

Figure 1 Different methods for analyzing microbial communities provide different levels of selectivity and functional description. These tools enable researchers to describe microbes from community capacities to individual metabolic tasks.

once again demonstrates that microbes incorporate new DNA when exposed to different nutrient sources and that these new acquisitions allow them to use multiple chemicals. *M. mobilis* displayed remarkable flexibility: for example, it used the formaldehyde-activation proteins to convert methylamine when grown on that substrate and incorporated the gene cluster *RuBisCo* in the presence of methanol. The movement and incorporation of DNA appears to be a fundamental strategy of microbial adaptation, and phage may hold the key to DNA transmission. As with all microbial metagenomes¹¹, Chistoserdova and colleagues¹ identified high coverage of bacteriophage; other studies have shown that viromes encode extensive metabolic capabilities⁷. This finding corroborates the notion that phage may act as a store of potentially useful DNA that enhances microbial growth and activity.

The new high-resolution technology described by Chistoserdova and colleagues¹ enables novel microbial functions to be identified without the need for culturing. By combining different metagenomic techniques, microbial function can now be described from the community level (by whole-community sequencing), to the individual level (by extracting active species via BrdU labeling⁹), to the level of single metabolic pathways (by

DNA-SIP) (Fig. 1)¹. These metagenomic tools will provide deeper insights into the productivity of microbial communities and the specification of individual metabolic tasks.

1. Kalyuzhnaya, M.G. *et al.* *Nat. Biotechnol.* **26**, 1029–1034 (2008).
2. Beja, O. *et al.* *Science* **289**, 1902 (2000).

3. Breitbart, M. *et al.* *PNAS* **22**, 14250 (2002).
4. Edwards, R.A. *et al.* *BMC Genomics* **7**, 57 (2006).
5. Dinsdale, E.A. *et al.* *PLoS ONE* **3**, e1584 (2008).
6. DeLong, E.F. *et al.* *Science* **311**, 496 (2006).
7. Tringe, S.G. *et al.* *Science* **308**, 544 (2005).
8. Dinsdale, E.A. *et al.* *Nature* **452**, 629 (2008).
9. Mou, X. *et al.* *Nature* **451**, 708 (2008).
10. McHardy, A.C. *et al.* *Nat. Methods* **4**, 63 (2007).
11. Sharon, I. *et al.* *ISME J.* **1**, 492 (2007).

Agrobacterium-mediated DNA transfer, and then some

Stanton B Gelvin

In addition to its plasmid DNA, *Agrobacterium tumefaciens* can transfer its chromosomal DNA to plant genomes.

The emergence of today's agricultural biotechnology industry stems largely from the discovery, ~30 years ago, that the pathogenic soil bacterium *Agrobacterium tumefaciens* can integrate so-called transfer DNA (T-DNA)

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on the tumor-inducing (Ti) plasmid into the genomes of most crops. In the late 1990s, scientists found that the transferred sequences can include regions of the Ti plasmid, or T-DNA 'binary vectors', outside the T-DNA borders¹. In this issue, Ülker *et al.*² show that the plant genome may also incorporate bacterial chromosomal DNA.

In the past decade, *Agrobacterium*-mediated transformation has been used to generate hun-

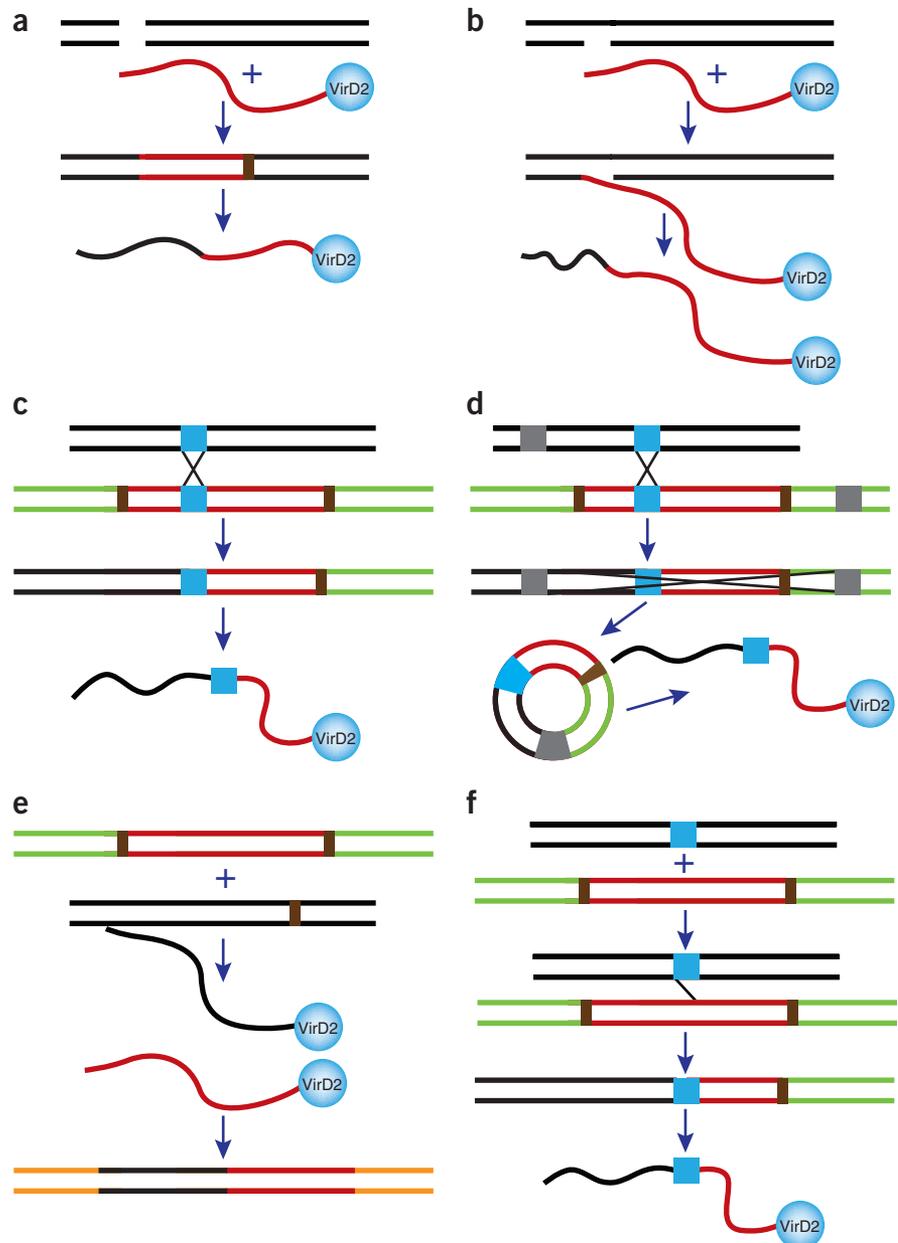
dreds of thousands of T-DNA insertion mutations in the genomes of model plant species, such as *Arabidopsis thaliana* (thale cress) and *Oryza sativa* (rice). These T-DNA insertion libraries, intended to saturate the genome with mutations, are important tools for forward and reverse genetic studies to understand plant gene function. As *Agrobacterium* can survive and reproduce in infected plants, many databases used to identify plants containing T-DNA flanking sequence tags (FST) in genes of interest are 'scrubbed' of T-DNA junctions with other T-DNAs, vector backbone sequences and *Agrobacterium* chromosomal DNA. These junction sequences are generally considered

unimportant for mutant gene identification or as experimental artifacts of the junction identification process.

Prompted by the serendipitous discovery that an *Arabidopsis* T-DNA insertion mutant contained T-DNA linked to *Agrobacterium* chromosomal DNA, Ülker *et al.*² set out to assess the frequency of T-DNA/*Agrobacterium* chromosomal (AchrDNA) junctions in primary, unfiltered data from many FST databases. They identified T-DNA/AchrDNA junctions at a frequency of ~0.4% of the sequenced junctions in each of these databases. Cloning and characterization of several of these junctions from plants revealed that

integrated *Agrobacterium* chromosomal DNA was more frequently associated with T-DNA right borders than with left borders, and could be >18 kb long. Of two ~18-kb inserts, one contained 16 complete bacterial open reading frames, and the other 18 *Agrobacterium* chromosomal genes. *A. tumefaciens* C58 (the base strain used to construct the T-DNA insertion libraries) contains both a linear and a circular chromosome^{3,4}. Sequences from both of these chromosomes associated with T-DNA integrate into the plant genome, with certain 'hot spots', such as the bacterial element IS426 and an ABC transporter gene, appearing at higher frequency.

Figure 1 Models for various mechanisms to link T-DNA to *Agrobacterium* chromosomal DNA. In each of these models, the VirD2 endonuclease nicks the T-DNA region or bacterial DNA at a border sequence and covalently attaches to the 5' end of the resulting single-stranded T-strand. **(a)** The T-strand integrates into a double-stranded break site in chromosomal DNA (perhaps generated by transposition of an IS element), is replicated to a double-stranded form and regenerates a border sequence. VirD2 subsequently nicks this new border, linking T-DNA with chromosomal DNA. **(b)** The T-strand directly links to a nick in the chromosomal DNA, generating a single-stranded T-DNA/chromosomal DNA hybrid molecule. **(c)** Homologous sequences within the T-DNA region and bacterial chromosomal DNA recombine. Subsequent nicking of the T-DNA border by VirD2 generates an Hfr-like molecule, which transfers to the plant. **(d)** Homologous sequences within the T-DNA region and bacterial chromosomal DNA recombine. Additional homologous sequences flanking the first region of homology recombine, forming an F'-like molecule. Nicking of the T-DNA border by VirD2 generates a hybrid molecule consisting of T-DNA linked to chromosomal DNA. **(e)** Two single-stranded molecules formed by VirD2 border nicking in the T-DNA region and in chromosomal DNA integrate into the same site in the plant genome. **(f)** An IS element on the chromosome inserts into the T-DNA region by replicative transposition, resulting in co-integration of the chromosome with the T-DNA region. A T-strand is generated by VirD2 border cleavage before resolution of the co-integrate. Parallel double lines, double-stranded DNA; single lines, single-stranded DNA; red lines, T-DNA region or T-strands; black lines, bacterial chromosomal DNA; light green lines, vector backbone sequences flanking the T-DNA region; orange lines, plant DNA; brown regions, T-DNA border sequence; light blue boxes, regions of homology; gray boxes, other regions of homology. The thin crossed lines in **c** and **d** (including the wide X between the two gray boxes in **d**) indicate recombination events. The thin single line in **f** indicates co-integration of the bacterial genome with the T-DNA region resulting from IS element replicative transposition.



Ancient T-DNA integration events have been found in plant genomes^{5,6}, and expression of these T-DNA sequences may have contributed to plant evolution^{7,8}. Similarly, the presence of *Agrobacterium* chromosomal DNA in plants suggests intriguing evolutionary possibilities and expands our knowledge of horizontal gene flow between species.

Whereas plasmid backbone transfer was somewhat predictable from studies on T-DNA processing⁹, the mechanism by which chromosomal DNA transfers to plants remains uncertain. The presence of insertion sequence (IS) elements in the AchrDNA in numerous T-DNA insertions in several insertion libraries led the authors to hypothesize that single-stranded T-DNA (the T-strand) processed from the T-DNA region may associate with nicks or breaks in bacterial chromosomal DNA resulting from IS element transposition. T-DNA integrated into these breaks would be 'relaunched' after reconstituting a new right border sequence (Fig. 1a). Such a border reconstruction could occur because sequences that serve as T-DNA borders can be somewhat degenerate¹⁰.

Other mechanisms for T-DNA linkage to bacterial chromosomal DNA are possible. For example, the T-strand could directly ligate to DNA at nick sites and transfer chromosomal DNA (Fig. 1b), or T-DNA could integrate into bacterial chromosomal DNA by homologous recombination, generating strains that behave like the Hfr (Fig. 1c) or F' strains of *Escherichia coli* (Fig. 1d).

Although the authors favor the T-DNA chromosomal integration and border reconstitution model, there are no characterized *Agrobacterium* enzymatic activities that would allow T-strand integration into the bacterial chromosome or direct T-strand linkage to nicks in bacterial chromosomal DNA. Moreover, it is likely that the VirD2/T-strand complex is exported to the plant as soon as it is generated, preventing access to and recombination with the chromosome. Creation of Hfr or F'-like intermediates would require homology between DNA sequences within T-DNA and sequences in the bacterial chromosome. Although duplicated sequences such as IS elements may exist among natural T-DNA regions and the chromosome, they are unlikely to exist in the numerous different 'engineered' T-DNAs used to generate the disparate T-DNA insertion libraries.

Several other mechanisms may enable linkage of T-DNA to bacterial DNA. T-strands from the T-DNA region linked to T-strands generated by nicking of border-like sequences in the chromosome may integrate into the same site in plant DNA (Fig. 1e). Linkage of two different T-DNAs has been described previously¹¹.

Alternatively, IS elements within the bacterial chromosome may link bacterial DNA with T-region DNA during a process similar to that of replicative transposition (Fig. 1f). These two latter models do not require homology between the T-region and the bacterial chromosome.

Whatever the mechanism, the introduction of bacterial chromosomal DNA into transgenic plants is not welcome news for regulators. The transfer of plasmid vector backbone sequences has raised the possibility that antibiotic resistance genes on the plasmid might be transferred to plants. T-DNA-encoded genes are transcribed from typical eukaryotic promoters. However, transcription in plants of bacterial chromosomal genes from prokaryotic promoters would be problematic.

The likelihood that approved transgenic crops might carry *Agrobacterium* chromosomal genes seems negligible. Genetically modified crops are routinely analyzed to determine the insertion site of T-DNA into plant DNA, and the presence of plasmid backbone or chromosomal DNA linked to T-DNA would be detected in these studies before field release. Moreover, as transgenic plants are routinely backcrossed many times to segregate out random mutations generated during T-DNA transfer and integration, any bacterial chromosomal DNA

unlinked to the selected T-DNA would likely be eliminated during this stage of the breeding process.

High-throughput sequencing technologies should allow the rapid identification of bacterial chromosomal DNA integrated into the plant genome and the elimination of such transgenic plants from breeding programs. In addition, mutant *Agrobacterium* strains that would mitigate T-region recombination with the bacterial chromosome could be used to generate transgenic plants, thereby reducing the probability of bacterial chromosomal DNA transfer to plants.

1. Kononov, M.E., Bassuner, B. & Gelvin, S.B. *Plant J.* **11**, 945–957 (1997).
2. Ülker, B. *et al. Nat. Biotechnol.* **26**, 1015–1017 (2008).
3. Wood, D.W. *et al. Science* **294**, 2317–2323 (2001).
4. Goodner, B. *et al. Science* **294**, 2323–2328 (2001).
5. Furner, I.J. *et al. Nature* **319**, 422–427 (1986).
6. Suzuki, K., Yamashita, I. & Tanaka, N. *Plant J.* **32**, 775–787 (2002).
7. Meyer, A.D., Ichikawa, T. & Meins, F. *Mol. Gen. Genet.* **249**, 265–273 (1995).
8. Ichikawa, T., Ozeki, Y. & Syono, K. *Mol. Gen. Genet.* **220**, 177–180 (1990).
9. Durrenberger, F., Cramer, A., Hohn, B. & Koukolikova-Nicola, Z. *Proc. Natl. Acad. Sci. USA* **86**, 9154–9158 (1989).
10. Rommens, C.M. *et al. Plant Physiol.* **139**, 1338–1349 (2005).
11. De Neve, M. *et al. Plant J.* **11**, 15–29 (1997).

Viral attenuation by design

Harriet L Robinson

A less-virulent poliovirus generated by altering codon usage suggests a new strategy for vaccine development.

Although live attenuated viruses provide the most effective vaccination strategy ever devised, current research has largely abandoned classical methods for vaccine development in favor of 'high-tech' approaches such as protein subunit and recombinant DNA vaccines. A recent study¹ in *Science* describes a method for reducing viral virulence that may eventually lead to a new class of live attenuated vaccines. Manipulation of codon usage has been exploited previously to increase the expression of select antigens in DNA vaccines²; Coleman *et al.*¹ apply it to opposite effect—to decrease gene expression in poliovirus by introducing underrepresented codon pairs (Fig. 1).

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Live attenuated vaccines have saved millions of lives and days of sickness by eliciting long-lasting antibody and T-cell responses³. Examples include the highly successful smallpox, measles, mumps, rubella, chicken pox and oral polio vaccines. Unlike inactivated, subunit or recombinant protein vaccines (such as those against diphtheria, pertussis, tetanus, hepatitis B virus and flu), which require multiple doses and regular boosting, live attenuated vaccines provided as single doses elicit long-lasting protection. Moreover, they are the most cost-effective form of vaccination. Manufacture requires only the growth of the virus. Purification is minimal because the vaccines, which amplify as they vaccinate, are effective at low doses. Live attenuated vaccines have the additional benefit of not requiring adjuvants: replicating pathogens provide the reservoirs of antigen and 'danger signals' needed to mobilize the immune response that