Genetic Engineering of Bacteria from Managed and Natural Habitats

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The genetic modification of bacteria from natural and managed habitats will impact on the management of agricultural and environmental settings. Potential applications include crop production and protection, degradation or sequestration of environmental pollutants, extraction of metals from ores, industrial fermentations, and productions of enzymes, diagnostics, and chemicals. Applications of this technology will ultimately include the release of beneficial agents in the environment. If safely deployed, genetically modified bacteria should be able to provide significant benefits in the management of environmental systems and in the development of new environmental control processes.

MANY DISTINCT FORMS OF BACTERIA EXIST IN NATURE, each with potentially useful or detrimental attributes. Several strategies can be used to modify bacteria for useful purposes. In some instances, one or more genes for undesirable traits have been targeted for removal. There are also circumstances when the survival of useful microorganisms may be improved by single gene transfer or by genetic selection for tolerance to toxic substances. Sometimes a trait or process that is restricted to a given strain may prove useful in a habitat that is not readily exploited by that species. Although the adaptations that enable bacteria to colonize or survive in specific habitats are generally unknown, it is likely that many characteristics collectively determine survival. Thus, it is presently difficult or impossible to transfer all the genetic determinants enabling a bacterium to survive in a habitat to which it is not already adapted. However, certain traits that may be desirable to have expressed in a given environment are conferred by single genes or gene clusters, which can be transferred to and expressed in a bacterial strain indigenous to that environment. Most studies have emphasized the introduction of genes for novel traits into bacteria indigenous to the habitat to be exploited. Because of the wide scope of genetic engineering targets, this review will focus on the modification of some bacteria that affect important natural and industrial processes.

Plant-Microbe-Pest Interactions

Most bacterial species that reside on plant surfaces are not harmful to the plant and may even protect it from pathogens, other deleterious microorganisms, and insects. Many such species, particularly strains of *Pseudomonas*, are well adapted for growth and survival on leaves or roots of plants, with population sizes of 10^5 to 10^7 cells per square centimeter of plant surface being common. Attempts have been made to modify these bacteria by the addition of single genes so that they might protect crops against insect pests. For example, many lepidopteran insects are susceptible to the delta endotoxin produced by various strains of *Bacillus thuringiensis* (*1*). This bacterial species is found in diseased insects or in soil and plant debris and can cause low levels of mortality in susceptible insects in natural settings (*1*). The genes conferring production of several different *B. thuringiensis* delta endotoxins with different insect host specificities have been cloned and partially characterized (*2*), and effort has been directed toward determining the functional domains within the toxin so that hybrid toxins with altered host ranges or enhanced potency can be made (*2*). Commercially produced cells of *B. thuringiensis* are effective insecticides, which are used on several agricultural and forest plant species. However, effective insect control requires repeated applications of *B. thuringiensis* since this species does not multiply on plants. Attempts have been made to overcome the spatial and economic limitations of foliar applications of commercially produced *B. thuringiensis* cells for insect control. For example, the delta endotoxin gene was introduced into the chromosome of *Pseudomonas fluorescens* (an effective colonizer of corn roots) in order to ensure stability of the gene and to minimize the risk of its transfer to other bacteria indigenous to corn roots (*3*). This recombinant strain attained population sizes similar to those of the parental strain on corn roots and did not differ from the parental strain in survival and dispersal characteristics as measured in laboratory studies. The modified *P. fluorescens* strain showed some toxicity to root cutworm but not to corn rootworm, which is a more important pest. There has not been a field test of the efficacy of this bacterium because environmental issues raised during an Environmental Protection Agency (EPA) review have necessitated additional research by the Monsanto Chemical Company, the initiator of this project. Whereas transfer of the *B. thuringiensis* endotoxin gene to root-colonizing bacteria may be potentially useful for increasing the number of habitats to which the toxin might be applied, its incorporation into an internal colonist of plants also has promise. *Clavibacter xyli* subspecies *cynodontis* is generally found inside Bermuda grass plants but can reach population sizes of $>10^8$ cells per gram of stem tissue when inoculated into other plant species, including corn (*4*). Such a bacterium could be an efficient vector for the expression of cloned genes inside plants, and the *B. thuringiensis* delta endotoxin gene has been incorporated into this species (*5*). Field studies have been initiated with recombinant *C. xyli* strains for the control of leaf- or stem-feeding lepidopteran insects (*6*).

Chitin, a polymer of *N*-acetylglucosamine, is a structural compo-

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nent of many plant pests, including fungi and insects. Many bacteria, notably species within the genera *Serratia, Streptomyces*, and *Vibrio*, produce extracellular chitinases. Biological control of some soil-borne fungal diseases by soil-borne bacteria has been correlated with the production of chitinases (7). Inactivation of a chitinase gene in a soil bacterium reduced the ability of the bacterium to lower the incidence of fungal disease (*8*). Chitinases have been cloned from several strains of *Serratia marcescens* and from other bacteria (*8, 9*) and have been transferred into efficient plant colonizing bacteria such as *P. fluorescens*. However, the effectiveness of the recombinant strains in controlling fungal disease has not yet been reported.

Some bacteria possess traits that make them harmful to plants. When the genetic determinants for such traits are cloned, it may be possible to replace the native gene with a homologous gene that has been inactivated in vitro. This approach has been successfully applied to the control of frost injury to plants. Ice nucleation (Ice^+) strains of *Pseudomonas syringae* are common on the leaves of many plants that cannot tolerate ice formation and are therefore an important cause of frost damage to these plants (*10*). The gene conferring ice nucleation in *P. syringae* was cloned, and internal deletions within the structural gene were produced in vitro (*11*). Reciprocal exchange of the modified *ice* gene for the native chromosomal gene was accomplished by homologous recombination. The resultant Ice^- mutants of *P. syringae* showed no difference in colonizing ability or survival on plants, or in other habitats, relative to the parental Ice^+ strains (*12, 13*). In both laboratory and field studies, the population size reached by Ice^+ *P. syringae* strains on leaves that had been previously colonized by Ice^- mutant strains was much lower than that reached on leaves without such competitors (*13, 14*). Preliminary results indicate that significant control of plant disease by avirulent mutants of pathogens is also possible (*15*). Preemptive competitive exclusion of deleterious bacteria by bacteria of similar genotype (and thus similar habitat resource requirements) may be a useful general method for biological control.

Molecular genetic studies should enable researchers to analyze the relevance of antibiotic production by plant-associated bacteria in the biological control of deleterious bacteria and fungi. Inoculation of plant parts with certain bacterial strains can disrupt the plant-associated microbial communities and subsequently enhance plant growth or reduce the incidence or severity of plant diseases (*16*). Many bacteria used as inoculants produce antibiotic-like substances that are inhibitory to plant pathogens in vitro. Antibiotic-nonproducing mutants (generated by insertion of the transposon Tn*5* or by chemical mutagenesis) of several bacterial strains have a reduced ability to antagonize deleterious fungi or bacteria on plants (*17, 18*). Similar genetic evidence for the inhibition of deleterious microorganisms by the production of efficient iron-sequestering agents (siderophores) has been obtained (*19*). In several cases, physical "tagging" of antibiotic biosynthesis genes by insertion of Tn*5* has permitted their cloning (*17, 20*); in other cases, the genes have been identified in cosmid clones that complemented chemically induced mutants (*18*).

The regulation and the temporal and spatial patterns of antibiotic biosynthesis in natural environments, such as on leaves or roots, can be investigated by fusing antibiotic genes with "reporter genes" such as *lacZ*, *lux*, *cat*, and *gus*, whose products can be measured in vitro (*21*). A *lacZYA* reporter gene has been used to determine the transcriptional activity of an antifungal antibiotic operon of a *P. fluorescens* strain in response to the nutritional status in culture, and *lux* fusions with this operon have been used for the same purpose on seeds (*22*). A promoterless *ice* gene may prove to be a sensitive indicator of transcriptional activity of bacterial genes in complex natural environments such as plant tissue or soil (*23*). Reporter genes can also be used in cloning studies to identify those gene promoters of indigenous plant-associated bacteria that are induced by a particular set of environmental conditions, such as in response to root exudates that may precede fungal infection of roots.

Most bacteria are not pathogens of higher organisms. Plants possess defense mechanisms that are rapidly activated in response to attempted infection and only a few microbes have the complex sets of genes encoding attributes that enable them to (i) establish a successful parasitic existence with the host (basic compatibility), (ii) to produce pathogenicity and virulence factors, and (iii) to avoid, or overcome, defense responses of the host (*24, 25*). Among the factors required for bacterial pathogenesis on plants are (i) enzymes, such as pectate lyases, proteases, phospholipases, and glycosidases produced by soft rot *Erwinia* species, some *Xanthomonas* species, and a few other pathogens, (ii) toxins such as those produced by some pseudomonads, and (iii) plant growth hormones, such as indoleacetic acid and cytokinins, produced in large quantity by pathogens that cause plant hyperplasias (*24*). Genes for several of the above enzymes, for the phytohormones, and for several toxins have been cloned and their specific role in pathogenesis had been established (*24*). A number of "pathogenicity genes" exist whose functions are not yet known. In *Pseudomonas*, *Xanthomonas*, and *Erwinia amylovora*, large contiguous clusters of genes (*hrp*) and other unlinked loci are required for pathogenicity (*24, 25*). Many of these genes also are required in conjunction with avirulence genes (*avr*) for triggering the hypersensitive reaction, which is not a pathogenic response but is connected with the expression of resistance to heterologous pathogens or to avirulent (incompatible) pathogen races that normally cause disease on other cultivars of the same host species (*25*). Other genes are responsible for symptom production but not for the elicitation of the hypersensitive response (*24, 25*). Many of these genes seem to be conserved within taxa of phytopathogens (*24*).

Defensive reactions of plants include the production of the antimicrobial agents termed phytoalexins and the rapid necrosis of plant cells (the hypersensitive response), which is closely associated with the accumulation of phytoalexins (*24*). The nature of bacterial substances that can elicit defense-related processes is unknown. Such substances do not generally have the same biological specificity as their producers, some of which have a broad host range, although most are highly specialized, infecting only a limited number of host plants or only one or a few cultivars of a given species. This specificity is much better understood at the genetic level. Although both negative and positive factors in bacteria and plants may collectively define host range, *avr* genes appear to be the main host range determinants in various pathovars of *P. syringae* and *Xanthomonas campestris* (*25*). These genes are genetically dominant in merodiploids and act in conjunction with functionally corresponding plant resistance genes (*R*), which in most cases are also genetically dominant. Such *R-avr* gene pairs control the activation of host resistance. Several *avr* genes have been cloned, and four have been sequenced (*25*). An extension of this concept concerns the genetic basis of nonhost resistance to pathogens (*26*). Interspecies transfer of genomic libraries of bacterial pathogens has revealed cryptic *avr* genes that restrict bacterial pathogenesis on nonhost species. The presence of these genes could not have been inferred from classical genetic studies because different species of pathogens or plants cannot be easily crossed. These findings suggest a common basis for resistance in host cultivars and nonhost species.

Understanding the molecular basis of microbial pathogenesis, elucidation of resistance mechanisms, and cloning of native plant resistance genes may have an impact on crop protection strategies in the long term. However, some short-term applications have been considered: nonpathogenic mutants of *P. syringae* for frost control; similar mutants of *Pseudomonas solanacearum* that may also degrade fusaric acid, a putative toxin produced by vascular wilt Fusaria, as

Table 1. In vivo engineering of bacteria for the degradation of xenobiotic and toxic wastes.

Bacterium	Substrate
Pseudomonas cepacia	2,4,5-trichlorophenoxyacetic acid (*67*)
P. putida and *Pseudomonas* spp.	2,2-dichloropropionate (*68*)
P. putida and *Pseudomonas alcaligenes*	Chlorobenzenes (*69*)
Pseudomonas sp.	Chloroaniline, chlorosalicylate, chlorobenzoate, dichlorobenzene, amino-naphthalene sulfonates, hydroxy-naphthalene sulfonates, and other chlorophenols (*70*)
Alcaligenes sp.	Dichlorophenoxyacetic acid, mixed chlorophenols, 1,4-dichlorobenzene (*71*)
Acinetobacter sp.	4-chlorobenzoate (*41*)

biological control agents of vascular wilts; and the construction of transgenic plants that express phytotoxin immunity genes derived from toxin-producing pathogens (*15, 27*).

Leguminous crops form symbiotic associations with *Rhizobium, Bradyrhizobium*, and *Frankia* species that fix atmospheric nitrogen in a form that can be used by the plant. The genes from these bacteria on which attention has focused include (i) the *nif* genes, which encode nitrogenase components, (ii) genes designated by various acronyms (*nod, hsn, fix, syr*) that collectively determine *Rhizobium* host range and nodule development and function, (iii) the *dct* genes, which are responsible for the energy-yielding metabolism of dicarboxylic acids in the nodule, (iv) the *hup* genes, which mediate the capture of hydrogen released as a consequence of nitrogenase function, and (v) genes for the biosynthesis of the phytotoxin rhizobitoxin by *Rhizobium japonicum*.

In the free-living nitrogen-fixing bacterium *Klebsiella pneumoniae*, 17 *nif* genes are organized into eight transcriptional units and occur as a cluster in a 24-kb region of the chromosome (*28*). Transfer of this cluster to *Escherichia coli*, a close taxonomic relative, confers nitrogen-fixing ability (*28*). Similarly, transfer of either Sym plasmids, which carry *nod* and host-range genes as well as *nif*, or certain cloned *nod* or *hsn* genes between rhizobia can extend, restrict, or have no effect on host range (*29*). Such a transfer also enables *Agrobacterium* to initiate nodulation on nonlegumes (*29*), which suggests that host range may be extended to nonleguminous hosts.

Attempts have been made to improve legume yields by modifying the expression of two specific genes in N-fixing symbionts. Strains of *B. japonicum* and *R. meliloti* that expressed the dicarboxylate transporter protein (DctA) and the *nif* activator protein (NifA), respectively, under nitrogen-regulated promoter control gave greater than 10% increase in biomass production on their respective hosts under greenhouse conditions (*30*).

A subset of *nod* genes form the *nodABC* operon, common to all *Rhizobia*, which is positively regulated by the *nodD* gene in the presence of plant-derived phenolic substances (flavones or isoflavones (*31*). Some phenolic components in the root exudate antagonize *nodD*-mediated activation of *nodABC* and some rhizobia have several copies of *nodD* with apparently different specificities for phenolic inducers (*31*). These findings can be exploited in useful ways; for example, the nodulation of legume varieties, or progeny obtained in breeding programs, will be able to be more rapidly screened (*32*). Interstrain exchange of *nodD* alleles with different inducer or anti-inducer specificities, modification of other host specificity genes, and engineering of the *Nod* protein are also envisioned.

In other experiments, a plasmid carrying the *R. meliloti dct* genes enhanced dicarboxylate uptake and nitrogenase activity under microanaerobic conditions in vitro (*33*). About 25% of natural isolates of *R. japonicum* are Hup^+ and can grow autotrophically on H_2 and CO_2, whereas other strains are phenotypically Hup^-. When isogenic Hup^+ and Hup^- strains were allowed to nodulate soybean roots in a contained N-free system, both the amount of total fixed N_2 and plant biomass produced were 10% greater in the Hup^+ strain (*34*). Finally, inactivation of phytotoxin biosynthesis by site-directed mutagenesis significantly improved strain performance (*35*).

The difficulty of introducing rhizobial strains into environments in which resident rhizobia are already present is a problem. Newly introduced strains encounter strong competition from resident strains (*36*). To establish effective nodulation in the rhizosphere, the inoculant must outnumber the indigenous population by at least 1000-fold. When indigenous *Rhizobium* populations in soil are high, this requirement leads to high inoculant costs. Little is known of the biochemical determinants of *Rhizobium* competitiveness, although a toxin (trifoliin) produced by the *Rhizobium leguminosarum* pathovar *trifolii* seems to be required for this strain to compete efficiently (*37*). Ultimately it may be possible to manipulate both host and rhizobial genes to obtain maximum efficiency of nodule formation and function and to tailor strains for unusual soil environments (*36, 37*).

Biodegradation of Xenobiotics and Toxic Waste Transformations

Genetic tools can be used to develop specific catabolic pathways for the degradation of xenobiotics in bacteria that can function under a wide range of environmental conditions. For example, the ability to degrade toluene was transferred from a mesophilic bacterium into the psychrophilic *Pseudomonas putida*, which could degrade toluate at temperatures as low as 0°C (*38*). Currently, the biodegradation effectiveness of recombinant bacterial strains at sites contaminated with toxic and hazardous waste has not been demonstrated; however, both successful (*39*) and unsuccessful (*40*) experiments with nonrecombinant bacteria have been reported. Some toxic chemicals may have structures that are resistant to microbial attack or may be present in mixtures that are incompatible for effective degradation, or in too low or too high a concentration. Successful biological treatment in situ will depend on (i) the introduction and establishment of microbes in the environment, (ii) an improvement in the rate and extent of xenobiotic degradation, and (iii) the resolution of problems inherent in heterogenous spatial distributions of pollutants, nutrients (including oxygen), and microorganisms (*39, 41*). The need to develop environmental processes to treat hazardous wastes is underscored by the fact that there are more than 900 designated hazardous waste sites in the United States, although the actual number is estimated to be closer to 10,000 (*40*).

Many bacterial genes for xenobiotic degradation have originated from strains isolated from contaminated waste sites and are often found on plasmids (*41, 42*). For plasmids encoding incomplete catabolic pathways, the degradation of recalcitrant chemicals as sole carbon and energy sources may require complementation of plasmid genes by host chromosomal genes to link the plasmid pathway with energy-yielding metabolism. Gene clusters subject to operon control are characteristic of most catabolic plasmids, and two or more regulons have been identified in some cases. Almost all of the plasmids characterized to date that have genes for xenobiotic catabolism are from gram-negative bacteria, predominantly *Pseudomomas* species (*43*).

Natural selection processes can be extremely slow in yielding improved xenobiotic degraders, especially when the acquisition of multiple catalytic activities is necessary, such as for the metabolism

of compounds having structural elements or substituents rarely found in nature (*41*). Critical to the evolution of new metabolic activities is the relaxation of substrate specificity of enzymes and regulators without a concomitant loss in function (*41, 44*). Laboratory selection can speed up this process and provide control not available under natural conditions (*42*). Metabolic pathways can be engineered in the laboratory by (i) long-term batch incubations, (ii) soil perfusions, (iii) chemostat selection, (iv) in vivo genetic transfers, or (v) selection for the evolution of new catabolic or regulatory functions or the assembly of a new pathway in vitro (*41, 42, 44*). The first four techniques have been used to derive bacteria able to degrade a variety of xenobiotic and toxic wastes (Table 1). The first multiplasmid-containing strain constructed in the laboratory (*45*) was capable of oxidizing aliphatic, aromatic, terpenic, and lower molecular weight polynuclear aromatic hydrocarbons. This strain grew faster on crude oil than any of the parental isolates, and although it was patented in a landmark case, which recognized for the first time that man-made microbes were patentable (*45*), it has not been used commercially. The metabolic diversity of this strain was fairly limited compared to the actual number of compounds present in crude oil (>3000), and it did not degrade higher molecular weight, condensed, or substituted hydrocarbon compounds, which tend to persist in the environment.

In vitro strain constructions require detailed genetic and biochemical information on the degradative pathways, which is nonexistent for many xenobiotics (*42*). When sufficient biochemical and genetic information has been available, genetic engineering of metabolic pathways for recalcitrant compounds was successful (*42, 44*). For example, broad-specificity enzymes were recruited to extend the chlorocatechol pathway in several bacteria so that the degradation of chlorinated compounds through the tricarboxylic acid cycle was enhanced. *Pseudomonas* B13 has a pathway for the complete degradation of chlorocatechols, but the first enzyme of its chlorobenzoate pathway, which is encoded by chromosomal genes, has narrow substrate specificity (*44*). Recruitment of a broad-specificity dioxygenase from a toluene catabolic plasmid enabled this strain to degrade a wider range of chlorobenzoates and additional chloroaromatics after deleting an enzyme that misrouted intermediates to the meta-ring cleavage pathway rather than to the normal ortho-ring cleavage pathway (*44*).

Biodegradation processes at contaminated waste sites may be limited if complex mixtures of xenobiotics are present, if the inducer of the degradative pathway is not present, or if the pathway is blocked by inhibitors. Although individual chemicals can be completely degraded in soil and wastewater treatment plants, mixtures sometimes cannot (*41*). For example, dead-end metabolites result when chlorocatechols are cleaved by a meta-fission pathway and when methylcatechols are cleaved by an ortho-fission pathway. This nonproductive routing of degradation products during simultaneous degradation of chloro- and methyl-substituted aromatics can actually destroy the functioning of the biodegrading community (*41*). Enzyme recruitment overcame this problem by constructing catabolic routes with only one ring-fission mechanism (ortho-pathway) for chloro- and methylaromatics (*41*). The control of catabolic pathways can also be modified by placing key biodegradative enzymes that require inducers, some of which are pollutants themselves, under the control of new regulatory regions. For example, genetic engineering has been used to uncouple the *Pseudomonas mendocina* toluene monooxygenase genes from toluene induction, to derive *Pseudomonas* transconjugants that constitutively express the 2,4-dichlorophenoxyacetic acid degradation pathway, and to derive *E. coli* recombinant strains with enhanced polychlorobiphenyl degradative activity in the presence of exogenous catabolite repressor substances compared to the wild-type *Pseudomonas* donor strain (*38, 46*). The cloning of genes for modified enzymes that have useful catabolic properties (such as relaxed substrate-specificities or enhanced induction capabilities) provides an important repository of genetic diversity for future research (Table 2).

Production of Chemicals and Fuels

Recombinant DNA technology is having an impact on the microbial production of industrial chemicals and fuels (*47*). Plans have been announced for the production of amino acids on a commercial scale with recombinant bacteria including *Bacillus amyloliquefaciens* and *Lactobacillus casei* (*47*). Genetic engineering has been applied to fermentation processes to enable bacteria to use a wider variety of feedstocks, to biosynthesize new products, to accumulate intermediate metabolites via blocked pathways, or to increase product yields by enhanced synthesis of special enzymes.

In many commercial fermentation processes the cost of raw materials is the most expensive component (*47*). Industrially useful bacteria can be modified to use cheaper feedstocks such as D-xylose, lignocellulose, or cellulose. *Zymomonas mobilis* transconjugants were able to use lactose when they contained the *E. coli* lactose operon under the control of a *Z. mobilis* promoter and the genes for galactose utilization from *E. coli* (*48*). The activity of cellulase synthesized in *Z. mobilis* was increased sixfold by placing a cellulase gene from *Cellulomonas uda* under the control of a strong *Z. mobilis* chromosomal promoter (*48*). An alternate approach for improving the economics of ethanol production has been to transfer *Z. mobilis* genes encoding the appropriate enzymes into other organisms, such as *E. coli* or *Klebsiella* (*49*). The genes encoding essential enzymes of the fermentative pathway for ethanol production in *Z. mobilis* were expressed at high levels in *E. coli*. The recombinant strain metabolized glucose and xylose to give almost the maximum theoretical yield of ethanol with significant decreases in the yields of formate, acetate, lactate, and butanediol (*49*). Similar results in strains of *Klebsiella planticola* have been obtained (*49*).

There are many bioprocessing applications for thermotolerant microbes and enzymes (*47, 50*), but genetic approaches with thermophiles have only recently been initiated (Table 2). The isolation of a highly transformable thermophile, *Bacillus stearothermophilus*, may facilitate advances in cloning thermophilic traits (*50*). Thermostable enzymes may also be produced by cloning their genes from thermophiles, provided selection for enzyme function can be achieved (*50*).

Mineral Processing

Most major copper mining companies use microbial extraction technology to obtain 10 to 20% of the world copper supply; microbial leaching of uranium is also used in Canada (*51*). Leaching rates are generally slow and metal recovery from the leachate is expensive. Commercial bioleaching operations in the mining industry are typically conducted outdoors with ores in heaps or pits, so many of the problems regarding introduction and establishment of improved bacterial strains apply to mining applications of biotechnology. Microbes responsible for the solubilization of metals tend to be acidophilic (*51*). Many are chemolithotrophs and obtain energy from the oxidation of iron or sulfur found in ores such as pyrite, arsenopyrite, or chalcopyrite. *Thiobacillus ferrooxidans* is thought to play the main role in most metal-leaching operations (*51*). Genetic technology as applied to this group of microbes is not as advanced as for other species largely because of the unusual conditions required to grow them (*51*). Recently, plasmids have been constructed that

may enhance the recovery of gold from arsenopyritic-pyritic ores by *T. ferrooxidans* and increase the resistance of *T. ferrooxidans* and *B. subtilis* to arsenite and arsenate (*52*). A *recA*-like gene from *T. ferrooxidans* has been cloned, and selectable shuttle cloning vectors have also been constructed for this bacterium (*51*). At least two companies plan to test genetically engineered organisms with enhanced bioleaching capabilities (*52*).

Wastewater Treatment Applications

Few aspects of municipal and industrial wastewater treatment processes are understood at the genetic and biochemical level. Some bacterial functions, such as the biodegradation of particular chemicals, may reside in one gene or gene cluster and be relatively easy to identify. Although it would be desirable to obtain improvements in other bacterial functions, such as better flocculation of heterotrophic bacteria in activated sludge, stronger attachment of bacteria to surfaces, increased growth rates of nitrifying bacteria, and decreased sensitivity to inhibitors, these are often complex and require multiple genes (*53*).

Many wastewater treatment processes require removal of biomass for disposal or recycling purposes. The most common example is the activated sludge process, which depends on the formation of aggregates of microbes for effective treatment (*53*). Although several microbes produce exopolysaccharides, which contribute to flocculation in aerobic wastewater treatment facilities, *Zoogloea* species have been implicated in particular (*53*). Recombinant DNA technology has been used to control biopolymer synthesis in *Zoogloea ramigera*. Several genes that are responsible for the production of different exopolysaccharides required for flocculation have been cloned from *Z. ramigera* strains (*54*). The ability to link the genes for biopolymer and surfactant production together with catabolic genes may improve wastewater treatment processes (*40*, *43*). This coupling would also provide a way of keeping plasmid-containing cells within the bioreactor during continuous-flow operations, as the nonflocculated, nonplasmid bearing cells would leave the system in the effluent through the overflow (*54*).

The cloning of the *Vitreoscilla* structural gene for the oxygen-carrying compound hemoglobin and its expression in *E. coli* may further improve bioprocessing and biomining operations that are oxygen-limited (*55*). Evidence indicates that floc-forming bacteria have a lower affinity for oxygen than do certain filamentous bacteria (*53*). The linking of genes for biopolymer synthesis in floc-forming bacteria with an enhanced ability to scavenge oxygen from the environment could result in significant improvement in wastewater treatment.

Genetic technology could also improve the sequestration of metals in bacteria by enhancing the absorption of metals to the microbial cell surface or by increasing intracellular uptake. This would be useful for the removal of metals from aqueous solutions for both pollution control and the recovery of precious metals (*51*). Metallothioneins are low molecular weight peptides that are induced in response to increased metal concentrations (*56*). Human metallothionein has been cloned into *E. coli* and it has been proposed that these engineered bacteria be used as an immobilized cell system for removing metals from wastewaters (*56*). When the metallothionein fusion protein is induced in the presence of $Cd^{2}+$ or $Cu^{2}+$, a direct correlation is found between the expression of the fusion protein and the bioaccumulation of $Cd^{2}+$ and $Cu^{2}+$. The use of immobilized cells with high metallothionein responsiveness could provide a waste treatment system naturally responsive to variations in heavy metal concentrations.

Bacterial metal-binding proteins can be used either to remove phosphorus in wastewater treatment systems, so as to prevent further eutrophication of waters that receive industrial and municipal wastewater discharges, or as regenerable adsorbants or biosensors (*51, 53, 57*). For example, the phosphate-binding protein of *E. coli* has been cloned and expressed in strains that secrete proteins directly into the medium, so as to minimize product recovery costs (*57*). The recovered phosphate-binding protein was immobilized on a support matrix as a thermally regenerable adsorbant and onto a

Table 2. Examples of genetically engineered bacteria.

Host bacteria	Altered trait	Genes transferred (source organism)
	Extension of substrate range	
Pseudomonas aeruginosa and *X. campestris*	Growth on whey for biopolymer production	Lactose metabolizing enzymes (*E. coli*) (*72*)
E. coli	Cellulose used as new feedstock	Various cellulose degrading enzymes (*73*)
Bacillus subtilis	Starch used as new feedstock	α-amylase (*B. subtilis*) (*74*)
	Thermophilic enzymes added	
E. coli	Cellulose degradation	Various cellulose degrading enzymes (*75*)
E. coli	Starch degradation	α-amylase (*Dictyglomus thermophilum* and *Bacillus licheniformis*) (*50*)
E. coli	Raffinose removal	α-galactosidase (*B. stearothermophilus*) (*76*)
	Biodegradation and waste treatment applications	
Pseudomonas	Altered pathway regulation	TOL plasmid catabolic enzymes (*44*)
E. coli	Enhanced degradation pathway for mono- and disubstituted chloroaromatics	Various genes (*67*) *Pseudomonas* spp. *Alcaligenes eutrophus*
E. coli	Metal removal from wastewater	Metallothionein (human) (*56*)
E. coli	Polychlorinated biphenyl metabolism	Entire pathway (*Pseudomonas* spp.) (*38*)
	New biochemistry added	
Methylophilus methylotrophus	Increased efficiency of methanol conversion to single cell protein	Alcohol dehydrogenase (*B. stearothermophilus*) and glutamate dehydrogenase (*E. coli*) (*77*)
E. coli	Indigo production	Naphthalene oxidation enzymes (*P. putida*) (*46*)
E. coli	Improved oxygen metabolism	Hemoglobin structural gene (*Vitreoscilla*) (*55*)
E. coli	Control of intra- and extracellular biopolymers	Exopolysaccharide and polyhydroxybutyrate biosynthetic genes (*Z. ramigera* and *A. eutrophus*, respectively) (*54*)

Table 3. Authorized release of live recombinant bacteria into the open environment as of 1989.

Bacterium	Altered trait	Site of test	Purpose of test	Year of first test
P. syringae	Deletion of *ice* g (*13, 14*)	California	Control of frost damage to plants	1987
P. fluorescens	Deletion of *ice* g (*14*)	California	Control of frost damage to plants	1987
P. fluorescens	Addition of *lacZY* to chromosome (*78*)	North Carolina	Assessment of spread of released bacteria	1987
R. meliloti	Additional copies of *nif* g	Wisconsin	To increase efficiency of N_2 fixation	1988
C. xyli	Introduction of *B. thuringiensis* delta endotoxin gene (*6*)	Maryland and France	Control of corn ear worm	1988
A. radiobacter	Deletion in *tra* g of Agrocin 84 plasmid (*62*)	Australia	Biological control of crown gall	1988
P. fluorescens	Addition of *lacZY* to chromosome (*78*)	Washington	To assess movement and survival of biological control agent of take-all disease of wheat (*17*)	1988

derivatized silicon chip to convert the binding of phosphate by the protein into an electronic signal for use as an on-line monitor of phosphate concentration (*57*).

Ecological Considerations for the Use of Genetically Engineered Bacteria

A large number of bacterial strains that are to be engineered for specific practical processes will need to be released into the environment, although some bioprocessing activities will likely be conducted in a confined and well-controlled setting, such as a bioreactor. Many genetically engineered bacteria to be released in the open environment will be descendants of indigenous environmentally competent bacteria. The potential environmental impact of released recombinant bacteria will need to be examined. This subject has been the focus of much attention and several international conferences since 1975 (*58*), and this review cannot adequately address the many concerns that have already been stated in other proceedings (*59*).

Regulations affecting the use of genetically engineered bacteria differ greatly between different countries (*60, 61*). One or more agencies in the United States have jurisdiction over a research activity or commercial biotechnology product, and excellent summaries of current agency jurisdiction in the United States have been published (*61*). The data required to support initial requests for field releases of recombinant bacteria have been formidable, and despite extensive documentation, not all requests for release of bacteria have been approved. Each experiment is currently addressed on a case-by-case basis.

Assessment of the environmental safety of released recombinant bacteria is rapidly taking advantage of advances in the methodology to sensitively and accurately detect specific bacterial strains or their genes. For example, bacterial DNA can now be efficiently extracted directly from environmental samples (such as soil) and identified and quantified by hybridization procedures (*62*). The polymerase chain reaction method for amplifying specific DNA sequences coupled with direct extraction of DNA from environmental samples increases the sensitivity of this method to as few as one cell per gram of soil (*63*). Information is also being generated on the frequency of gene exchange among bacteria in natural and managed environments (*64*). Several approaches to reduce or eliminate the potential persistence of modified bacteria also have been evaluated (*65*). For example, the *hok* gene, which encodes a protein that causes lethal collapse of the transmembrane potential of cells, has been placed under the control of the inducible *lac* promoter (*66*). The *hok* gene product can be induced to kill cells, if necessary, by application of inducer, thereby eliminating the recombinant cells from that environment. However, mutations that result in insensitivity to the *hok* gene product are strongly selected. Combinations of several conditional lethal blocks have yet to be tested for effectiveness and possible interference with intended biological performance.

Whereas tools now exist to genetically modify bacteria and to detect, disable, or measure cell activity in natural environments, a consensus has yet to be reached on what constitutes a safe release. There is need for better integration of research both on the ecology and molecular biology of bacteria and better focus on relevant questions that can be addressed by scientific methods. Modified bacteria, when properly applied, can become an important component of our environmental protection strategies in the future.

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Construction of Large DNA Segments in *Escherichia coli*

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Recombinant DNA clones containing large pieces of DNA are useful in the study of large genetic units, but these are difficult to make in most bacterial cloning vectors. A strategy is described that uses general and site-specific recombination to construct large pieces of eukaryotic DNA from smaller cloned segments. The large clones are propagated on F factor–based plasmids in *Escherichia coli*. They can be easily modified to introduce mutations or rearrangements. These techniques were applied to the construction of large DNA segments from the bithorax complex of *Drosophila*.

GENES IN HIGHER EUKARYOTES ARE SURPRISINGLY LARGE and complex. The bithorax and Antennapedia complexes of *Drosophila* contain transcription units of 75 and 100 kb (*1*), respectively. Certain mammalian genes are even larger. The current record holder is dystrophin (*2*), whose transcribed region may exceed 2000 kb. The noncoding DNA of some large loci contains regulatory sequences critical for appropriate spatial and temporal regulation. For example, the *Ultrabithorax* (*Ubx*) gene is regulated by sequences up to 50 kb away from the messenger RNA start site, both upstream and within the introns (*3*). Whereas the human β-globin gene has a transcription unit spanning only a few kilobases, sequences as far as 50 kb upstream appear to be needed to obtain full expression in transgenic mice (*4*).

For such large genes, a method is needed that allows rapid cloning and manipulation of large functional units, so that the whole unit can be tested by transformation into the appropriate organism. Cosmid vectors are limited to a narrow size range, typically 40 to 45 kb of insert DNA. More recently, a new vector system has been developed (*5*), based on the creation of yeast artificial chromosomes (YAC clones). This allows the cloning of much larger pieces of DNA (up to several hundred kilobases), but the yeast chromosomes are more difficult to work with than bacterial plasmids.

We have developed a method, called "chromosomal building," that allows rapid construction in bacteria of large pieces of defined DNA, in F factor–based vectors. It relies on a combination of general and site-specific recombination to join large pieces of DNA from smaller, overlapping cloned segments in vivo. The replication and partition systems of the F factor (*6*) ensure stable maintenance of the resulting large plasmids. The vectors permit the large clones to be further modified. They can be rearranged, mutations can be introduced, or selectable markers can be added for transformation into various organisms. The product is a supercoiled, circular molecule that is resistant to shearing.

Basic Building Strategy

The basic building strategy is a process in which the F plasmid serves as the recipient of DNA transferred from the shuttle plasmid by recombination. This transfer is repeated a number of times; with each repetition the F plasmid increases in size by an amount roughly equivalent to the size of the insert in the shuttle plasmid. The process begins with the cloning of a DNA fragment (designated A-B-C in Fig. 1) into a polylinker cloning site in the F plasmid vector, pMBO132 (*7*). The F factor origin maintains the plasmid at one to two copies per cell, which enhances its stability. The plasmid also carries the resolution site for the F factor's site-specific recombination system, *rfsF* (*8*).

A second DNA segment (designated C-D-E in Fig. 1) is then cloned into the polylinker cloning site of the shuttle plasmid, pMBO96 (*7*). The second DNA segment is chosen to overlap the first by 500 bp or more, depending on the convenience of restriction sites. The origin of replication for the shuttle plasmid is temperature-sensitive. At 30°C the plasmid can replicate, conferring tetracy-

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