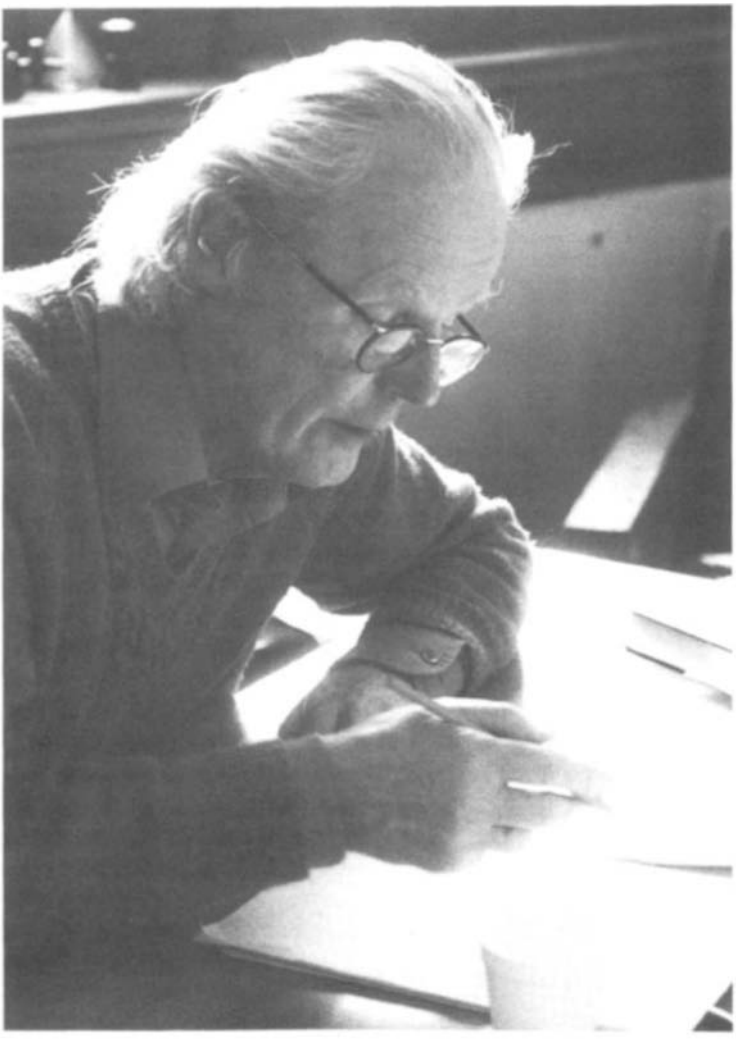

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THE EVOLUTION OF PROKARYOTES: DOES SEX MATTER?

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For many years, my lecture course on population genetics began with an account of the Hardy-Weinberg ratio. That is, I assumed that the typical evolving population could be approximated by an infinite random-mating population of sexual diploids. The effects of finite population size, demic structure, and assortative mating were treated later as departures from this ideal state, rather as a physicist might first describe an ideal gas, and then describe deviations from it due to Van der Waals forces. This clearly won't do for prokaryotes, but we are not yet clear what alternative image we should have in mind. In particular, is it sensible to see bacterial populations as composed of reproductively isolated clones? How far do processes such as conjugation, transformation, and transduction modify this picture? At the opposite extreme, is it better to take a wholly gene-centred view of bacterial evolution, and regard the bacterial cell—or, rather, the bacterial chromosome—as merely a temporary alliance of genes, analogous to a European football team, composed of players from many different countries, all liable to be transferred at any time? I think that the answers to these questions are beginning to emerge.

One of the first conclusions to emerge from a study of bacterial evolution,

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in particular the evolution of drug resistance, was that bacterial populations can adapt to sudden changes in their environment, not, as eukaryotes do, by the selection of chromosomal mutations, either newly arisen or already present in the population, but by acquiring plasmids carrying the requisite genes. Plasmids can benefit their hosts by conferring resistance to antibiotics, drugs, or heavy metals, by producing toxins, by coding for restriction enzymes, and by utilizing novel substrates: In addition many "cryptic" plasmids exist, which probably have a function, even if unknown. Given time, plasmid-born genes can be transferred across wide taxonomic boundaries: For example, a plasmid-born β-lactamase that breaks down penicillin was first observed in bacteria related to *E. coli* and *Salmonella* but is now found in the distantly related bacterium, *Neisseria,* which it reached via an intermediate host, *Haemophilus*. This is true although, as discussed below, it is still not the common mechanism of penicillin-resistance in this genus. Of course, the gene coding for β-lactamase must have arisen in the first instance by selection and mutation, but, having once arisen, it can be acquired by a wide range of host bacteria.

When it was realized that plasmid-born genes can be transferred in this way, the idea arose that chromosomal genes might also be readily transferred between taxonomically distant species, giving rise to the "football team" model of bacterial evolution.

For example, the ability to fix nitrogen is today found in bacteria that are taxonomically diverse and phylogenetically unrelated. This patchy distribution led to the suggestion (19) that the battery of genes required to fix nitrogen has been transferred horizontally between unrelated bacteria. This suggestion, plausible enough at the time, now seems less likely. An analysis (9) of the sequences of *nif* genes, and of 16S rRNA genes from the same bacteria, shows that the two data sets are compatible with the same phylogenetic tree, suggesting that the observed distribution is to be explained, not by horizontal gene transfer, but by the loss of *nif* genes in most lineages.

This discussion of the evolution of nitrogen fixation indicates how the football team model can best be tested. If it is true, then the phylogenetic tree for a set of bacteria deduced from one class of molecules will bear no similarity to the tree deduced from a different molecule, whereas if distant transfer of genes is rare, the phylogenetic trees deduced from different molecules will be similar. The matter has recently been discussed by Woese (29), who concludes that different molecules do as a general rule give similar trees—for example, rRNA and cytochrome *c* of the purple bacteria give trees with the same topology. Of course, this does not rule out the possibility of occasional distant transfer, or that the genes serving some functions are transferred more readily than others: As Woese remarks, we do not know

what fraction of functions in a bacterial cell are subject to interspecific gene transfer, or which ones they are.

How far is a tree, and the associated hierarchical classification, the appropriate image for classifying natural objects? Clearly, it is not always so—Mendeleyev's name would not be remembered if he had insisted on classifying the elements cladistically. A tree is the appropriate image only if the objects to be classified have arisen by a branching process. The oddest thing about the pattern cladists is that they wish to combine the idea, silly but not illogical, that the pattern of the living world must be fully described before questions about its evolution can be addressed, with the assumption (illogical in the absence of an evolutionary hypothesis) that the appropriate method of description is hierarchical. For the members of an asexually reproducing population, a tree is the appropriate image: If reproduction is by binary fission, the tree is dichotomously branching. For the members of a sexual population, the appropriate image (which, unhappily, cannot be drawn on a flat piece of paper) is a multidimensional net. At higher taxonomic levels, however, a tree is again the appropriate image (although dichotomous branching cannot be justified), provided that reproductively isolated species exist. This suggests a distinction between a "fractal tree," which is tree-like at all scales of magnification, down through individual reproduction and cell division to DNA replication, and a "large-scale tree," which is tree-like only at low magnification. For sexual eukaryotes, the appropriate image is a large-scale tree. Is a fractal tree appropriate for prokaryotes (at least, if we forget about their plasmids)? Surprisingly, there has been little discussion of how one might answer such a question by looking at the objects to be classified (as opposed to studying their reproduction)—but see Eigen et al (6) and Maynard Smith (11).

Before discussing the evidence, it is important to mention a third possible type of tree, which I will call a "local continuum tree." Imagine a "genus" of sexually reproducing plants (the oaks and the potentillas of North America may approximate to this image) with the following properties:

(i) Any individual can cross successfully with others that are genetically not too distant from it (if dioecious, the partners must obviously be of opposite sex), but cannot cross with genetically more distant members of the group.

(ii) There are few discontinuities, so that, even if A cannot mate with E, A can mate with B, B with C, C with D, and D with E. Hence, gene flow can occur throughout the whole taxon. However, such local continuity could be combined with occasional discontinuities, arising for accidental historical reasons, so that the living world would be divided into a number of large taxa

between which gene flow could not occur but within which gene flow is possible via a series of closely related intermediates.

Why is the world not like this? It is, after all, what one would expect if the only barrier to hybridization were developmental breakdown when the two parental sets of genetic information were too disparate. Bateson (1) argued that "species" represent the different possible stable states of living matter—a view that has been resurrected recently by proponents of "laws of form." I can see little to recommend the idea. Dobzhansky sometimes wrote as if there existed a finite number of discrete ecological niches, so that "species" would represent adaptations to preexisting environmental discontinuities. I can see even less to recommend this. A third possibility lies in an intrinsic disadvantage of rarity in morphospace. This is the explanation favored in the only recent discussion of the issue known to me (2). However, my reason for raising the issue here is not so much to discuss why eukaryotes, typically, do not form a locally continuous tree, but to raise the possibility that prokaryotes in fact do so.

There is recent evidence, due largely to Selander and his colleagues (22), that the structure of bacterial populations is clonal. This comes mainly from protein electrophoreseis. For example (23), *E. coli* is highly polymorphic: 94% of loci are polymorphic, compared to 33% in humans, and mean genetic diversity per locus is 0.34–0.54, compared to 0.063 in humans. Hence, considering only this electrophoretic variability, an immense number of different genotypes could be distinguished. In practice only a small fraction of these are found, and some "electrophoretic types" (ET's) have been found repeatedly, and in different continents. This implies that recombination, resulting in crossing over between gene loci, is a relatively rare event. It does not, of course, prove that the members of a single ET are genetically identical at all loci.

This clonal pattern of variation seems to be general in prokaryotes. It is found also in bacteria that, unlike *E. coli,* habitually undergo transformation in nature—for example, *Haemophilus* (17) and *Neisseria* (13). However, as we shall see in a moment, there is equally strong evidence that recombinational events do occur in bacteria. This has led Milkman & Stoltzfus (15) to propose a modified clonal model of population structure. They suggest that a favorable chromosomal mutation will occasionally occur and will spread throughout the "species." If there were no recombination, all bacteria with the new mutation would be genetically identical (except for new mutation). But since there is some recombination, the new mutation will be associated with a "segmental clone," that is, with a chromosomal region that has not yet been broken up by recombination. The older the favorable mutation, the shorter the segmental clone, and the greater the variation that will have arisen within it by

subsequent (neutral) mutation. In effect, this is an application to prokaryotes of the idea (12) that, in sexual species, each favorable mutation will create a window of genetic homozygosity by hitchhiking.

The idea of a segmental clone is attractive, but I have one reservation. This concerns the nature of recombination in prokaryotes. The model assumes that recombination has consequences similar to those of genetic crossing over in eukaryotes: that is, that it causes the replacement of one large block of genes by another. This may not be so. The evidence suggests that the important events may be much more local: A small block of DNA, of a few hundred or thousand bases, from one individual is inserted into the chromosome of another. "Local" and "global" recombination have quite different consequences for population structure. If all recombination is global, we would expect different strains of, for example, *E. coli* to have the structure of a web or net, but we would expect the genes at a single locus, if sequenced from the same set of strains, to be tree-like. In contrast, if all recombination is local, we would expect the strains to reveal a tree-like structure, but the pattern of individual genes to be net-like. Of course, the truth may lie between these extremes.

In reviewing the evidence, it is appropriate to start with *Streptococcus pneumoniae*. It was in this bug, then called *Pneumococcus,* that Griffith (7) discovered bacterial transformation and in so doing set in train the process that led to the discovery of the structure of DNA and to molecular biology. It is only recently, however, that the evolutionary significance of transformation has begun to emerge. Most bacteria evolve resistance to penicillin by acquiring a plasmid coding for β-lactamase. *Pneumococcus* has evolved resistance in a different way. Penicillin kills bacteria by binding to several high molecular weight proteins that are needed in cell wall synthesis. These proteins are misleadingly called "penicillin-binding proteins", or PBP's: Their function, of course, is not to bind to penicillin. Part of one of these proteins, PBP2B, has been sequenced from 6 sensitive and 14 resistant strains of *Pneumococcus*. The results (3) are summarized in Figure 1.

The interpretation of these results is as follows. Sensitive *Pneumococcus* are very uniform. The five "class 2" resistant strains are genetically identical to one another. They differ from the sensitive strains by the introduction, presumably by transformation, of a block of DNA, including the initial nucleotides of the sequenced region, and an unknown number of nucleotides prior to this, differing from *Pneumococcus* by 7.5% sequence divergence. The fact that the five strains, recovered from Britain and Spain over a five-year period, are identical implies that the clone, once it arose, spread without further recombination, at least in this region. The nine "class 1" strains are more complex. They are characterized by six successive amino acid substitutions: It is known from site-directed mutagenesis that these

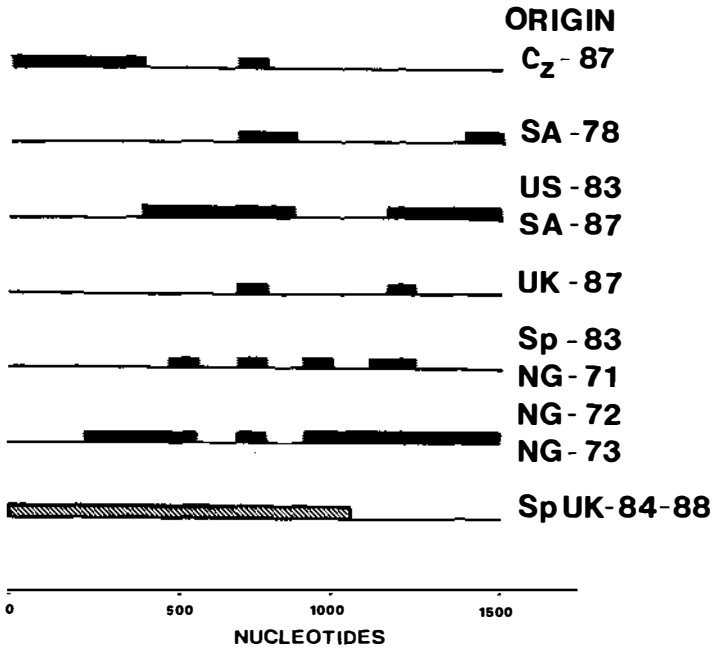

Figure 1 Mosaic structure of PBP2B gene in penicillin-resistant strains of *Streptococcus pneumoniae*. —— , regions similar to the sensitive strains (<5% sequence divergence); ▬ , regions differing from the sensitive strains at 14% of sites (where these regions overlap in different strains, they differ from one another at less than 4% of sites); ▨ region differing from the sensitive strains at 7.5% of sites, but not resembling the other resistant strains in sequence. Places of origin: Cz, Czechoslovakia; SA, South Africa; US, United States; Sp, Spain; UK, Britain; NG, Papua New Guinea.

changes alone are sufficient to confer a high degree of resistance. However, different strains have additional blocks of introduced DNA, differing from *Pneumococcus* by 14% sequence divergence. The most likely explanation is that a block of DNA, covering the whole sequenced region, was originally introduced, by transformation, from an unknown source. This may have happened in the 1950s in Papua New Guinea, when penicillin was used extensively to treat various respiratory diseases. The varying block structures of different strains have arisen in subsequent transformation events, as resistance has spread by selection. We cannot, however, rule out the possibility that the different block structures represent different original transformation events, from the same donor species.

The species that donated the resistance gene to *S. pneumoniae* is as yet unidentified. This difficulty has been overcome in the case of penicillin resistance in *Neisseria* (25, 26). There are two pathogenic species, *N. meningitidis* and *N. gonorrhoea*, whose names are based on their extended

phenotypes. The PBP2B gene has been sequenced from sensitive and resistant strains of these two species; from a naturally resistant commensal (i.e. harmless) species, *N. flavescens;* from sensitive and resistant strains of a second commensal species, *N. lactamica;* and from a resistant strain of a fifth species, *N. polysaccharae*. The results are summarized in Figure 2 and Table 1.

The following conclusions can be drawn:

(i) The sensitive strains of the two pathogenic species are very similar (1–2% divergence), although our symptoms, if humans are infected by them, are admittedly very different.

(ii) Both pathogens have evolved resistance by acquiring blocks of DNA from the naturally resistant species, *N. flavescens*. The ends of the blocks, indicating crossover points, are in some cases sufficiently similar to suggest a common origin of different resistant strains.

(iii) A second commensal species, *N. lactamica,* has acquired resistance by the introduction of DNA from a resistant strain of *N. meningitidis,* consisting in part of *N. flavescens* DNA and in part of *N. meningitidis* DNA.

(iv) Perhaps surprisingly, the sensitive *N. lactamica* strain also reveals a mosaic structure. The region between sites 728 and 1260 differs from *N. meningitidis* by 12% sequence divergence, but the next 720 nucleotide sites differ by only 2.9%.

(v) There is evidence (for example, in *N. meningitidis* strain 5) of introduced DNA from an as yet unidentified species.

(vi) One resistant strain of *N. gonorrhoea* differs from the sensitives only by the insertion of a single additional codon, which by itself is sufficient to confer resistance. It is not clear whether this was an independent mutation, or whether it represents the introduction of a short block of DNA (perhaps of the order of 20 nucleotides) from the anonymous donor species, which also has an additional codon at the same position: The amino acid is the same, but the codon is different.

The details are confusing, but it is clear that blocks of DNA have been exchanged between at least six "species" (one as yet unidentified), differing by up to 20% sequence divergence. However, the frequency of exchange has not been so high as completely to randomize the sequences, which would have destroyed all evidence of the recombinational events that have occurred. It is also likely that the frequency with which new gene sequences, arising by recombination, have been established in natural populations has been increased by the strong selection pressure imposed by penicillin. The locus is not unique, however. Similar local recombination events are known to have affected the evolution of two other genes in *Neisseria*—the *iga* gene (8), coding for an extracellular enzyme that cleaves human *IgA* protein, and the

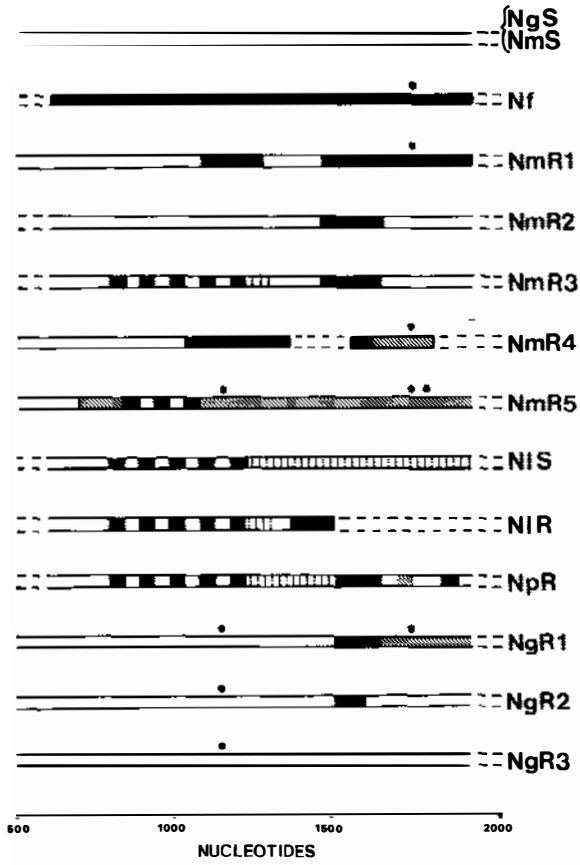

Figure 2 Mosaic structure of PBP2B genes in *Neisseria*. NgS, NmS, penicillin-sensitive *N. gonorrhoeae* and *N. meningitidis;* Nf, *N. flavescens* (naturally resistant); NmR1–NmR5, resistant strains of *N. meningitidis;* N1S, N1R, sensitive and resistant strains of *N. lactamica;* NpR, resistant strain of *N. polysaccharae;* NgR1-NgR3, resistant strains of *N. gonorrhoeae*.

, DNA similar to sensitive *N. meningitidis*

, DNA similar to *N. flavescens*

, DNA similar to *N. lactamica,* and differing from N. meningitidis by 12% sequence divergence

, DNA similar to *N. lactamica,* and differing from N. meningitidis by 3% sequence divergence

, DNA of unknown origin

• , single additional codon

Table 1 Mean sequence divergence (percent nucleotide sites) for the PBP2B gene in *Neisseria*

	N.m.	*N.f.*	*N.l.*	Anon
N. meningitidis	—			
N. flavescens	21.9			
N. lactamica	12.0	20.9	—	
Anonymous	13.6	13.8	12.9	—

N.gonorrhoeae differs by 2% from *N.meningitidis*. The values for *N.lactamica* are for nucleotides 728–1260, and for "Anonymous" are for nucleotides 1259–1947 in *N. meningitidis* resistant strain 5. (unpublished data from Dr. Brian Spratt).

pilE gene (21), coding for the protein of the highly antigenic pili. Both these genes, too, are likely to be under strong and fluctuating selection.

It is natural to ask whether these observations on *Pneumococcus* and *Neisseria* are peculiar, either because the genes concerned have been under particularly strong selection, or because these genera have evolved a special capacity to undergo genetic transformation. Levin (10) has argued that transformation is an evolved trait, enabling a competent cell to take up single-stranded DNA from the medium, and, provided sufficient sequence homology exists, to incorporate it by homologous recombination. It is not clear whether this function has evolved because it makes possible the repair of double-stranded DNA damage (14, 30), or because it facilitates evolutionary novelty, as it certainly has done in the examples described above. As always, the mere fact that an "organ" (the machinery needed for transformation) has some effect does not by itself establish the cause of its evolution.

E. coli is not a bacterium competent for transformation, and there is no reason to think that the genes that have been sequenced from several strains are under specially strong selection. There are three such loci: *gnd* (5), *trp* (15), and *phoA* (4). In every case, there is evidence of a mosaic gene structure, although it is not so obvious as in the cases of *Pneumococcus* and *Neisseria*. In the latter two examples, the mosaic structure is striking, and it is easy to invent statistical tests to demonstrate its significance, although there is always an element of doubt about the exact positions of the crossover points. The *E. coli* data call for a more sophisticated analysis (20, 27). The mere fact that the nucleotide differences between two strains, A and B, are concentrated in particular regions of the gene does not demonstrate recombination: It is to be expected if mutations in particular regions of the gene are more frequent, or more likely to be selectively neutral (admittedly, it is not easy to see why silent substitutions are more likely to be neutral in one region of a gene than another). Suppose, however, that we have sequenced several strains, A,B,C.

. . . We can then list all polymorphic sites and ask of a particular pair of strains, say A and B, whether the polymorphic sites at which they differ occur in runs. Essentially, this is the approach adopted by Sawyer (20). In the case of the *phoA* gene, the mosaic structure is more obvious, and Du Bose et al (4) reconstruct the history of their eight strains, suggesting a "tree" with four crossovers. In addition to the three genes mentioned above, there is evidence of recombinational events at the *pap* gene cluster (18), and for a 3500 bp region close to the *trp* locus sequenced by Stoltzfus et al (28).

It seems, then, that local recombination is also occurring in *E. coli,* probably mediated by conjugation plasmids, or by temperate phage vectors. If most recombination events are local, they would not destroy the clonal structure revealed by electrophoresis. However, a hard question remains unanswered. It is clear that a "fractal tree" is not an appropriate image: but is the appropriate picture a "large-scale tree" or a "local continuum tree"? In other words, is there something corresponding to the "species" among eukaryotes? In sexual organisms, the species has two aspects. First, a species is a population whose members can exchange genes with one another: Second, the members of a species cannot exchange genes with members of other species. In bacteria, it seems that the evolving unit, between whose members genetic exchange is possible, is somewhat wider than the named "species," such as *S. pneumoniae* or *N. meningitidis.* Admittedly, my microbiological colleagues feel that the named entities do correspond to real groupings, but I suspect that this may mean only that they would know whether they had meningitis or the clap. To an evolutionist, the relevant entity seems to be closer to the genus *Neisseria,* or to *Escherichia* plus *Shigella.*

There remains the question whether there is anything corresponding to the isolating mechanisms found in sexual eukaryotes. The necessity for homologous recombination imposes an upper limit on the genetic distance over which at least some kinds of exchange can occur. If this were the only limit on exchange, the appropriate image would be a local continuum tree. What other isolating mechanisms are possible? Geographical isolation is not a candidate: A peculiarity of bacterial species is that their distributions are world-wide. In the case of symbiotic species, host specificity is a possibility. This is the only context in which Dobzhansky's idea of preexisting ecological niches makes some sense. However, both *Streptococcus* and *Escherichia* strains seem to cross host-species boundaries rather easily. One process that could in principle give rise to discontinuities is the action of restriction endonucleases, which can exclude foreign DNA. However, even with nucleases that recognize four-base sequences, the typical fragment length after cleavage will be 250 bp, which is long enough to explain most of the mosaic structure observed.

The evidence is at present contradictory—or I find it so. One study that suggests discontinuity is the analysis (24) of the differences between the genes

of *E. coli* K12 and *Salmonella*. Some 60 genes have been sequenced in both species. Some loci show, in both species, a high bias in codon usage. These are "highly expressed genes"—that is, genes producing a lot of mRNA and protein. A plausible explanation is that, for such genes, it is selectively advantageous to use codons for which the corresponding tRNA is present in large amount. Other, less highly expressed, genes show less bias in codon usage. It turns out that, comparing the two genera, genes with a high codon bias are more similar (and, presumably, have evolved less rapidly) than genes with a low codon bias. This is precisely the result one would expect if gene substitution occurs only, or mainly, at those sites at which selection is weak. However, the result also implies that there has been little gene exchange between the two genera for a considerable time—either directly, or via intermediates as might occur on the continuum hypothesis.

Snags arise when one looks in detail at one particular locus, *gnd,* that has been sequenced from nine *E. coli* strains and from *Salmonella* (5). If one was shown these ten sequences and asked which sequence belongs to a reproductively isolated group, I do not think one could answer. There is a group of seven genes (including *E. coli* K12) that are rather similar to one another (4–6% divergence), so clearly none of these can be the odd man out. But if we compare K12, the two remaining *E. coli* strains, and *Salmonella,* all six differences are in the range 14–18%, and no strain is characterized by an unusually high number of unique nucleotides (in fact, *Salmonella* does not have the largest number of unique bases). These facts argue against discontinuity between *E. coli* and *Salmonella* (a different conclusion would be reached if the *trp* locus was examined; for these sequences *Salmonella* is an obvious outlier).

It seems that it is too early to say whether a "large-scale tree" or a "local continuum tree" is the more appropriate image of bacterial evolution, or, equivalently, whether anything corresponding to a reproductive isolating mechanism exists among bacteria. What is clear is that evolution is strongly influenced by local recombination between closely related cells: a "fractal tree" would not be an appropriate image.

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