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Santa Rosalia revisited: Why are there so many species of bacteria?

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Abstract

The diversity of bacteria in the world is very poorly known. Usually less than one percent of the bacteria from natural communities can be grown in the laboratory. This has caused us to underestimate bacterial diversity and biased our view of bacterial communities. The tools are now available to estimate the number of bacterial species in a community and to estimate the difference between communities. Using what data are available, I have estimated that thirty grams of forest soil contains over half a million species. The species difference between related communities suggests that the number of species of bacteria may be more than a thousand million. I suppose that the explanation for such a large number of bacterial species is simply that speciation in bacteria is easy and extinction difficult, giving a rate of speciation higher than the rate of extinction, leading to an ever increasing number of species over time. The idea that speciation is easy is justified from the results of recent experimental work in bacterial evolution.

Introduction

One can imagine the American ecologist, G. Evelyn Hutchinson, sitting next to a pool just outside the church of Santa Rosalia on a hill overlooking the city of Palermo. In this pool teem vast numbers of two different species of water-bugs of the genus *Corixa*. One can imagine him asking the questions: Why are there just two species in this pool? Why not a single species? Why are there such an enormous number of animal species? Why are a majority of these insects?

In his presidential address to the American Society of Naturalists on December 30, 1958, Hutchinson tried to answer some of these questions in terms of food chains and niche requirements. One of his conclusions is that there will be many more species of small organisms than of large ones. Given that bacteria are much smaller than insects, how many million species of bacteria might there be? Surely many more than the three to four thousand well described species. In this paper I would like to provide an estimate of the number of species of bacteria there might be in the world and provide reasons why there are so many.

Estimation of number of bacterial species in the world

Species definitions

I will use a definition of species which in this context is both useful and conservative. The formal definition is as follows: 'The phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5 °C or less Δt_m. Both units must be considered.' (Wayne et al 1987). Thus two strains are different species if less than 70% of their DNA will reassociate and the decrease in melting temperature of the DNA that does reassociate is more than 5 °C ($\Delta t_m > 5$ °C). This definition is conservative because species defined this way are likely to be separate species by any criteria, while groups that are different species by other criteria can be defined as within the same species by this criterion.

For example, *Neisseria gonorrhoeae* and *N. meningitidis* are considered separate species even though the percentage of DNA that reannealed was 93% (Hoke and Vedros, 1982), well above the 70% criterion used to distinguish species. By an ecological criterion, i.e.

niche differentiation, these are separate species – one lives in the urogenital tract of man and the other lives in the upper respiratory tract. By a genetic criterion, they are separate species – a dendogram of the *glnA* gene sequences from eleven *N. gonorrhoeae* strains and eight *N. meningitidis* strains separates the strains from the two species onto two different branches (Spratt et al., 1995). These species are distinct by both an ecological criterion, they occupy different niches, and a genetic criteria, there is little or no genetic exchange between them, but are considered part of the same species by the criterion of DNA hybridization. By DNA hybridization, not only would *N. gonorrhoeae* and *N. meningitidis* be considered a single species, but *N. lactamica* and *N. polysaccharea* would also be included in this species (Hoke and Vedros 1982, Maynard Smith 1995).

It is clear that species as defined by DNA hybridization are certainly separate species given other definitions of species. A decrease of the melt temperature of 5 °C implies that the DNA has a sequence difference of more than 7–8% (Caccone et al., 1988). This amount of divergence indicates that genetic transfer between the species is rare to non-existent. The more divergent two strains are, the larger the effect any recombination has on homogenizing them (Cohan 1994, 1995). Also, the rate of recombination between homologous DNA decreases as the divergence increases (Shen and Huang 1986) such that as the divergence increases, any residual recombination will become less and less likely.

This sequence difference can be translated into a time difference. Ochman and Wilson (1988) have estimated that synonymous sites change at a rate of 1%/million years and nonsynonymous sites about 20 fold slower. Since most of the homologous DNA in bacterial genomes is coding sequence, one can estimate that the rate of change is about 1%/2.7 million years. This implies that two lineages that have a ΔT_m of 5 °C will have been separated for 20 million years. If these two species occupy the same niche and consequently not different species by the ecological criterion, one would have eliminated the other over a period of 20 million years by competative exclusion. Thus we do not expect two species to be different by DNA homology, but not to be different species by an ecological or genetic criteria. Consequently, defining species in terms of DNA homology is conservative, i.e. it will underestimate the number of species.

Defining species in terms of DNA homology can be used to estimate the number of species within an ecosystem without characterizing isolated strains. Only about 0.1% to 1% of the cells from most natural ecosystems can be grown in the laboratory and these represent a biased sample of the species in the ecosystem. Thus, any estimation of the number of species of bacteria in any ecosystem must be indirect.

Estimation of number of species in a community

Reassociation kinetics can be used to estimate species diversity (Torsvik et al., 1990a). The rate of the reassociation of single stranded DNA with its homologue depends upon the number of different types of DNA in a solution or on the complexity of the DNA. The more complex the DNA, the longer it will take homologous strands to pair. The reassociation kinetics are measured in terms of the concentration of DNA in moles per liter (C_0) times the time in seconds (t). This is the Cot value. If the concentration of DNA is held constant, then the number of molecules of each unique sequence in the genome decreases as genome size increases. For example, if the concentration of DNA is 12 pg, a solution will contain 4000 copies of a genome of 0.003 pg but only 4 copies of a genome of 3 pg. In this example, it will take on average 1000 times longer for the DNA in the large genome to find its homologue, since there are many fewer copies of the larger genome. The Cot value when half the DNA is associated gives an estimate of the genome size (Figure 1). If we think of the bacteria in a natural community as a single species of bacteria, how large would its 'genome' be? The number of species in the community can be estimated by dividing this 'genome' by the average size of a bacterial genome.

Torsvik, Goksøyr and Daae (1990a) isolated 30 grams of top soil from a beech forest north of Bergen, Norway. This soil contained 1.5×10^{10} bacteria/gm of dry soil by microscope observation, but the colony count was only 4.3×10^7. As usual, the colony count was less than 1% of the observable cells. The bacterial fraction was isolated from the soil and DNA extracted from the bacteria. The DNA was sheared, single stranded DNA removed, and the DNA melted. The reassociation was done at 25 °C less than T_m, where T_m is temperature at which half of the native DNA melted. As seen in Figure 1, the reassociation does not follow the same kinetics as *E. coli* or calf thymus. These two curves in Figure 1 are the same; one is just moved to the right of the other. The difference between the reassociation of the DNA from the soil bacteria and from the calf thymus shows that the various species in

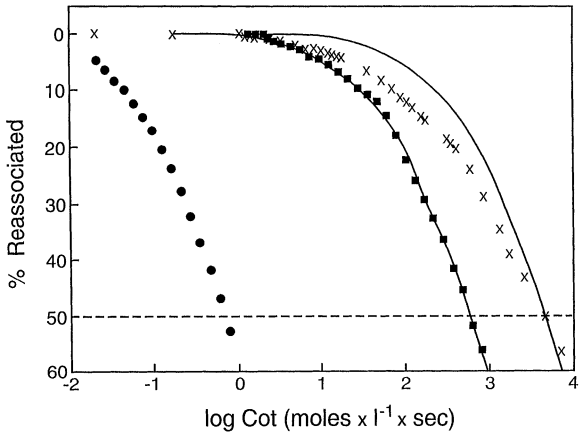

Figure 1. The reassociation of soil bacterium (X), Calf thymus ($\int$) and *E. coli* B ($\bigcirc$) DNA. A curve was drawn through the calf thymus DNA and then shifted to the right such that the curve passes through the soil bacterium when 50% is reassociated. This figure is derived from Figure 3 of Torsvik et al., (1990a).

the soil are at different frequencies. The reassociation of the bacterial DNA is about ten times slower than calf thymus DNA where the two curves cross the dotted line in Figure 1. The estimated heterogeneity of the bacterial DNA at this point is 2.7×10^{10} bp (Torsvik et al., 1990a). If the average size of the genome of soil bacteria is 6.8×10^6 bp (Torsvik et al., 1990b), then there are about 4,000 completely different bacterial genomes in the sample. Figure 1 shows that the first hybridization is taking place at about the same time as the calf thymus. The complexity of calf thymus is equivalent to about 500 bacterial genomes. If this first reassociation is due to the most common species in the soil, then less than one percent of the individuals or 3×10^7 bacteria/gram of soil belong to this species. This is a very different picture of diversity than the one obtained from 16s RNA cloning and sequencing. Usually at least one species is isolated more than once within a sample of 100 or less.

However, the definition of species does not require that the genomes be completely independent and non-hybridizing – it requires that only 30% of the DNA does not hybridize. The melt curve of the DNA that did hybridize shows that much of hybridizing DNA is from separate species. The ΔT_m is about 18 °C (Torsvik et al., 1990a). The curve suggests that all but about 10% of the reassociated DNA melted by the time the temperature had risen to 5 °C less than the T_m of the native DNA. Thus 90% of the reassociating DNA belongs to different species and we have underestimated the number of species by nine fold. This implies that the number of species is 36,000.

Fortunately, there is another way to estimate this correction. Two hundred and six strains were isolated from the same soil (Torsvik et al., 1990a). While these were not checked by the species definition above, the phenotypic diversity of the population was high. The rehybridization of these strains when they were in equal frequency gave an estimate of 20.6 separate species. If we assume that these are all different species, then the reassociation estimate is a ten fold underestimate, which is close to the nine fold estimate as determined above. I feel the assumption to treat these 206 strains as separate species is valid, even though many of the strains are phenotypically identical by the tests used (Torsvik et al., 1990b). Jimenez (1990) has shown that strains from subsurface soil that are phenotypically the same by the tests used are often different species by percent DNA hybridization and G+C content. Thus we will estimate 40,000 species in the sample. Clearly, this conversion factor will have to be estimated with more precision if this method is to be used regularly.

In natural ecosystems, species are not equally frequent. Some are common with many individuals present and other are rare with only a few individuals present. The reassociation kinetics of the DNA from soil bacteria do not follow the typical second order reaction kinetics. Rather the reaction rate slows faster than expected as the reassociated progresses. This is exactly the pattern expected if the species are not equally frequent. The estimate of the number of species is made from the 50% of the individuals belonging to the most common species, with the other 50% of the individuals belonging to rare species. Thus 40,000 would be a considerable underestimate of the number of species present. Animal and plant ecologist have enumerated the number of each species in a sample of individuals from a community. If we divide the sample into equal numbers of individuals with the individuals from common species in one part and the individuals from rare species in the other, how many rare species are there for every common species? Table 1 shows this value for various studies. This value is not constant but covers a wide range. With the high values, one finds one or two species that are excessively common. About half of the individuals belong to these very common species. With the low values one finds fewer rare species than expected. Presumably this is because species of large animals with few individuals are likely to go extinct. With the soil bacteria considered here, we clearly do not have an excessively

Table 1. The ratio of rare to common species within diverse communities

Taxonomic group	No. of species	No. of individuals	Ratio (rare/common)	Reference
Birds	50	365	6.8	Preston 1948
Birds	80	14,353	12	Saunders, 1936
Insects and mites	52	822	25	Pielou and Matthewman 1966
Moths	240	15,609	30	Fisher et al 1943
Moths	277	87,110	150	Preston 1948

common species (shown above). On the other hand, as explained below, we would not expect high extinction rates for bacteria even when rare. Thus we will take 25 as the factor we will use since it is the modal estimate. Clearly, this correction could be much too large if the number of individuals per species are more evenly distributed than with animals. Or, this correction could be much too small if there are many rare species within each community. This correction factor is unsatisfactory because one is dividing a large number (the number of rare species) by a small number (the number of common species). I checked this ratio using data from Patrick (1968). In 1965 she set up eight semi-natural populations of diatoms and in 1966 an additional four populations. In 1966, the ratios ranged from 15 to 25 with an average of 19. Thus the ratio is fairly constant for the same group of organisms in the same ecosystem. However, in 1967, the ratios ranged from 66 to 76 with an average of 69. The difference between the years is that in 1966 there was one common species and in 1967 there were two common species. Clearly, this correction factor is unsatisfactory, and a more robust one will have to be devised.

But if we accept for the time being a correction factor of 25, we estimate that there are about 20,000 common species and 500,000 rare species in a small quantity of soil or about a half million species. I contend that even this is an underestimate. Animal and plant ecologists deal with samples of hundreds or thousands of individuals, not numbers in the range of 10,000,000,000. The larger the population, the larger the chance of rare species being present, the more rare species, and the larger the number of rare species for each common species.

Estimation of the number of bacteria communities

Bacteria are found everywhere. There are ten times more individual bacteria on you than there are cells in your body. There are communities of bacteria at 600 meters down into the earth. There are bacteria found in the rocks in Antarctica where they are frozen except for less than one day a year. There are bacteria that live in hot springs. Bacteria are everywhere, perhaps even on Mars. How many different communities might there be?

The measurement of differences between communities is much easier than attempting to measure the number of species within a community. For example, using rRNA sequence diversity, one can recover similar types from the same or similar habitats, thereby defining different ecosystems (Stahl 1995). For example, the two types of *Fibrobacter succinogenes* isolated from the intestine of a horse were clearly different from the three types isolated from the rumen of a cow (Lin and Stahl, 1995). While these five types are considered subspecies because of no clear physiological differentiation, genetically they are different enough to be classified as separate species, perhaps separate genera (Lin and Stahl, 1995).

DNA-DNA hybridization has been used to define different bacterial communities. This method measures both species composition and relative diversity. The DNA from one community is bound to a filter and hybridized with radioactivity labeled DNA from the same and different communities. The similarity index is then the comparison of the amount of bound labeled DNA from the different community normalized by the bound labeled DNA from the same community. The reciprocal cross-hybridization (DNA from community one as a probe hybridizing to DNA from community two compared to DNA from community two as a probe hybridizing to DNA from community one) will give the same results only when the two communities are equally diverse. Otherwise, the more diverse community used as probe will give a higher similarity value.

Griffiths et al. (1996) tested four local soils. The order of the diversity in the communities from most to least diverse was sandy loam, sandy clay loam, loamy sand, and clay loam. There was considerable similarity

or shared species between clay loam and sandy clay loam, between sandy clay loam and sandy loam and between sandy loam and loamy sand. But, there was little three way overlap. For example, those species in sandy clay loam that are also in clay loam are not found in sandy loam. These data suggest that the four soil types represented at least two or three different non-overlapping communities.

Lee and Fuhrman (1990) showed that bacteria isolated from a coral reef lagoon, from Long Island Sound, from the Caribbean Sea, and from the Sargasso Sea are all different communities. There was overlap only between the communities from the Caribbean and Sargasso Sea.

Another approach is to isolate a group of bacteria from diverse locations and then to compare the divergence of these strains using dot blot hybridization. Holm et al., (1996) isolated 1,199 pure cultures of *Hyphomicrobium* from a sewage treatment plant and its adjacent receiving lake. These strains were divided into six morphologically different types. Of the 755 strains which could be used for dot hybridization (the 436 isolates from morphological type 6 grew too slowly to be used), 671 isolates were assigned to 30 hybridization groups. The dot blot hybridization were done at a temperature 10 °C lower than the melting temperature of the DNA. Thus, strains which belong to different hybridization groups belong to different species by the DNA homology definition of species. Only one of the hybridization corresponded to one of the 14 known species tested. Of the 30 groups, 12 were found in the sewage plant, 14 in the lake and only 4 groups found in both. Two of these four groups were found mostly in the sewage plant with only a single isolate from the lake. These single isolates probably represent transients from the effluent from the sewage plant. Thus the overlap between these communities represent only two out of thirty species.

We can conclude there are many different communities of bacteria. Clearly, much more work will have to be done before we know how many. Are the bacterial communities in the soil of a beech forest different from the communities in an oak forest or a maple forest? How closely related are the bacterial communities as one descends into the subsurface soil? How closely related are communities in different types of desert soils? How closely related are the communities in beech forests in Norway and Michigan? Then we have all the communities in fresh water and in the ocean. We have communities on every living person. How close are the communities on a person and a robin? Clearly, I do not have an answer to these questions, but I will take a guess that there are more than a 2,000 communities that are different from each other and as complex as the Norwegian forest soil. This would imply there are more than a thousand million species of bacteria and this is probably a considerable underestimate. I would guess that the number of bacterial species might be as high as a trillion given all the underestimations that I have done.

Why are there so many species of bacteria?

One simple answer is that new species of bacteria form faster than old ones disappear by extinction. Even in metazoans, the rate of speciation normally appears to be faster than the rate of extinction, since, between periods of mass extinction, there seems to be a gradual accumulation of metazoan diversity (Sepkoski 1984).

Low extinction rates

I would expect the extinction rates of bacteria would be lower than metazoans. Bacteria don't starve to death as do you or I. Bacteria don't die of old age as do you or I. Bacteria don't need sex to reproduce as do you or I. Bacteria are much smaller than you or I and so there are a lot more of them than you or I. When conditions are bad, bacteria can sit and do nothing as they do in stab tubes. The range of environments that bacteria can live in far exceeds the range of environments metazoans can live in. Bacteria are able to stand harsher conditions. All this might lead to a lower extinction rate over geological time. If bacteria avoid mass extinctions, they could have undergone continual expansion in the number of species for 3 billion years. However, the large number of species is likely to be due to an unusually high speciation rate rather than an unusually low extinction rate.

High Speciation Rates

Laboratory studies in experimental evolution have provided evidence that speciation is easy and likely in bacteria. I am changing the definition of species and speciation away from a DNA based definition to an ecological definition. Two strains are different species if they occupy different niches and the same species if they occupy the same niche. This definition implies that different populations of the same species can easily replace each other, but that populations of differ-

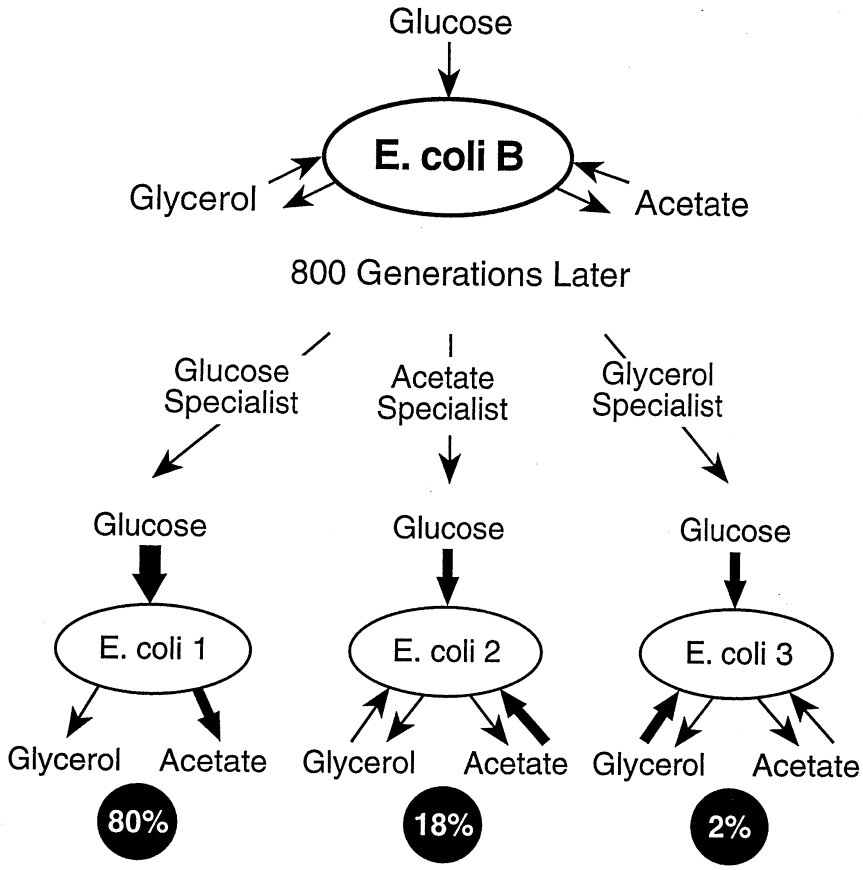

Figure 2. Evolution of one species into three. The width of the uptake and excretion arrows shows the evolutionary changes in the derived species. The dark circles at the bottom give the approximate proportion of each type in the mixed culture. This figure is derived from the data of Helling et al. (1987) and Rosenzweig et al. (1994).

ent species can not: each species has an advantage on certain parts of the resource base and consequently remains extant for some extended period of time. If this time is long enough, the species become genetically distinct enough that one can apply a DNA based rule to define species.

It is often assumed that species multiply to fill available niches, so that there can be no more species than there are available niches. By this assumption, niches are given in the environment and then species fill them. However, niches are not out there in the environment waiting to be filled. Originally an environment is undivided, but as species develop, they divide the environment into separate niches. Species create niches. Different niches have different amounts of resource and are differentially stable. If a new niche occupied by an incipient species is too small or too unstable, the species will go extinct and the niche will disappear. Its resources will be incorporated into other niches occupied by other species. This will be clarified by the discussion of the experiments discussed below.

Three niches from one; three species from one

Helling, Vargas and Adams (1987) did a simple experiment. They grew a single strain of *Escherichia coli* in a glucose-limited chemostat for a long time. The chemostat provides a homogeneous constant environment with only one nutrient limiting. The cells are competing only for glucose, and all other nutrients are in excess. The cultures transferred periodically into fresh chemostats to make sure that the glass walls of the chemostat did not provide another niche. Thus they grew a single strain in a zero dimensional niche, the least complicated environment anyone could imagine. A zero dimensional niche can be thought of as a point

in environmental space. In this niche there is no environmental variation, such as changing temperature, or environmental gradient as a pH gradient which can be divided to form multiple niches.

They planned to investigate how an isolated lineage would evolve over time, but the experiment did not work as planned. Instead of one strain evolving over time, the one strain evolved into at least three strains which coexist. The one niche was partitioned into three niches. As shown in Figure 2, just adding the bacteria complicated the environment. *E. coli*, while metabolizing glucose, excretes acetate and a little glycerol. These compounds are later taken up again and used. Over the eight hundred generations in the chemostat, a strain evolved which was a specialist on glucose. This strain excreted a lot of acetate and some glycerol, but could no longer take up and metabolize either of these compounds. Two other strains arose each of which specialized on these compounds while retaining the ability to utilize any glucose they could acquire (Rosenzweig et al., 1994). Thus the bacteria have created three niches where before only one existed and the three coexisting strains, none of which can become extinct within this system, can evolve into separate species over time. One might even consider them separate species already by the ecological definition of species.

Environmental partitioning

Bennett, Lenski and Mittler (1992) have shown that bacteria can partition the temperature dimension of the niche very finely. An ancestor *E. coli* culture was adapted for 2,000 generations to 37 °C (Lenski et al., 1991). This culture was divided into six replicate lines for each of three temperatures, 32, 37, 42 °C. The 18 cultures were then adapted for another 2,000 generations. At each temperature, there was significant adaptation to that temperature, but no adaptation or loss of adaptation to another temperature (Bennett et al., 1992). For example, the culture evolved at 32 °C was significantly fitter than the ancestor at 32 °C, but not significantly different in fitness from the ancestor at either 37 or 42 °C. When the adaptations were tested across a range of temperatures, it was found that cultures adapted to the three temperatures partitioned the niche dimension of temperature into three parts (Bennett and Lenski, 1993). Clearly, one could partition *E. coli* into at least three species along a temperature dimension.

Natural variation for niche partitioning

When naturally occurring samples of the *lac* operon of *E. coli* are extracted from natural populations, different operons have different fitness on various β-galactosides (Silva 1992, Dean 1995). For example, the *lac* operon from ECOR16 (Ochman and Selander 1984) is fitter than the operon from K12 on lactose, galactosyl-glycerol and methyl-galactoside while the reverse is true on lactulose and galactosyl-arabinose. When two sugars are mixed in a chemostat, the relative fitness of two strains can be predicted for any given mixture by the equation:

$$w = \frac{\mu_1}{\mu_2} \cong w_{R1}\left[\frac{R_0}{R_0+S_0}\right] + w_{S1}\left[\frac{S_0}{R_0+S_0}\right] \quad (1)$$

where w is the relative fitness on the mixture of sugars, μ_1 and μ_2 are the growth rates of the two strains, R_0 and S_0 are the concentration of the two sugars in the reservoir, and w_{R1} and w_{S1} are the relative fitness on R and S respectively (Dykhuizen and Dean 1994). Figure 3 shows an experimental conformation of this equation. Silva (1992) has shown that this applies not only to sugars that are used by two different pathways, but also by sugars that are used by the same pathway (Figure 4). As shown in this figure, the *lac* operon from ECOR16 has a selective advantage when the lactose concentration is higher than about 60%, but the other operon from K12 has the advantage when the lactose concentration is below this level. At a mixture of lactose and lactulose of about 60/40, one expects a stable equilibrium. If the numbers of cells with the *lac* operon from ECOR16 drops, the relative concentration of its preferred substrate, lactose, will increase in the chemostat and there will be selection for ECOR16. If the other strain becomes rare, the relative concentration of lactulose will increase and it will be selected for. Thus the system is stable and, over time, these two strains could evolve to specialize on their preferred substrate and form separate species. Thus the genetic variation is available in populations to allow specialization and speciation given that the environment is suitable for partitioning.

In the laboratory, niche division and specialization are easy and happen quickly. Sexuality or lateral transfer of DNA between lineages and diploidy will slow the process described above. Since bacteria are haploid and generally have lower rates of gene exchange that sexually reproducing metazoans, the speciation rate is expected to be faster in bacteria. While it is usually assumed that the environment is much more variable in

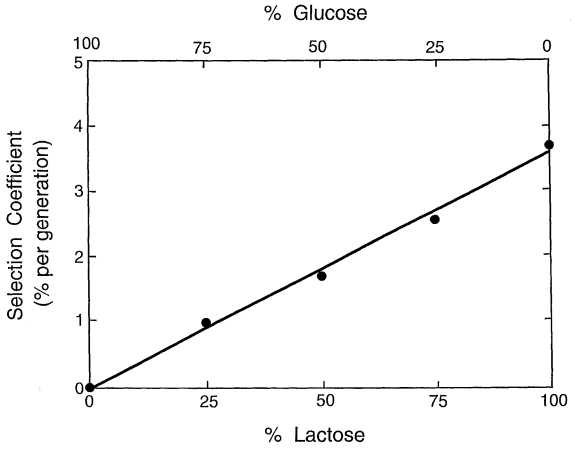

Figure 3. Fitness on mixed sugars. The competition is between TD2 ($lacI^- Z^+ Y^+ A^+$) and TD4 ($lacI^- Z^{mut} Y^+ A^+$). The *lac*Z mutation in TD4 has 1.1% of the activity of wild type. This figure is derived from Figure 4 of Dykhuizen and Dean (1994).

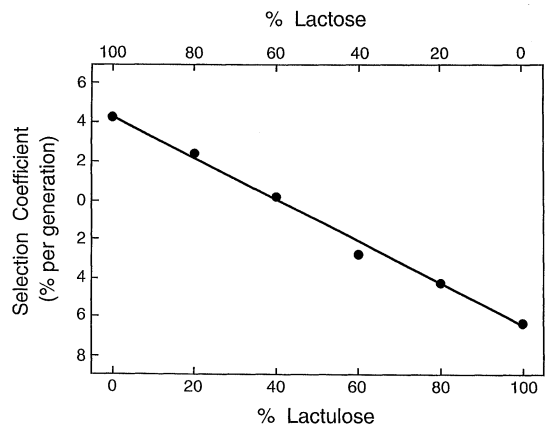

Figure 4. Fitness on a mixture of two β-galactosides. The competition is between two strains isogenic except for the lactose operon. The two lactose operons are both wild type, having been isolated from nature. This figure is derived from Figure 6.2 of Silva (1992).

nature than in the laboratory and niche division will be much more difficult, these examples suggest that even short periods of environmental stability could lead to speciation. Speciation is easy.

Epilogue

In this paper I want to suggest that there are a tremendous number of species of bacteria in the world. The numbers estimated in this paper are clearly best guesses for now. But it is clear that as the techniques are refined and much more work is done comparing the species composition of various communities, much better estimates of the number of bacterial species will be made. If bacteria communities have structure like metazoan communities (Preston 1962, Sugihara 1980), then a much more accurate estimate of the numbers of species in a community can be derived from the deviation of the Cot curve from the expected curve as shown in Figure 1. If the number of species is anything like I suggested, the cloning and sequencing 16s RNA types from nature will never give an adequate picture of the species diversity. This method will only provide information on some of the common species of the community.

While we might not want to define and name each and every species as we have done with birds and mammals, we need to have an idea of world-wide bacterial diversity and good ways of assessing this diversity to make sure that the microbial world does not become unstable as man changes the environment. The methods are available and now need refinement and use.

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