PERGAMON

Soil Biology & Biochemistry 34 (2002) 285–296

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Review

The role of selective pressure and selfish DNA in horizontal gene transfer and soil microbial community adaptation

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Received 14 November 2000; received in revised form 7 June 2001; accepted 6 September 2001

Abstract

Recent advances in genome sequencing and horizontal gene transfer in soil have led to new insights on soil microbial community adaptation. In this review, we document and evaluate the role of selective pressure and selfish DNA in propagating horizontal gene transfer in soil through the use of a model system involving the organic pesticide 2,4-dichlorophenoxyacetic acid and the metal cadmium. This review provides a theoretical framework for microbial adaptation, wherein it is the selfish nature of DNA that provides the initial stimulus for adaptation rather than the host cells themselves. Subsequent to selfish DNA transfer, if useful to host cells, the transferred DNA may become integrated into the host chromosome. Following these events, ultimately the growth of more fit individuals within the newly created ecological niche allows for adaptation of the soil microbial community. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Co-contamination; Cadmium; 2,4-Dichlorophenoxyacetic acid; Horizontal gene transfer; JMP134(pJP4); Microbial adaptive evolution; Selfish operon

1. Overview

Genetic exchange between soil organisms is a process that can potentially increase an organisms' ability to adapt to its environment and thus could increase its survival potential (Cohan, 1996; Slater, 1985). Such horizontal gene transfer has occurred over evolutionary time and has led to the current diversity within soil microbial populations (Hill and Top, 1998; Dröge et al., 1999). Studies on horizontal gene transfer are essentially retrospective and at this point do not explain the dynamics of the process. Nevertheless, the data from genome sequencing projects has had an immediate and major impact on our view of prokaryotic evolution, revealing the importance and prevalence of horizontal gene transfers. Horizontal gene transfer, by implication, challenges the very existence of an evolutionary classification of prokaryotes. Furthermore, bacterial adaptation to xenobiotics has undoubtedly been influenced by horizontal gene transfer. The focus of previous studies in contaminated soil has been on the adaptation and fitness of microbial organisms upon application of selective pressure. Events of horizontal gene transfer were therefore thought of as an adaptive strategy benefiting the recipient cells. However, the selfish DNA theory (Doolittle and Sapienze, 1980; Orgel and Crick, 1980) views plasmids and transposable elements as genetic parasites, whose sometimes beneficial effects on the long-term evolution of prokaryotic hosts are coincidental.

Common to antibiotic or heavy metal resistance determinants and genes encoding functions for degradative pathways, is their organization into operons mostly on conjugative plasmids or other mobile elements. This organization allows for efficient horizontal transfer of genes otherwise susceptible to loss by genetic drift according to the 'selfish operon' concept (Lawrence and Roth, 1996). Briefly, the selfish operon theory implies that the proximity of genes provides no selective benefit to the individual organism but does enhance the fitness of the gene cluster itself, as clusters can be efficiently inherited horizontally as well as vertically. The 'fitness' of selfish operons is increased because the operon may provide the novel functions necessary to invade novel ecological niches. In contrast, changes such as nucleotide substitutions are so modest and incremental in the encoded product, that they would only rarely confer novel functions. Consequently, selfish operons enable rapid, effective and competitive exploration of novel niches, whereas novel functions arising by mutational processes allow only slow, initially inefficient exploitation of new resources (de Visser et al., 1999). It thus seems that horizontal gene transfer of the selfish operon is one of the driving forces in bacterial adaptation within soils.

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PII: S0038-0717(01)00183-3

In addition to selfish operons, there are other parameters that can influence horizontal gene transfer within soil microbial communities. Often, genes of interest are contained on plasmids or other mobile elements, the nature of which influences the rate of gene transfer. For example, the size of the plasmid, the presence and regulation of Tra^+ or Mob^+ functions, and the nature of the compatibility group of the plasmid are all important factors that have been well documented in the literature (Zatyka and Thomas, 1998). Genes on mobile elements are not always organized as operons initially, but rather indicate recent events of gene capture. Plasmid-mediated capture of chromosomal traits was observed during conjugation mediated by different plasmids carrying a transposable element (Szpirer et al., 1999). However, possible subsequent integration into the host chromosome favors operons over dispersed genes since only a single event is required.

A question of interest is "what is the rate of genetic exchange within soils, and how do human activities influence such exchange?" Specifically, it has been shown that the addition of contaminants be they organic xenobiotics or metals, can influence rates of genetic exchange, and alter the soil microbial gene pool. In this review, we will document and evaluate the role of selective pressure and selfish DNA in propagating horizontal gene transfer in soil through the use of a model system involving the organic pesticide 2,4-dichlorophenoxyacetic acid (2,4-D) and the metal cadmium (Cd). The addition of 2,4-D to soils usually results in its degradation by only a few of the many genera of microorganisms present in soil. Likewise, the addition of Cd to soil can select for microbes with variable metal resistance (Roane and Pepper, 2000). Thus contaminants have the potential to stimulate or inhibit specific segments of the soil microbial community. Concomitant with these processes, the selective pressure might also influence horizontal gene transfer and subsequent soil microbial community adaptation. At this point we are not able to delineate gene transfer events from subsequent growth of transconjugants. However, one study demonstrated multiple gene transfer events after addition of 2,4-D (DiGiovanni et al., 1996). Newby et al. (2000a,b,c) showed at least 40 different transconjugants and culturable transconjugants comprising 10% of the total culturable population (10^7 transconjugants/g soil). This review provides a theoretical framework for such adaptation, wherein it is the selfish nature of DNA that provides the initial stimulus for adaptation rather than the host cells themselves. Subsequent to selfish DNA transfer, if useful to host cells, the transferred DNA may become integrated into the host chromosome. Following these events, ultimately the growth of more fit individuals within the newly created ecological niche allows for adaptation of the soil microbial community.

2. Degradative pathways of 2,4-D

Degradation of 2,4-D in soil has been extensively examined. Numerous diverse organisms capable of such degradation have been isolated from a wide variety of soils (Fulthorpe et al., 1996). In many cases, the catabolic genes were determined to be plasmid encoded, and specifically, often within conjugative plasmids (Don and Pemberton, 1981; Maë et al., 1993; Top et al., 1995b). Catabolic genes for 2,4-D degradation have also been found to be chromosomally encoded (Matheson et al., 1994; Suwa et al., 1996).

Plasmid pJP4 is the most studied 2,4-D degradative plasmid. This plasmid can be maintained within a variety of hosts; however, it may have a reduced size or exist as a multimer depending upon the host strain (Friedrich et al., 1983). In addition, pJP4 has been shown to co-exist with other large plasmids (Springael et al., 1993). Plasmid pJP4 is a broad host range, self-transmissible, IncP1, low copy number, catabolic plasmid. It is 80 kb in size, and encodes for the degradation of 2,4-D to 3-oxoadipate which is then degraded to succinyl-CoA and acetyl-CoA (Laemmli et al., 2000). The degradation pathway encoded by pJP4 for 2,4-D is shown in Fig. 1 (Laemmli et al., 2000). Succinyl-CoA and acetyl-CoA can enter the tricarboxylic acid (TCA) cycle and subsequently be mineralized to CO_2. Although 2,4-D is generally not considered toxic to microorganisms, at high concentrations, the intermediate 2,4-dichlorophenol (2,4-DCP) can be toxic. Additional pJP4 genes code for the degradation of 2-methyl-4-chlorophenoxyacetic acid and 3-chlorobenzoate, while others encode resistance to mercuric ions and phenyl mercury acetate (Don and Pemberton, 1981). In order to be able to confer 3-chlorobenzoate degradative capabilities to its host, pJP4 undergoes a genetic rearrangement in the presence of this compound. This rearrangement involves the insertion of a 24.5 kb inverted random duplication of a segment encoding catabolic functions (Ghosal and You, 1988). Plasmids for 2,4-D degradation such as pJP4 are a good model to observe the evolution of new pathways. Existing sets of genes acquired from different organisms can be assembled into new structures. This assembly is catalyzed by mobile DNA elements such as the IS element ISJP4 in plasmid pJP4 (Fulthorpe et al., 1995; Don and Pemberton, 1981).

The genetic arrangement of the 2,4-D degradative genes is also of interest. The genes in pJP4 are arranged as shown in Fig. 2. Top et al. (1996) suggested that this arrangement of the *tfd* genes of pJP4 is indicative of recruitment of genes, specifically *tfdA* and *tfdB*, during the evolution of the catabolic pathway. This group demonstrated the successful recruitment of genes from soil microbes with a high degree of homology to the *tfdA* gene of pJP4 through the use of a derivative of plasmid pJP4 (pBH501aE) from which *tfdA* had been deleted. A comparison of different 2,4-D degrading microorganisms show that the order and origin of the *tfd* genes can be quite variable indicating different sources of recruitment.

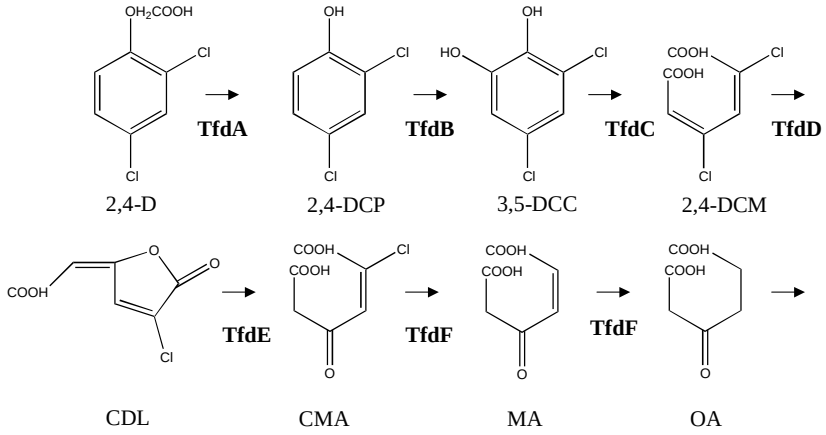

Fig. 1. Pathway for 2,4-D degradation of 2,4-D. Genes within plasmid pJP4 encode the first six steps in the pathway (Laemmli et al., 2000). TfdA, 2,4-D α-ketoglutarate dioxygenase; TfdB, chlorophenol hydroxylase; TfdC, chlorocatechol 1,2-dioxygenase, TfdD, chloromuconate cycloisomerase; TfdE, dienelactone hydrolase; TfdF, (chloro)maleylacetate reductase. Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4-DCP, 2,4-dichlorophenol; 3,5-DCC, 3,5-dichlorocatechol; 2,4-DCM, 2,4-dichloromuconate; CDL, *cis*-chlorodiene lactone; CMA, chloromaleylacetate; MA, maleylacetate; OA, 3-oxoadipate.

3. Mechanisms of cadmium resistance determinants

Microorganisms need to take up metal cations such as Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} because they are required as micronutrients for vital cell functions. Other metal ions such as Cd^{2+} or Pb^{2+} are not beneficial to the cell but compete with essential metal ions for uptake. However, all of these metal cations are also toxic in high concentrations, making homeostatic resistance mechanisms necessary (Rosen and Silver, 1987). The most common way to prevent metals from reaching toxic levels is active efflux catalyzed by metal resistance/homeostasis determinants. Most often these determinants are members of one of three major transporter groups: P-type ATPases, ABC multi-component ATPases, and membrane potential-driven non-ATPase transporters (Silver and Phung, 1996). Whereas active efflux of excess metals is the most common method of detoxification, other mechanisms which impart resistance to heavy metals have been identified (Ji and Silver, 1995). These are: (i) prevention of uptake, e.g. Cu^{2+} (Lutkenhaus, 1977); (ii) intracellular sequestration of the metal by binding proteins, e.g. Cd^{2+} and Zn^{2+} (Robinson et al., 1990; Roane and Pepper, 2000); (iii) extracellular sequestration, often by extracellular polysaccharides on the cell wall, e.g. Pb^{2+} (Gadd and Griffiths, 1978) and Cu^{2+} (Cooksey, 1994); and (iv) enzymatic conversion of the metal to a less toxic form, e.g. Hg^{2+} and CH_3Hg (Misra, 1992). Many of these heavy metal resistance mechanisms are encoded by genetic determinants, which have been extensively studied, and have been reviewed elsewhere (Silver and Phung, 1996).

In recent years, it has become obvious that the differentiation between plasmid-borne resistance mechanisms and intrinsic chromosomal determinants responsible for metal homeostasis and trafficking is not as sharp as it once seemed (Axelsen and Palmgren, 1998; Rensing et al., 1999). What has been learned is that all organisms thus far studied have genetic determinants responsible for intrinsic tolerance to heavy metals. Heavy metal resistance is a variation of a theme that confers additional resistance. The increased number of completely sequenced microbial genomes illustrate close homologs on the chromosomes to previously identified plasmid-borne resistance determinants. For example, CadA, a Cd(II) (also Pb(II) and Zn(II)) transporting P-type ATPase from *Staphylococcus aureus* plasmid pI258 is a homolog of ZntA, a Pb(II)/Cd(II)/Zn(II)-translocating P-type ATPase from the *Escherichia coli* chromosome (Rensing et al., 1997a; Rensing et al., 1998; Nucifora et al., 1989) (Fig. 3). Sequence comparison of these transporters shows that sequences cluster by cation specificity and function, and do not follow the overall phylogenetic patterns of the particular microbes, as determined by sequence comparison of small subunit ribosomal RNAs. Rather, soft metal translocating P-type ATPases are an example of where genes appear to have been exchanged by lateral gene transfer in the course of evolution (Axelsen and Palmgren, 1998). Since all microorganisms have mechanisms to regulate heavy metal concentrations inside

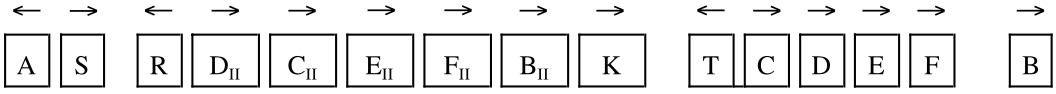

Fig. 2. Diagram showing the genetic arrangement of the *tfd* genes encoded by pJP4. Gene structure and promoters regulated by Tfd(R)(S) (modified from Laemmli et al., 2000).

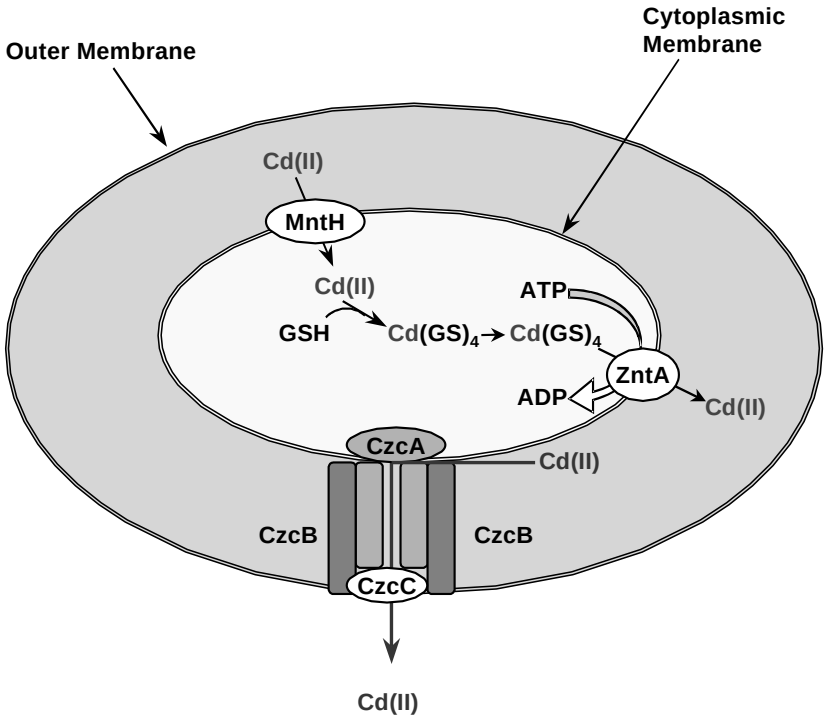

Fig. 3. Cadmium pathways in Gram-negative bacteria. Cadmium is taken up by the manganese uptake system MntH in *E. coli* and *S. typhimurium* (Kehres et al., 2000). In the cytoplasm Cd^{2+} binds to thiolates such as in glutathione. Cd^{2+} is transported into the periplasm by ZntA, a soft metal transporting P-type ATPase (or homologues such as CadA or ZiaA in *H. pylori* or *Synechococcus* PCC 6801, respectively). Cd^{2+} is not transported as a sulfhydryl complex; however, the presence of thiolates stimulates Cd^{2+} transport at physiological pH. The Czc system, a chemiosmotic proton/cation antiporter, is an additional Cd^{2+} resistance mechanism, transporting Cd^{2+} from the cytoplasmic membrane over the outer membrane into the extracellular medium. Czc might transport Cd^{2+} bound to phospholipids.

the cell, the term heavy metal resistance is rather subjective. However, heavy metal resistance determinants that give additional resistance have been described on plasmids, transposons and integrated into the chromosome. While ZntA and homologous transporters are required to prevent accumulation of toxic concentrations inside the cell, other systems can confer additional resistance. A well-studied example is the *czc* determinant from *Ralstonia metallidurans* CH34, formerly *Alcaligenes eutrophus*. The large plasmids of the soil chemoautotroph *R. metallidurans* CH34 have numerous heavy metal resistance determinants (Mergeay et al., 1985) [in strain CH34, these include three for mercury resistance, one for chromate resistance, and two for divalent cations called *czc* (for Cd^{2+}, Zn^{2+}, and Co^{2+} resistances) and *cnr* (for Co^{2+} and Ni^{2+} resistances)]. The *czc* determinant encodes three structural proteins, CzcA, CzcB and CzcC. Czc is an efflux pump that functions as a chemiosmotic divalent cation/proton antiporter (Nies et al., 1989; Nies, 1999; Rensing et al., 1997b). The proteins involved have become the prototype for a new family of three-component chemiosmotic exporters, including members that efflux toxic cations or organic compounds (Diels et al., 1995). Homologous metal transporting systems have been found in *Pseudomonas*, *Synechococcus*, *Salmonella* and *E. coli*. They seem to be widespread in Gram-negative bacteria, and probably confer additional resistance to certain heavy metals. A tentative model predicts that a P-type ATPase such as ZntA pumps metals such as Cd from the cytoplasm into the periplasmic space. Upon saturation of periplasmic cadmium stores, it is anticipated that the *czc* system mediates Cd export across the outer membrane (Fig. 1). Another family of divalent cation transporters that might be responsible for Cd transport is the cation diffusion facilitator (CDF) protein family, which occurs in all three domains of life (Paulsen et al., 1997). However, only CzcD has so far been implicated in Cd transport (Anton et al., 1999).

4. Theory of selfish DNA

Plasmids are extrachromosomal DNA elements found in species from the domains Archaea, Bacteria, and Eukarya. Since plasmids can be introduced into new hosts by a variety of mechanisms such as conjugation and transformation, they can be considered to be a pool of extrachromosomal DNA, which is shared among populations. They can also incorporate and deliver genes by recombination or transposition, thus favoring genetic exchanges in bacterial populations. Additional ubiquitous mobile genetic elements such as insertion sequences, transposons and retrotransposons are also found in all domains of life. However, the distinction

between plasmids, phages, and transposons is somewhat artificial even on functional grounds. For example, when they are in bacteria, the prophage of the phage P1 is a plasmid and that of Mu is a transposon. Although many mobile elements carry additional genes that provide a selective advantage to the host (e.g. antibiotic resistance), some elements appear to be cryptic and are usually considered genomic parasites or selfish genes (Doolittle and Sapienza, 1980; Orgel and Crick, 1980). In this review, we focus on plasmids since most of the work describing horizontal gene transfer in soil involved conjugative plasmids and we also consider plasmids to be selfish but have to broaden the definition of 'selfish DNA'. Many traits favoring the survival of the cell are located on plasmids making the term selfish DNA seem wrong. However, the mobile elements themselves are under selective pressure so that those carrying genes beneficial for the host have a better chance to proliferate. Selective pressure will enhance the transfer of broadly favored but narrowly available genes and former elements through mobile accessory sequences. Since this transfer might not be beneficial to the donor, this behavior indicates that the selfish nature of the mobile elements and the genes they carry are driving horizontal gene transfer. Whether the mobile elements bearing these genes will be phages, conjugative or non-conjugative plasmids, or transposons or whether those genes would be acquired by transformation or conjugation will depend on a variety of historical, genetic, and ecological factors that are specific to the bacteria and habitats involved. Additionally, plasmids have evolved circuits to minimize the metabolic and phenotypic load on the host, while optimizing the benefits to the plasmid of possessing a transfer apparatus (Zatyka and Thomas, 1998). Control is often designed to ensure propagation of the plasmid and the transfer of plasmids via conjugation can often be considered selfish. This transfer is tightly regulated (Zatyka and Thomas, 1998; Kado, 1998), and requires multiple plasmid-encoded genes. For example, transfer of a number of enterococcal plasmids respond to pheromones. Plasmid-free recipients secrete multiple sex pheromones that trigger expression of transfer functions in the donor cells. Thus, the accumulation of pheromones indicates to donors that recipients are close by and propagation of the plasmid is ensured. When a plasmid is acquired, secretion of the related pheromone is prevented (Clewell, 1993; Wirth, 1994). Other plasmids exert different types of control, and show that different environmental conditions influence gene transfer frequency. The transfer process can be split into two stages: mating pair formation and DNA transfer. The possession of such a machinery for these systems places a burden on the host arising either from the energy expended in creating and maintaining the apparatus or from the associated properties such as bacteriophage sensitivity (Del Solar et al., 1998). The tailoring of gene-encoded functions by plasmids is often aimed at maximizing the survival of the bacterial cell in which plasmids reside. To create such molecular niches, novel genes are integrated into each plasmid during events of gene capture and horizontal gene transfer resulting from differing selective pressures (Kado, 1998). Organisms, which survive the best, are those containing plasmids that give the bacterium the ability to withstand adverse conditions or the ability to utilize an unusual compound. However, under some circumstances plasmids show a different kind of behavior not beneficial to the host. For example, *Ralstonia* sp. CH34 undergoes plasmid rearrangements and mutation after a shift from 30 to 37 °C (not the optimal temperature) on rich medium (Mergeay et al., 1986). This event is not beneficial for the plasmid-bearing cells but rather causes high mortality. This phenomenon was termed 'temperature-induced mutagenesis and mortality' (TIMM) (Diels and Mergeay, 1990; Taghavi et al., 1997). However, this event clearly benefits the plasmid and the encoded heavy metal resistance operons because the resulting plasmid is derepressed for self-transfer. This results in a 4–5 orders of magnitude of higher frequency of plasmid transfer as compared to the original plasmids (Taghavi et al., 1997).

5. Evolutionary aspects of horizontal gene transfer

Microbial genome analysis has made it apparent that genetic material is readily exchanged in the course of evolution by lateral gene transfer making the concept of prokaryotic species seem arbitrary for some researchers. However, microbial genomes consist primarily of core sequences, which encode housekeeping functions and which carry gene clusters that show very little mutational change. In addition to the core genome there are sequences with different G + C content and codon usage that appear to have been acquired by horizontal gene transfer. For example, a recent study suggests that 755 of 4288 ORFs have been introduced into the *E. coli* genome in at least 234 lateral transfer events since this species diverged from the *Salmonella* lineage 100 million years ago (Lawrence and Ochman, 1998). These regions encode accessory functions such as antibiotic resistance, additional metabolic activities, or additional heavy metal resistance. In the natural environment, gene dissemination through lateral gene transfer involves many different elements such as plasmids, phages, transposons and integrons utilizing different mechanisms such as homologous and site-specific recombination, transposition, conjugation, transformation and transduction (Dröge et al., 1999). Conjugation seems to be the most important way of gene transfer in most circumstances. Additionally, the ability of a plasmid to mediate retrotransfer (to capture genes that could bring an advantage to its host) is shared by many conjugative plasmids, which possess this ability to a greater or lesser extent. Retrotransfer may have great evolutionary significance as an additional possibility of promoting horizontal gene transfer (Szpirer et al., 1999; Top et al., 1995a). There

are a number of well-documented examples in which bacterial adaptation has been influenced by horizontal gene transfer including antibiotic resistance, degradative pathways for xenobiotic compounds and heavy metal resistance (Datta and Hughes, 1983; De Rore et al., 1994; Harwood and Parales, 1996; Hughes and Datta, 1983; Mazel and Davies, 1999; Osborn et al., 1997; Bogdanova et al., 1998).

6. Strategy for survival: cell adaptation through propagation of the selfish operon

The focus of previous studies in contaminated soil has been on the adaptation and fitness of microbial organisms upon application of selective pressure. Events of horizontal gene transfer were therefore thought of as an adaptive strategy benefiting the microorganisms. However, it does not necessarily benefit the donor if genes that give a selective advantage to the donor are transferred to possible competitors. Rather, it suggests the selfish nature of the mobile elements and the genes they carry are driving horizontal gene transfer. We have broadened the term selfish DNA to include mobile elements possessing useful genes and view these elements as being mainly concerned with self-propagation. However, these mobile elements themselves are under selective pressure, as natural selection will operate on mobile elements through organismal phenotype. Those mobile elements carrying genes that make the host more competitive in the environment will be favored. In order to better understand these conflicting views on adaptive evolution in soil, we first look at the structure of genetic elements conferring an adaptive advantage from the 'genes' point of view. Common to numerous antibiotic or heavy metal resistance determinants and genes encoding functions for degradative pathways, is their organization into operons mostly on conjugative plasmids or other mobile elements. This organization allows efficient horizontal transfer of genes otherwise susceptible to loss by genetic drift according to the 'selfish operon' concept (Lawrence and Roth, 1996). Selfish operons may provide the novel functions necessary to invade novel ecological niches. A sudden environmental change, such as contamination with Cd or 2,4-D, would put selective pressure on the microbial community. Horizontally transferred DNA includes selfish operons, which can provide novel functions, such as additional Cd resistance or the ability to degrade 2,4-D, immediately upon introduction. In contrast, changes such as nucleotide substitutions are so modest and incremental in the encoded product that they would only rarely confer novel functions. Consequently, selfish operons enable rapid, effective and competitive exploration of novel niches whereas novel functions arising by mutational processes allow only slow, initially inefficient exploitation of new resources (de Visser et al., 1999). Strikingly, at present no phenotype distinguishing *E. coli* and *Salmonella* can be attributed to the differentiation of ancestral genes by point mutation, rather, all described phenotypic differences can be attributed to the gain or loss of genes. Novel functions can evolve through duplication and divergence and also through structural reorganization, probably when selection is not intense. For example, a novel function might evolve to benefit an organism in an existing ecological niche where selection for this function is not critical: the novel function would not be employed to invade a novel ecological niche, where selection for function would be intense. Accordingly, functions can evolve under weak selection in a donor species, but allow niche exploitation and survival under strong selection only following horizontal gene transfer. Therefore, horizontal gene transfer serves to decouple speciation, from metabolic differentiation by mutation and adaption (Lawrence, 1997). It thus seems that horizontal gene transfer of the selfish operon is the driving force in bacterial adaptation and subsequent evolution.

As we have pointed out previously, operons are often transferred on mobile elements such as transposons or plasmids that can also be considered selfish. However, often genes required for novel functions, such as 2,4-D degradation, are not initially organized as an operon. Rather, genes are recruited from different sources since it does not alter the 'fitness' of the plasmid whether the genes are organized as an operon or not. Recent results on gene capture suggest that genes for degradative functions can be rapidly assembled (Top et al., 1995b; Poelarends et al., 2000). On the other hand, it has also been suggested that bacteria possess evolution genes, the products of which act for the benefit of the biological evolution of the population of organisms (Arber, 2000). These genes encode functions enabling bacteria to generate and modulate genetic variation. A striking example appears to be the presence of integrons. Integrons are gene expression elements that acquire gene cassettes and convert them to functional genes. The insertion of a gene cassette takes place by site-specific recombination between the circularized cassette and the recipient integron. The essential components are an integrase gene (*intI*) and a linked attachment site (*attI*) required for the efficient site-specific integration of the gene cassettes into the integron structure (Recchia and Hall, 1995). In the light of these results, it has been suggested (Levin and Bergstrom, 2000) that, if the mobility of host-adaptive genes has a cost, that mobility will be lost eventually. Plasmid and other mobile elements, or the genes they carry will give up their mobile lifestyle and become integrated islands. Integration also explains why operons would be favored over single genes. Integration of an operon only requires a single event and favors the existence of operons versus single genes over extended periods of time. Thus, genes originally carried on accessory elements are in a continuous state of flux with respect to their mobility and within-host stability. As the habitat of a bacterial population becomes more stable and/or the opportunities for its mobile elements to move to uninfected populations decline,

selection will favor the incorporation of those elements or the genes they carry into the chromosome. Thus, evolution genes influence the kind and the frequency of genetic variation occurring in a population of organisms and may ensure a certain degree of genetic isolation. These postulates are not exclusive but rather describe some of the dynamics of bacterial evolution.

7. Concept of bacterial speciation on microbial community structure

The role of selective pressure and subsequent horizontal gene transfer has the potential to affect microbial diversity within soils. However, first one must define what a bacterial species constitutes. We do not wish to cover this problem in depth but take a practical approach as to illustrate our point. Speciation involves the establishment of genetic barriers between closely related organisms. A species may be defined as a population of organisms capable of sharing their gene pool through mating and genetic recombination. The inability to undergo genetic recombination with each other isolates related species independently of geographic isolation. The structural basis of the barrier to genetic recombination on the molecular level is the difference in their DNA sequences (Vulic et al., 1997). Although sequence divergence is clearly the structural basis for the genetic barrier, the effectiveness of this barrier is under the control of cellular systems, particularly those responsible for the initiation of genetic recombination, and those responsible for editing recombinational intermediates, SOS and mismatch repair, respectively (Matic et al., 1995). A number of environmental and physiological factors have been found to affect the state of the SOS and mismatch repair systems in bacteria (Feng et al., 1996). Under conditions of metabolic stress, the SOS system is induced, whereas the mismatch repair system is inhibited, which leads to an increase in point mutations, intragenomic plasticity, and horizontal gene transfer, all resulting in rapid genetic diversification. When an environmental stress is overcome by an adaptation, then the return to mismatch repair proficiency and repressed SOS maintains genomic stability and separates different genetic lineages by restricting gene exchange. The genes involved in this process have also been referred to as evolution genes, and describe the intrinsic ability of bacteria to control genetic variation (Arber, 2000).

An event such as co-contamination of a soil with Cd and 2,4-D would create a sudden change in the environment, and as such a new ecological niche to be conquered. Only organisms having the necessary genes would thrive in this new environment. Since it is a new ecological niche, speciation could occur over time. Dykhuizen (1998) estimated that thirty grams of forest soil contain over half a million species, and speculated that the reason for this abundance of bacterial species is that speciation in bacteria is easy and extinction difficult. Speciation will continue until the number of species is large enough that, even with a low extinction rate, the extinction and speciation come into equilibrium. The features that discriminate closely related bacterial taxa probably reflect sets of selective pressures inherent in their individual lifestyles: each bacterial species occupies a distinct ecological niche that provides selection for essential, niche-specific functions. It therefore appears that much of the speciation and sub-speciation in bacteria can be explained as the result of macroevolution events mediated by horizontal gene transfer, an alternative to mutational processes. Further adaptation to the newly conquered niche will occur by successive point mutations and genetic rearrangements, and only after millions of years will the new species be recognized by hybridization or 16S RNA sequencing. Therefore, we have to distinguish between the radical speciation event by horizontal gene transfer, and instillation of the new 'variant' as a recognized species by chromosomal divergence.

If many bacterial speciation events can be traced to the acquisition or loss of specific sets of genes as a result of horizontal gene transfer, speciation by horizontal gene transfer may not be a specific response to defined challenges, but a global evolutionary response of bacterial populations (De La Cruz and Davies, 2000). Studies of genes and genomes are indicating that horizontal transfer of operational genes is a continual process (Jain et al., 1999). Thus, we can model prokaryotic, operational genes as semi-autonomous agents within a global superorganism (Doolittle, 1999). Conjugative plasmids and other mobile elements are therefore important for the survival and spread of genes conferring, in our example, Cd resistance or the degradation pathway for 2,4-D, enabling them to act as independent agents within the global superorganism. The need of the microbial community to adapt opens the window of opportunity for these determinants to spread while subsequent speciation aids in ensuring propagation. In summary, we conclude that the selfish nature of the genes and of the mobile elements themselves is one driving force in bacterial evolution. The other is the intrinsic ability of microorganisms to generate and modulate genetic variation and to integrate formerly mobile elements or the genes they carry into their chromosome.

8. Role of gene transfer in the dispersal of genes in soils

The potential for gene transfer from an introduced donor in the soil environment depends on the survival and transport of introduced organisms as well as of transconjugants in the ecosystem. Survival and transport of organisms in the environment directly impacts the potential for gene transfer. At the macrolevel, bacteria can be transported by wind or water. At the microlevel, transport of bacterial cells through soil is governed by numerous factors including, but not

limited to the following: adhesion processes, filtration effects, physiological state of the cells, porous medium characteristics, water flow rates, predation, and intrinsic mobility of the cells. Newby et al. (2000c) briefly reviewed some of the numerous studies that have been conducted to assess the influence of these factors. The first two factors, adhesion processes and physical straining/filtration of the cells, significantly reduce cell transport. Limited transport of inoculum cells significantly hampers bioaugmentation efforts. Gene transfer to indigenous populations may make it possible to distribute genetic information more readily through the soil by establishing a stable and diverse array of plasmid hosts.

Studies have addressed the transport potential of transconjugants generated by transfer of a plasmid from an introduced donor to indigenous populations. In a soil column study involving a donor surface inoculum, Daane et al. (1996) found that transconjugants were limited to the top 5 cm of the column. However, when earthworms were also introduced into the column, not only did the depth of transport of donor and transconjugants increase, depending on the burrowing behavior of the earthworm species, but also the number of transconjugants found increased by approximately two orders of magnitude. In a related study, the presence of earthworms was found to facilitate transport of donors and recipients, as well as transfer of plasmid pJP4 between spatially separated donor (*A. eutrophus*) and recipient (*Pseudomonas fluorescens*) bacteria in non-sterile soil columns (Daane et al., 1997). No transconjugants were observed in the absence of earthworms.

In a separate study, Lovins et al. (1993) examined the transport of a genetically engineered *Pseudomonas aeruginosa* strain that contained plasmid pR68.45 and the indigenous recipients of this plasmid in non-sterile, undisturbed soil columns. The surface of the column was inoculated, and unsaturated flow conditions were maintained. Transconjugants survived longer in the columns and were found to have leached farther down the column than the donor. The greater survival rate of transconjugants would be expected because these organisms had previously adapted to the particular conditions of the soil. The increased transport was hypothesized to be the result of plasmid transfer to smaller, more mobile bacteria. In addition, consecutive gene transfer events between indigenous microbes may be a mode of transporting genes through soil. This would be especially feasible when microbes are present in high densities, such as stationary microbes growing within a biofilm on soil surfaces or in the rhizosphere.

9. Gene transfer of pJP4 and microbial soil community adaptation in response to the selective pressure of 2,4-D and Cd additions to soil

Plasmid pJP4 is a well characterized 80 kb catabolic, low copy number, broad host range plasmid which belongs to the incompatibility group IncP1(Don and Pemberton, 1981). Genes for the degradation of 2,4-D to 3-oxoadipate are located on this plasmid and have been sequenced (Perkins et al., 1990; Perez-Pantoja et al., 2000). The transfer and expression of plasmid-borne degradative genes is not necessarily the same as the transfer of antibiotic and heavy metal resistance genes, since they may be growth enhancing. pJP4 genes are not useful when transferred into organisms which lack the 3-oxoadipate pathway which is vital for the organism's survival on 2,4-D as a sole carbon source (Kinkle et al., 1993). Don and Pemberton (1981) monitored transfer of pJP4 in pure culture and found that the 2,4-D degradative genes were functionally expressed in *A. eutrophus*, *Variovorax paradoxus* and *Pseudomonas putida*, but not in *E. coli*, *Rhodospeudomonas sphaeroides*, *Agrobacterium tumefaciens*, *Rhizobium* spp., *P. fluorescens* or *Acinetobacter calcoaceticus*. However, resistance to mercury, which is also encoded by pJP4 was expressed in all of the above organisms. Although plasmid pJP4 is frequently used as a model, particularly in gene transfer studies, it is important to note that several 2,4-D catabolic plasmids differing from pJP4 have also been described (Don and Pemberton, 1981). For example, Top et al. (1998) examined the conjugative transfer of two plasmids, pEMT1k and pEMT3k, to indigenous soil bacterial populations. These plasmids differ in sequence as well as gene organization from pJP4. They also confer different rates of 2,4-D degradation to the host microorganism *Ralstonia eutropha* JMP228. This research group observed different levels of plasmid transfer to indigenous populations depending upon the plasmid being transferred, the presence of 2,4-D selective pressure, and the soil type.

Early studies of pJP4 were conducted with the donor *A. eutrophus* JMP134, now known as *R. eutropha* JMP134. More recently, other donors of pJP4 have been utilized including *E. coli* and *P. putida*. The influence of pJP4 on the rate of degradation of 2,4-D amended soil has been variable. For example, Dejonghe et al. (2000) showed that pJP4 transfer from the donor *P. putida* UWC3 and enhanced degradation of 2,4-D occurred in the B horizon of a soil, whereas in the A horizon, although transfer occurred, the rate of 2,4-D degradation was not enhanced. Interestingly, when the same donor was used with plasmid pEMT1 (Top et al., 1999), which carries the same degradative genes but probably belongs to a different incompatibility group than pJP4, transfer rates were similar to those with pJP4 (Dejonghe et al., 2000). These data show that transfer rate of the plasmids was independent of the kind of plasmid (pJP4 or pEMT1) and soil type (A or B horizon).

Our own laboratory has concentrated on gene transfer of pJP4 in contaminated soils using different donor organisms to introduce pJP4 into the soil community. A relevant question is "What is the role of horizontal gene transfer in soil adaptation, and how does the selective pressure of contaminant addition influence gene transfer?" A further query is "What is the role of soil bioaugmentation in altering

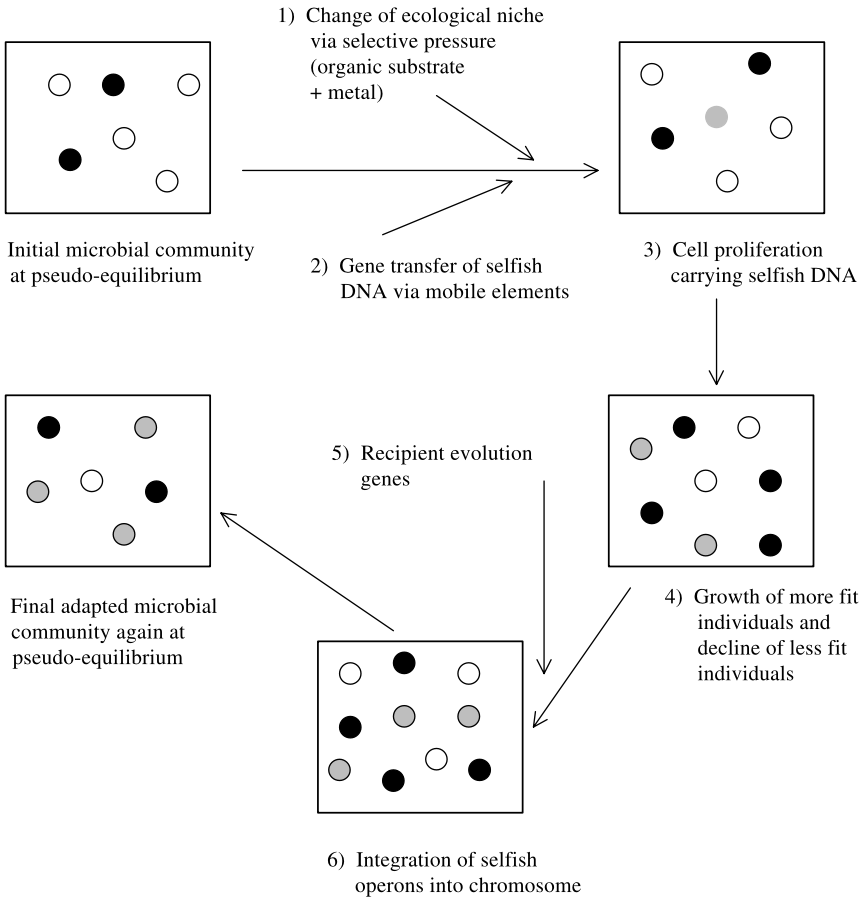

Fig. 4. A conceptual model for soil microbial community adaptation.

the soil microbial gene pool?" In stable, undisturbed soils, microbial soil communities are probably relatively stable. Bacteria are haploid, reproduce clonally and generally are thought to have low rates of gene recombination within bacterial populations (Levin and Bergstrom, 2000). However, horizontal gene transfer is a mechanism for producing sources of genetic variation within these populations. Gene transfer via accessory elements such as plasmids is primarily dependent on two key factors. The first is simply the number of bacteria within the soil environment. This was illustrated by Neilson et al. (1994) who showed that in pure culture, gene transfer between *A. eutrophus* JMP134 and *V. paradoxus* occurred extensively. A transfer frequency of approximately $1/10^3$ donor and recipient cells was observed on solid agar media, decreasing to $1/10^5$ in sterile soil, and finally $1/10^6$ in 2,4-D-amended non-sterile soil. In soil, the decreased gene transfer was most likely due to spacial separation within soil, and also biotic competition in the case of the non-sterile soil. Because bacterial numbers are important in dictating gene transfer rates, in an undisturbed soil where microbial substrates would be relatively rare, most organisms would exist under limited starvation. Thus, active metabolism would not exist for many communities, reducing gene transfer rates.

The second major factor is the number of mobile accessory elements within the population. Genes carried on plasmids are likely to be in a continuous state of flux with respect to their mobility and within-host stability. If the environmental habitat (in this case soil) is stable, the opportunities for plasmid transfer to new populations decline. Concomitantly, genes important to the host are likely to be sequestered within the chromosome, with resulting decreased potential for mobility. In such a stable situation, gene transfer rates are probably low but consistent, resulting in subtle genetic shifts between populations. Genetic shifts can also occur via the processes of mutations, including deletions and additions.

Now consider the impact of adding a selective pressure onto the soil microbial community (Fig. 4). If the selective pressure is a potential organic substrate, as in the case of the addition of 2,4-D, microbes with the appropriate degradative genes will be at a selective advantage over those microbes not possessing such genes. Typically, if 2,4-D is added to a soil, which has not had prior exposure, degradation does not occur immediately. This lag phase is generally referred to as the 'adaptation' period for the soil microbial community, but what is it that actually happens during this period? The classical theory of soil adaptation relied on two possibilities. The first scenario assumed that a few bacterial cells already contained the appropriate degradative genes,

and that during adaptation, there was merely growth of these cells, and a resulting increase in cell concentration to the point where effective degradation occurred. The second possibility is that random mutations could potentially occur within the community, resulting in selective adaptation, and following growth of these new 'fit' individuals, enhanced rates of degradation.

A more likely explanation for soil adaptation is the role of horizontal gene transfer mediated by selfish DNA. Following the amendment of the soil with 2,4-D, selection will favor mobile elements such as plasmids. In this case, horizontal gene transfer is likely to be vital to the adaptation of the soil microbial community, and can occur within and between indigenous soil bacterial populations. If a soil is bioaugmented through the introduction of appropriate plasmid bearing bacteria, then gene transfer events can be enhanced substantially. This was the case in the study by DiGiovanni et al. (1996), where the introduction of *A. eutrophus* JMP134 resulted in significant new transconjugant populations. These transconjugant populations were not only at levels of 10^6 per gram of soil, but also changed over a six week, very short time period. Three different species of transconjugants within two genera were detected sequentially. Whether transfer occurred from the donor organism to three different soil recipients, or whether transfer occurred from one population of transconjugants to different soil recipients is unclear. Also, the number of discrete gene transfer events versus growth following gene transfer could not be discerned.

Newby et al. (2000a) utilized donor counter selection to enhance pJP4 gene transfer detection. In this study, plasmid pJP4 was introduced into a donor microorganism, *E. coli* ATCC 15224 by plate mating with *R. eutropha* JMP134. The *E. coli* donor (D11) lacked the chromosomal genes necessary for mineralization of 2,4-D, allowing presumptive transconjugants obtained in gene transfer studies to be selected by plating on media containing 2,4-D as the sole carbon source. Use of this donor counter selection allowed detection of plasmid pJP4 transfer to indigenous populations in soils when transfer rates were low. This donor counter selection produced transconjugant populations of up to 10^8 per gram of Madera soil, when amended with 1000 μg 2,4-D per gram of soil. The dominant genera of these populations was *Burkholderia* or *Pseudomonas*. This *E. coli* donor also produced transconjugants in two other soils, whereas previous studies with *R. eutropha* JMP134 as the donor produced no transconjugants.

There is also indirect evidence in the literature, for the role of horizontal gene transfer mediated by selfish DNA. Newby et al. (2000b) compared gene transfer events in a soil when inoculated with one of two donors both of which contained plasmid pJP4. When the donor was *E. coli* (unable to mineralize 2,4-D), transconjugant populations were 10^7 per gram of soil, or 10% of the culturable heterotrophic population. In contrast, when JMP134 was utilized, much less gene transfer was detected. Of interest is the fact that the *E. coli* donor cells decreased in number, whereas the JMP population remained high. This would appear to be a quintessential example of the role of selfish DNA in soil adaptation. Note that in the study of DiGiovanni et al. (1996) the donor organism also died off (or at least became non-culturable) while gene transfer was occurring vigorously.

If the selective pressure is a metal such as Cd, a different situation exists than when the selective pressure is an organic substrate. All microbes have some tolerance level to metals, and therefore the real issue is the level of resistance that a particular population may have. Thus a gradient of metal resistance can exist within a microbial community, with the potential for enhanced resistance to be passed along via plasmid-encoded genes. In a co-contaminated soil containing both organic and metal selective pressures, the situation becomes even more complex. Our laboratory has studied such a system involving 2,4-D and Cd (Josephson and Pepper, unpublished data). With increased Cd levels, 2,4-D degradation was delayed as the soil 'adapted'. Whether selfish DNA in the form of degradative plasmids or metal resistance plasmids was involved in this adaptation is unclear. However, when pre-exposed to Cd prior to 2,4-D amendment, degradation rates did increase, showing the potential for adaptation to metal stress prior to adaptation to organic stress.

In this same study, bioaugmentation with a metal resistant 2,4-D degrader overcame the need for adaptation, with 2,4-D degradation occurring without any appreciable lag phase. This bioaugmentation effect was also cell concentration dependent. With high inoculant cell concentrations, 2,4-D degraded rapidly and gene transfer occurred. At lower cell concentrations, lower rates of degradation occurred, and no gene transfer was detected.

Overall, these studies suggest a critical role for selfish DNA in propagating horizontal gene transfer and subsequent adaptation of soil microbial communities. They further suggest that selective pressures influence the rate of horizontal gene transfer and subsequently bacterial speciation since many speciation events can be traced to the gain or loss of specific set of genes. Clearly, numbers and diversity of soil microbial gene pools are directly influenced by genetic transfer mechanisms within a particular environment. Although the mechanisms for genetic exchange are well documented, the rates of gene transfer are relatively unknown in environmental samples. Direct microscopic observation often indicates spacial separation of bacteria within soils hindering free genetic exchange. Thus, in undisturbed soils genetic recombination events between similar microbes may occur relatively infrequently. It remains to be seen how selective pressure and selfish DNA impact gene transfer rates and subsequent soil microbial community adaptation.

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