculture in which either methanogenesis or the reduction of tetrachloroethene to 1,2-*cis*-dichloroethene could serve as the electron sink (*101*).

This is by no means an exhaustive list of even presently identified anaerobic aromatic degraders and new species are being discovered at what seems to be an accelerating pace. The rates of degradation and the

ultimate fates of various aromatic substrates will depend on several factors, including:

1. The composition of the local anaerobic microbial community and the potential for its acclimation;
2. The availability of suitable electron donors, cometabolites, and inducers of key enzymic components; and
3. The nature and relative concentrations of oxidants that can be used as respiratory electron sinks.

Yet despite the surprising number of obligate anaerobes that are able, either partially or completely, to degrade simple aromatic ring compounds, it remains the case that availability of oxygen is an important factor in determining rates of hydrocarbon biodegradation in soils and natural waters (*102*). Under anoxic conditions, benzene derivatives bearing oxygen-containing substituent groups are more vulnerable to biodegradation than are substrates whose ring structures carry no such substituents. Furthermore, the larger (more complex) is the substrate molecule, the more resistant it generally is to anaerobic breakdown. This is particularly true of the complex polymeric lignins that are evidently not very susceptible to anaerobic degradation; in anaerobic environments cellulose utilization (fermentation) takes precedence over the degradation of aromatics (*61*).

Dehalogenation

Chlorinated aliphatic hydrocarbons and products of their partial dehalogenation are among the most common and insidious pollutants of groundwaters. Whereas trichloethene (TCE), *cis*- or *trans*-1,2-dichloroethene (DCE), 1,1-dichloroethene (1,1-DCE), vinyl chloride and trichloromethane (i.e., chloroform, CF) can be transformed aerobically (e.g., *103–106*), there is currently no evidence for wholly aerobic transformations of hexachloroethane (HCA), tetrachloroethene (PCE), or tetrachloromethane (CTC) (*105*), while 1,1,1-trichloroethane is transformed only slowly by aerobic processes (*106*). Reductive dehalogenation, which mainly, but not exclusively, occurs under anaerobic conditions, is presently the only known means of initial attack on these compounds (*45,106*), as is also the case for some highly chlorinated pesticides (*45,107*). Reductive dehalogenation is also an initial step in the degradation of most aryl halides (*108,109*).

One of two processes is involved (*45*), either hydrogenolysis in which a halogen atom is replaced by a hydrogen atom, or vicinal reduction (also known as dihaloelimination) whereby two halogen substituents are removed from adjacent carbon atoms. Both processes are dependent on the provision of a suitable electron donor (reductant), but aryl halides are susceptible only to the hydrogenolytic mechanism of anaerobic dehalogenation (*45*). Compounds that are only sparsely halogenated (and especially

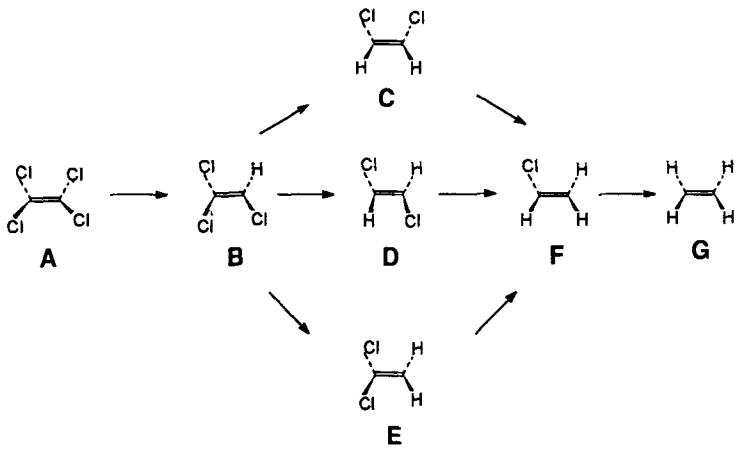

Fig. 4. Stepwise anaerobic reductive dehalogenation of tetrachloroethene with consequent possible accumulation of partially dehalogenated intermediates (especially *cis*-dichloroethene): (**A**) tetrachloroethene, (**B**) trichloroethene, (**C**) *cis*-dichloroethene, (**D**) *trans*-dichloroethene, (**E**) 1,1-dichloroethene, (**F**) monochloroethene, (**G**) ethene.

monochlorinated compounds) are generally less easily dehalogenated reductively than oxygenatively. Thus the less chlorinated *ortho*-substituted biphenyls that result from stepwise reductive dechlorination of PCBs in anaerobic environments can thereafter be mineralized via aerobic bacterial activities (*107*). Accordingly, partial dehalogenation products of chlorinated alkyl solvents may accumulate in anoxic polluted environments. Thus *cis*-dichloroethene is frequently the major contaminant when an anaerobic aquifer has been polluted by tetra- or tri-chloroethene (Fig. 4). That this need not necessarily occur has been demonstrated by the finding that tetrachloroethene was completely transformed to ethane in an anaerobic column of Rhine river sediment supplemented with a granular sludge (*110*). This occurred when a great (150-fold) excess of reducing equivalents was supplied in the form of lactate, with the halogenated substrate being infiltrated at a relatively low concentration. The requirement for excess reductant was also illustrated by the successful employment of gaseous hydrogen as supplementary electron donor for the reductive conversion of tetrachloroethene to vinyl chloride and ethene (*111*).

Anaerobic digestor sludge supplied with acetate and nutrients, after a period of acclimation to pentachlorophenol (PCP) could progressively dehalogenate this compound (alongside its continuing methanogenesis) to yield dichlorophenols and 4-chlorophenol (*112*). More recently (*113*), it has been reported that microbial aggregates developed in a laboratory-scale anaerobic upflow sludge blanket reactor when supplied with acetate, propionate, butyrate, and methanol could completely remove PCP when this was supplied at 40–60 mg/L, the PCP being wholly converted to methane and carbon dioxide. When supplied together with a cosubstrate, PCP was degraded by acetogenic anaerobes such as *Acetobacterium woodii* and

Eubacterium limosum and by sulfate-reducing bacteria such as *Desulfovibrio vulgaris* (*114*).

For complete anaerobic degradation, the polyhalogenated substrate must evidently be supplied at concentrations that do not (by themselves or as degradative intermediates) pose toxicity problems for component members of the anaerobic bacterial consortium (*115*). Accumulation of partially dechlorinated intermediates could again be due to an insufficient rate of supply of "reducing power." Furthermore, consortia obtained from diverse locations can differ markedly in the relative rates at which they can degrade the different members of a complex mixture of halogenated organic compounds, these rates and specificities also being determined by what other electron acceptors are available to the consortium (*116*).

To ensure that less highly halogenated, but possibly more toxic, intermediates do not accumulate, it has often been recommended that alternating anaerobic and aerobic periods of bacterial treatment be employed to degrade polyhalogenated substrates. The imposition on a well mixed bacterial system of cycles of full aeration/deaeration could be problematic, since the viability of the obligately anaerobic constituents could thereby be imperiled. Thus, the more usual procedure is to expose the substrate first to an anaerobic consortium and then to an aerobic bacterial system (*105,117,118*). The combination of an anaerobic fluidized bed followed by an aerobic trickling filter or aerated lagoon has been employed, for example, to treat chlorophenolic waste from the paper pulp bleaching process. On a laboratory scale, however, attempts have been made to combine the two processes in a single system, for example, by coupling reductive and oxidative reactions during the degradation of DDT and 4-chloro-2-nitrophenol by a mixture of facultatively anaerobic and obligately aerobic bacteria entrapped in calcium alginate beads (*119*). Gerritse and Gottschal, however (*120*), made use of their knowledge of the behavior of anaerobes in natural environments when they attempted to effect the complete mineralization of the herbicide 2,3,6-trichlorobenzoic acid (236TBA) by a coculture of anaerobic and aerobic bacteria. From an anaerobic enrichment culture they had obtained a mixture of obligate anaerobes that removed chlorine from the *ortho* position of 236TBA to give 2,5-dichlorobenzoic acid that, in turn, could aerobically be utilized as a carbon and energy source by *Pseudomonas aeruginosa* JB2. Recognizing that in natural situations, e.g., soil crumbs, sediments, stratified water columns, it is possible to recognize oxic and anoxic zones (sometimes as microniches) that are bridged by gradients of decreasing oxygen tension, these investigators were also very aware that under the conditions of oxygen limitation that prevail at the interfaces of these zones, microbial activity is often particularly marked. In part this is the consequence of the reductants generated by the anaerobes (e.g., sulfide ions, reduced organic products of fermentation, hydrogen, methane) being highly suitable electron donors for a range of aerobic respirers. Armed with this knowledge they were able to devise conditions of low dissolved oxygen concentration that supported

growth of a stable, continuous flow coculture of their reductively dehalogenating anaerobes together with the aerobic *Ps. aeruginosa* JB2. This they achieved by restricting the rate of aeration of the culture so that the dissolved oxygen concentration was nowhere and never greater than 0.3–0.5 μM and by providing a nidus for the obligately anaerobic components in the form of vermiculite particles contained within a nylon net suspended in the culture. When this culture was not aerated the 236TBA was completely converted to 2,3-dichlorobenzoate, but when it was aerated in the properly restrained manner the 236TBA was completely mineralized.

Although there have been many reports of degradation by anaerobic methanogenic and other consortia of haloaromatics, including chlorinated benzenes and toluenes (*121*), only a few bacteria that are capable of reductive dehalogenation of aryl halides have so far been grown in monoculture. Certain of these are aerobes, e.g., species of *Flavobacterium* and *Rhodococcus*, and although there have been reports of anaerobic metabolism of 3-chlorobenzoate, 4-chlorobenzoate, and 2,4-dichlorobenzoate by dentrifying bacteria including *Alcaligenes denitrificans* (*122*), and of transformation of chlorinated phenol by a fermentative *Clostridium* (*123*), possibly the most interesting of all obligate anaerobes that to date have been reported to metabolize halogenated aromatic compounds is *Desulfomonile tiedjei* (*124*). This bacterium was first encountered as a necessary participant in an anaerobic methanogenic enrichment culture capable of mineralizing 3-chlorobenzoate. The essential component organisms when isolated from this enrichment culture consisted of:

1. *D. tiedjei*, which initiated attack on the substrate by effecting its reductive dehalogenation;
2. An anaerobic, rod-shaped bacterium BZ-2 that fermented the ensuing benzoate; and;
3. A *Methanospirillum* species that scavenged any hydrogen liberated from the benzoate fermentation in excess of that consumed by the *D. tiedjei* as the electron donor for dehalogenation (*125*).

These three organisms formed a 3-chlorobenzoate-consuming anaerobic consortium stabilized by their mutual syntrophism, with the two hydrogenotrophs being cooperative rather than competitive in their usage of the hydrogen produced by the hydrogenogen (BZ-2). In pure culture *D. tiedjei* could employ 3-chlorobenzoate as terminal electron acceptor in the ATP-yielding respiratory utilization of hydrogen (*126*) or formate (*127*). Its dehalogenating activity was inducible and required the provision of 1,4-naphthoquinone or menadione in the growth medium. The activity was also preferentially, though not exclusively, directed to the *meta* position of aromatic substrates. Cells induced by exposure to chlorobenzoates during growth on pyruvate, or formate plus acetate or CO_2, could reduce chlorophenols, though the latter would not induce the dehalogenating activity. Furthermore, whereas tetrachloroethene would not induce *D.*

tiedjei to undertake its dehalogenation, this could be accomplished by cells that had been preinduced by exposure to chlorobenzoate. The organism also illustrates the common finding that the range of halogenated substrates that are capable of transformation by a single species is largely unpredictable. Thus *D. tiedjei* can attack monohalogenated benzoates and benzamides but not monochlorinated phenols; yet more highly chlorinated phenols are liable to be reductively transformed.

Desulfomonile tiedjei is additionally capable of channeling hydrogen consumption to the reduction of sulfate, sulfite, and thiosulfate. The latter two sulfoxy anions inhibit the reductive dehalogenation of 3-chlorobenzoate by the organism though, in certain media, this can occur concurrently with sulfate reduction (*128*). *D. tiedjei* is therefore an anaerobically respiring anaerobe that exploits a wide range of electron acceptors but a narrower range of electron donors. It is particularly intriguing that its reductive dehalogenation is free-energy conserving via the usual respiratory mechanism of transmembrane proton pumping, for this suggests that the enzyme(s) for dehalogenation are membrane-associated. Furthermore, *D. tiedjei* can grow fermentatively on pyruvate plus CO_2, with the oxidation of pyruvate to acetate plus CO_2 being made possible by the concurrent reduction of CO_2 to acetate via the acetylCoA pathway. It is therefore not so surprising to find that *D. tiedjei* resembles the homoacetogenic anaerobes (that also operate the reductive acetylCoA pathway) in being able anaerobically to *O*-demethylate methoxybenzoate to form hydroxybenzoates. Thus it both dehalogenates and *O*-demethylates 3-chloro-4-methoxybenzoate. Its versatility makes it well suited to life in a complex anaerobic microbial community with companion organisms that can supply a variety of reductants and oxidants and that can efficiently remove end products of its metabolism (e.g., sulfide ions, benzoate, acetate). A Gram-negative anaerobic bacterium (strain PER-K23) has recently been isolated that also conserves free energy from the anaerobic respiration of hydrogen or formate with a chlorinated organic compound serving as the terminal electron acceptor, though in this instance the electron sink is tetrachloroethene, which is reduced via trichloroethene to *cis*-1,2-dichloroethene (*129*).

In some instances anaerobic dehalogenations occurring in locations heavily populated by bacteria might actually be accomplished by "nonbiological" means. Transition metal complexes, associated with enzymes that are particularly prevalent in some obligate anaerobes, have been found to catalyze both alkyl and aryl dehalogenations when suitable reductants are available. Cobalt-containing corrinoids and nickel-containing factor F_{430} have been shown to catalyze reductive dehalogenations, and there have therefore been questions raised as to what fraction of the dehalogenation activity of methanogenic consortia might be attributable to such mechanisms (*130*). Similar questions have been asked in respect of the transformations of tetra- and tri-chloromethane to CO_2 by *Acetobacterium woodii, Clostridium thermoaceticum,* and *Desulfobacterium autotrophicum* (*131*). Whatever the answers, a thriving anaerobic bacterial consortium will sup-

ply the necessary components (reductants and catalysts) for all processes, whether enzyme-based or not. Indeed, removal of halogenated aromatic compounds from industrial effluents may better be done anaerobically than aerobically (*1*).

Both anaerobic dehalogenation and the anoxic degradation of aromatic compounds have been surveyed in some detail because both processes demonstrate many of the basic features of anaerobic bacterial transformations. First, though at one time it appeared inherently unlikely that either process could occur in nature to any extent, we now know that they are widespread and can be undertaken by a great variety of both obligate and facultative anaerobes. Second, the ability of an anaerobic bacterium to degrade a given substrate easily may be overlooked if that substrate is not itself an inducer of the requisite enzyme activities, e.g., cometabolism of either *ortho*-cresol or *ortho*-xylene with toluene. Third, anaerobic microbial consortia may more readily undertake the required degradation than any pure culture, even though a substantial period of acclimation may still be required.

These features are also discernible in other types of anaerobic degradation processes, of which several have now been described that were formerly deemed unlikely. For example, ether linkages can be broken under anoxic conditions, and not only when a phenol is one of the bonded partners. Thus anaerobic enrichment cultures were obtained that were capable of degrading polyethylene glycol, and from these were isolated obligately anaerobic bacteria that could attack this substrate by shifting the ultimate hydroxyl group to the penultimate carbon atom, thus transforming the ether linkage into a half-acetal bond (*132*). Another reaction that can be achieved anaerobically is the demethylation that is accomplished when trimethylamine is consumed by methanogens capable of anaerobic growth on methanol (*133*), or when betaine is utilized by *Acetobacterium carbinolicum* (*134*).

Given our dependence on subterranean (or submarine) stocks of fossil fuels, it is fortunate that anaerobic degradation of fully saturated aliphatic compounds, as well as some of the aromatic components of petroleum, is evidently very difficult and hence slight under natural conditions. It is therefore particularly interesting that a sulfate-reducing anaerobe has been isolated that can grow on C12–C20 alkanes, completely oxidizing them to CO_2 via the carbon monoxide dehydrogenase pathway (*135*). Also intriguing is the recent report (*14*) that among the hyperthermophilic bacteria present in deep oil reservoirs there were organisms that grew anaerobically at 85°C as "disk-shaped cells" in enrichment cultures in which sterilized artificial sea water was supplemented with the hydrophobic fraction of crude oil as the sole source of carbon and energy. The presence of side chains or of cyclopropane components can make saturated substrates even more refractory to anaerobic breakdown. On the other hand, the presence of one or more double bonds in the hydrocarbon evidently renders it more vulnerable to anaerobic attack. This is particularly

so when the double bond is in a subterminal position; thus 1-hexadecene was readily converted by a methanogenic enrichment culture to 1-hexadecanol, which was in turn degraded to acetate, and eventually to methane and carbon dioxide (*136*). Even so, many unsaturated compounds, including some carotenoids, sterols, and hopanoids are not easily degraded under anoxic conditions.

Pollutants are not invariably organic in nature and obligate anaerobes can undoubtedly also play a useful role in purifying effluents contaminated by inorganic materials. It has, for example, been suggested that uranium dissolved in effluents as U(VI) might be subject to anaerobic reduction to U(IV), and hence to precipitation, by bacteria such as *Geobacter metallireducens, Shewanella putrefaciens*, or, in marine waters, some sulfate-reducers (*137*). It has even been proposed that other radioactive metals such as plutonium and technetium, which exist in multiple redox states and which are insoluble in their reduced forms, could also be anaerobically removed in a like manner.

BIOSYNTHESIS/BIOPRODUCTION

Anaerobic fermentations are not well suited to the production of single cell protein since the efficiency of conversion of substrate into biomass is considerably less than that achievable by aerobically respiring microbes. The valued products of such fermentations are generally therefore the excreted end products of reduction of the terminal organic electron acceptors employed in the fermentation. These substances, which include short-chain fatty acids and alcohols, are most often low unit value, high volume commodities, and to be commercially attractive it would be necessary to produce them using very large scale, high productivity processes based on an abundant and cheap feedstock. Furthermore, bacterial fermentations accumulate their end products in relatively low concentrations; indeed, they are usually somewhat toxic to the producer organism. Thus attempts to enhance the resistance of producer strains to elevated concentrations of the fermentation product run parallel with attempts to improve the efficiency and economics of its downstream processing. Both of these approaches have been taken with the most famous of all large-scale anaerobic bacterial fermentations, namely the solventogenic fermentation of starchy grains or molasses by *Clostridium acetobutylicum* (*36,37*). This batch process was employed on a large scale to produce acetone and butanol in both World Wars, but in the UK and US it was not able thereafter to compete with chemical synthesis of these solvents from petroleum. It is therefore somewhat poignant to find that C. *acetobutylicum* has also been suggested for possible *in situ* use in microbial-enhanced oil recovery (*138*) since in a water-flooded oil reservoir the organism could liberate both gas and oil-mobility-enhancing metabolites.

Another clostridial fermentation that holds out promise of commercial scale exploitation is the synthesis of acetic acid by the thermophilic homoacetogen *C. thermoaceticum*. Even if conventional strains of this organism were to be used, it was calculated some years ago (*139*) that calcium-magnesium acetate (a highway deicer) could be produced by fermentation of hydrolyzed cornstarch at a price close to that currently being charged for the chemically synthesized compound. Were strains of the organism to be obtained that were tolerant of higher concentrations of acetic acid at a lower culture pH (*140*), then the equation might work out even more in favor of the anaerobic bioproduction of this compound. There may also be the prospect of some premium price being charged for organic acids of natural biological, as opposed to synthetic chemical, origin. In such circumstances an organism such as *Clostridium thermobutyricum* that undertakes an almost homobutyric fermentation could prove valuable (*141*). More widespread interest has, however, been expressed in the possibility of utilizing cellulolytic, thermophilic anaerobes for the production of fuel alcohol(s) or fuel solvent additives (*1,142*). One of the anticipated advantages of employing thermophiles is the supposedly diminished risk of process contamination and the lesser costs of culture cooling coupled with the prospect of on-line (possibly vacuum) stripping of the product(s) from the growing culture. A mixed culture might very well combine the best features of several of its component organisms. For example, a coculture of *Clostridium thermocellum* (with high cellulase activity) with *Thermoanaerobacter ethanolicus* (formerly *Clostridium thermohydrosulfuricum* 39E. of high ethanol productivity) could be grown at or around 60°C with a relatively cheap feedstock. Various species of *Clostridium* (including *C. butyricum* and *C. perfringens*) have additionally been appraised as potentially useful sources of H_2 gas (e.g., in biological fuel cells), but biogas production by large scale methanogenic digestion of liquid and semisolid wastes (including land-filled putrescible wastes) is by far the most important anaerobic bacterial process for fuel generation from renewable biomass.

Diversion of the normal electron flow in a fermentation (particularly when this follows a branched route) can sometimes be provoked by changes in culture conditions, including provision of alien electron acceptors or limitation of substrate or of some other nutrient. In turn, this can redirect the fermentative carbon flow, or even open up new outlets leading to the accumulation of unusual products. For example, a partially successful attempt to cause *Clostridium propionicum* to manufacture acrylate was based on the provision of an alternative electron sink to acrylylCoA (*143*), phosphate limitation promoted the formation of R(−)-1,2-propanediol by *Clostridium sphenoides* when this was fermenting glucose (*144*), and *Clostridium thermosaccharolyticum* could also, under certain growth conditions, form this same diol plus acetol as a consequence of the rerouting of intermediates of sugar fermentations via a methylglyoxal bypass (*145*). Should the economics of the various processes hereafter favor usage of biomass for

the production of synthetic chemicals rather than fuels, then some of the more unusual products of anaerobic fermentations (natural or distorted) could be most valued as chemical synthons.

BIOTRANSFORMATIONS

Many obligatory anaerobic bacteria are capable of degrading polymeric molecules, generally relying for this purpose on an array of secreted hydrolytic enzymes. In some instances, these may be employed by pathogenic organisms as invasive agents (toxins, virulence factors), as in the case of collagenases, hyaluronidases, lecithinases of various pathogenic clostridia (*146*). However, the degradative enzymes secreted by nonpathogens and especially by thermophilic species have attracted most attention. They include amylases, pullulanases, glucosidases, cellulases, pectinases, chitinases, proteases, and lipases that have been purified from cells and culture supernatants of a wide range of thermoanaerobes (*1,10,47–50,142*). Possibly more from an academic than a commercial standpoint, particular interest has been aroused by the multifunctional, multienzyme complex of *Clostridium thermocellum* (the cellulosome), which is located in protuberances on the cell surface and which, besides displaying both cellulase and endoglucanase activities, helps the bacterium to adhere to the crystalline cellulose substrate (*147,148*).

The very great diversity of free-energy conserving processes (especially of fermentations) that have been developed by obligate anaerobes is reflected in the great array of singular enzymes that they possess, many of which could well be candidates for exploitation as catalysts of reactions that would be difficult to achieve by chemical means. Furthermore, their enantiomeric specificities would be especially valued in the production of chiral synthons. For example, β-methylaspartate glutamate mutase is a B_{12}-dependent enzyme that catalyzes the unusual rearrangement whereby L-glutamate is converted into (2S, 3S)-3-methyl-L-aspartate as the initial step in the fermentation of glutamate that is carried out by *Clostridium tetanomorphum* (*149*). The enzyme has been purified and employed for the synthesis of ^{13}C-labeled β-methylaspartate to be used as a precursor in the synthesis of β-lactams. Among unusual dehydratases may be instanced that from *Clostridium sporogenes* that forms (E)-cinnamate from (2R)-phenyllactate (*150*) and the enzyme from *Clostridium microsporum* that reversibly converts (R)-2-hydroxyglutarate to (E)-glutaconate (*151*). The L-lysine decarboxylase of *Clostridium cadaveris* is employed in an enzyme electrode used to assay L-lysine (*152*) and creatinine iminohydrolase, which is obtainable from several anaerobic bacteria (*153*), can be used for the determination of serum and urinary creatinine. It has been suggested that the 4-hydroxylaminobenzoate-degrading enzyme from *Comamonas acidovorans*, because it can form oxygen-labile catechols under anoxic conditions,

could enable valuable catechols to be made not only from aromatic hydroxylamines but, in cooperation with an aromatic nitroreductase, from cheap nitroaromatic substrates (*154*).

These examples could be multiplied manyfold (*36,40*), yet, it is in the field of specific (often enantioselective) reductions that obligately anaerobic bacteria have hitherto chiefly been employed as agents of biotransformations. It is notable that many anaerobes achieve benefits from being able to dispose of excess reducing equivalents to oxidants in their environment. The reductases employed for this purpose are often of such broad substrate specificity that they can accept xenobiotic compounds as electron acceptors. Indeed, it has been suggested that this ability, namely of effecting the reductive cometabolism of alien oxidents, could have been of evolutionary advantage since the organisms would benefit by being enabled thereby to form more highly oxidized end products of fermentation (a circumstance usually associated with a greater than normal specific yield of ATP). This could be the explanation of the great variety of reductive elimination reactions (dehydroxylations, dehalogenations, deaminations) that are accomplished by fermentative anaerobes (*41*). Interestingly, by adopting the same general strategy it was possible in chemostat culture to select a mutant strain of *Clostridium tyrobutyricum* that was greatly enhanced in its ability to reduce ketones alien to its normal metabolism (*155*).

A large number of reductive enzymes has thus been isolated from anaerobes and several have been found to display features that could make them useful agents of specific biotransformations. The NADP-linked secondary alcohol-ketone oxidoreductase from *Thermoanaerobacter brockii* (*156,157*), the lactate dehydrogenase of *Thermotoga maritima* (*158*), or the low-potential, tungsten-iron-sulfur-containing aldehyde-ferredoxin oxidoreductase of *Pyrococcus furiosus* (*159*) are just examples of the many enzymes that have been proposed to have properties that could make them commercially useful catalysts. However, although single-step bioreductions can be catalyzed in vitro with purified enzymes, it can prove expensive to provide stoichiometric quantities of the natural electron donor, and sometimes quite elaborate means have to be devised to recycle the reduced cofactor that serves as the immediate reductant (*160*). There are substantial benefits to be had therefore from carrying out the same biotransformations even more simply and economically by using whole organisms (semipermeabilized if necessary) in suspension or immobilized in, or on, some suitable support material. In some instances, the reducing equivalents are supplied by the cofermentation of a suitable substrate, but in others the possession by the organism of an uptake hydrogenase enables H_2 to be supplied as the primary reductant; with some bacteria formate is equally conveniently employed. Washed suspensions of whole cells have been employed, for example, to effect specific reductions of aldehydes and ketones (*161,162*), production of (R)-2-hydroxycarboxylic acids by reduction of 2-oxo acids (*162*), reductions of azo- and of nitro-compounds (*163–165*), reductive dehalogenations (*45*), reduction of *N*-allylhydroxylamines to *N*-

Where:

X = COOH, CHO

R = Me, Eth, halogen, OMe, SMe, NHCHO

R = almost no limitation

R = must not be too bulky

Fig. 5. Reactions catalyzed by the 2-enoate reductase of *Clostridium tyrobutyricum* (*see* ref. *176*).

allylamines (*166*), reductive splitting of the N-O bond of dihydrooxazines (*167*), hydrogenation of C=C double bonds (*162,168,169*), and various bioconversions of bile acids and steroids, including specific epimerization, dehydroxylation, nuclear dehydrogenation, and desmolytic reactions undertaken by species of anaerobic bacteria, e.g., *Clostridium* and *Eubacterium* from the human gut (*170–172*).

There are applications wherein to use whole organisms would be inappropriate but to employ expensively purified enzymes would be unnecessary. Crude cell extracts of anaerobes may be found to undertake the required bioconversion, and extracts of different organisms may even be mixed to produce the desired catalytic cocktail. Thus a cell extract of *Clostridium kluyveri* is particularly rich in hydrogenase activity and can be mixed with a cell extract of aerobically-grown *Enterobacter agglomerans* to catalyze the anaerobic hydrogenation of 3-hydroxy-3-methylbutan-2-one to yield (S)-2,3-dihydroxy-2-methylbutane (*162*). The extract of *C. kluyveri* of itself was incapable of undertaking this reduction, whereas the *E. agglomerans* extract was not able to utilize H_2 but could accomplish the desired reaction using the NADH which was continuously regenerated by the *C. kluyveri* extract plus H_2. Beside supplying hydrogenase, *C. kluyveri* is also the source of a commercially available NAD(P)H diaphorase, which is widely used in enzyme-linked assays of various analytes, formation of reduced pyridine nucleotide being coupled by the diaphorase to the reduction of a suitable dye to yield a colored product (*173*).

Simon and his colleagues (*162,174–176*) have particularly promoted interest in the use of anaerobic bacteria as agents of regio- and enantiospecific bioreductions, by their demonstrations that several of their reductases have particularly broad specificities and, furthermore, are able to accept artificial electron mediators in place of their natural cofactor(s). Thus the 2-enoate reductases of *C. kluyveri* and *C. tyrobutyricum* catalyzed the reduction of a surprisingly great variety of nonactivated 2-ene carboxylic acids and aldehydes (Fig. 5). The attack on C-3 was always *trans* to

that on C-2 so that different products are formed from E and Z isomers of a substrate. Furthermore, the specificity of the reduction was such that with an enoate that carried a nitrophenyl substitutent on its β-C the double bond was hydrogenated without concurrent reduction of the aryl nitro group. Though normally NADH-dependent the enzyme could employ reduced methylviologen (MV) in its place, thus opening up the possibility of effecting the desired reduction either by biohydrogenation (with H_2 as the electron donor to MV) or electromicrobially (with MV being reduced at the cathode of an electrochemical cell). Suspensions of whole cells of suitably grown *C. tyrobutyricum* could be employed in place of purified 2-enoate reductase and suspensions of *C. ghonii* or *C. sordellii* were used to prepare (4S)-[4-^{2}H]NADH by electromicrobial reduction of NAD in ^2H_2O. Suspensions of *C. kluyveri* were similarly used to prepare (4S)-[4-^{2}H]NADPH, whereas, again using MV as mediator, suspensions of permeabilized cells of *C. sporogenes* catalyzed the synthesis of several 2-oxo acids by bioelectrical reductive carboxylation (*177,178*). In the presence of viologens and H_2, anaerobic bacteria such as *Methanobacterium thermoautotrophicum* and *C. kluyveri* reductively split the N–O bond of dihydrooxazines in a chemoselective manner (*167*). A pyridine nucleotide-independent resorcinol reductase has also been described (*179*) that catalyzes the conversion of resorcinol to cyclohexane-1,3-dione using reduced viologens as artificial electron donors.

Simon and his colleagues (*175,180*) also reported that the acetogenic anaerobes *C. thermoaceticum* and *C. formicoaceticum* were able to reduce nonactivated carboxylic acids to their corresponding alcohols when carbon monoxide or formate were supplied as primary reductants. The aldehyde oxidoreductases implicated in this reaction operate at low redox potentials and are not pyridine nucleotide-dependent. Instead, they can evidently accept electrons from reduced viologen dyes. The enzymes from the two clostridia are very different, but both contain tungsten and a pterin with iron sulfur clusters (*181–183*). Remarkable though it is, the capability to reduce carboxylic acids in this manner is evidently more widespread among obligate anaerobes than was first thought. Thus the activity has been demonstrated in *C. aceticum* and *C. thermoautotrophicum*, whereas other acetogens like *Butyribacterium methylotrophicum* and *Eubacterium limosum* were also able to emply CO and formate to reduce propionate to propanol. The previously mentioned aldehyde oxidoreductase from the anaerobic archaebacterium *Pyrococcus furiosus* is another low potential, tungsten-containing enzyme (*159*). Yet *C. formicoaceticum* in addition to its tungsten-containing aldehyde oxidoreductase also possesses a less oxygen-sensitive molybdenum-containing enzyme that, with reduced tetramethyl-viologen ($E_o' = -550$ mV), can reduce various aliphatic and aromatic carboxylic acids to their aldehydes (*184*).

The carbon monoxide dehydrogenase of anaerobes such as the acetogenic clostridia is another enzyme whose activity is unusual and could be

exploited. For example, whole cells of *C. thermoaceticum*, or cell extracts of this organism, in the presence of viologens or copper sepulchrate catalyzed the reduction of CO to methanol (*185*). Indeed, carbon monoxide-utilizing bacteria such as *Butyribacterium methylotrophicum* (*186*), *Carboxydothermus hydrogenoformans*(*187*), and *Peptostreptococcus productus* (*188*) promise to be of considerable biotechnological interest (*189,190*).

Now that the molecular genetics of obligately anaerobic bacteria, including species of *Bacteroides* (*191*), *Clostridium* (37), and methanogens (*54*), is making rapid progress, there are many reports of genes specifying interesting enzymes being cloned in various hosts, including *E. coli*. For the reasons outlined above this may not prove necessary so long as adequate screening is performed to ensure that the most appropriate anaerobic bacterial species is chosen, and that the organism is grown in a suitable medium and manner, with the cells being harvested when the desired activity is at its peak. As an example, once it had been discovered that 2-enoate reductase was present in both *C. kluyveri* and *C. tyrobutyricum* the specific activities of the enzyme in both organisms were followed during growth in various media to determine the optimal conditions for production of the enzyme. Thus with *C. tyrobutyricum* the enoate reductase was best produced during its growth on crotonate, though on this substrate the hydrogenase activity of the cells was not high. On the other hand growth on glucose produced high hydrogenase levels (especially during the early exponential phase of batch culture growth) but very low levels of enoate reductase. In the event the compromise was chosen of growth in batch culture on crotonate with the cells being harvested while the hydrogenase was at its peak (*192*). We return therefore to the point that was made regarding the potential use of anaerobic bacteria for *in situ* bioremediation. One cannot be content with demonstrating that the desired activity is expressed by laboratory-grown cultures; it must be expressed by the organisms in the situation in which they find themselves. This is one of the reasons why there is currently a resurgence of interest in the properties and activities of very slow growing bacteria and of activities that continue to be displayed (or are singularly only displayed) by bacterial cultures that have attained the so-called stationary phase of batch growth.

As we become better acquainted with obligately anaerobic bacteria, and more informed regarding their normal habits and habitats, we will discover many more highly practical ways in which to exploit their industry and versatility.

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