**MINI
REVIEW****The causes of *Pseudomonas* diversity**

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The diversity

The genus *Pseudomonas* encompasses arguably the most diverse and ecologically significant group of bacteria on the planet. Members of the genus are found in large numbers in all of the major natural environments (terrestrial, freshwater and marine) and also form intimate associations with plants and animals. This universal distribution suggests a remarkable degree of physiological and genetic adaptability.

Evidence of physiological diversity came from early biochemical studies of Stanier, Doudoroff and others at Berkeley. These scientists studied the remarkable capacity of *Pseudomonas* strains to degrade a wide range of substrates including aromatic compounds, halogenated derivatives and recalcitrant organic residues. From this work grew the need for a sound taxonomic framework and so, assisted by Palleroni, Stanier and Doudoroff began one of the most comprehensive studies of *Pseudomonas* diversity ever undertaken. The paper, published in this journal in 1966 (then *Journal of General Microbiology*), was 112 pages in length and reported growth characteristics of 267 strains on 146 different organic compounds, plus a wide range of associated tests (Stanier *et al.*, 1966). Since publication, it has been cited close on 1000 occasions. The significance of the work and its enduring legacy is attributable to the fact that these authors finally made sense of a group of organisms that had previously proved intractable. Its success stemmed largely from the fact that the work was underpinned by a solid understanding of the diversity of *Pseudomonas* physiology – the groupings therefore reflected the population biology of the bacteria.

Since this seminal work, many others have contributed to the development of a rational taxonomic framework for dealing with the diversity of *Pseudomonas* [Palleroni (1993) provides a thorough historical account; see also Yamamoto *et al.* (2000)]. DNA analysis and progress toward the description of monophyletic groups have recently seen a number of species moved to different genera, but the core groupings identified by Stanier *et al.*

(1966) remain robust, and *Pseudomonas sensu stricto*, every bit as diverse as originally reported.

Diversity within the genus is not limited to physiological traits. The diversity of phenotypes is also reflected at the genetic level, and evidence is mounting to suggest that the diversity of genome architecture (of both chromosomes and accessory genetic elements) is of particular significance. Fingerprinting studies performed by many workers have revealed a remarkable degree of restriction fragment polymorphism among strains of a species – and even among strains that are closely related on phenotypic grounds (see, for example, Ginard *et al.*, 1997; Grothues *et al.*, 1988; Rainey *et al.*, 1994). Genome sizes vary widely, ranging from 3.7 Mbp for *Pseudomonas stutzeri* (Ginard *et al.*, 1997) to 7.1 Mbp for *Pseudomonas aeruginosa* (Schmidt *et al.*, 1996), with the genome sizes of *Pseudomonas fluorescens* SBW25, *Pseudomonas syringae* pv. *phaseolicola* and *Pseudomonas putida* KT2440 being 6.7 Mbp (Rainey & Bailey, 1996), 5.6 Mbp (De Ita *et al.*, 1998) and 6.1 Mbp (C. Weinel, personal communication), respectively. In addition, studies of multiple isolates from a single species have shown substantial genome size polymorphism; *P. stutzeri* spans from 3.7 to 4.7 Mbp (Ginard *et al.*, 1997) and *P. aeruginosa* spans from 5.2 to 7.1 Mbp (Schmidt *et al.*, 1996). Interestingly, this extensive diversity appears not to be reflected at the level of DNA sequence, as might be expected. For example, a sequence analysis of housekeeping loci from 19 environmental and clinical isolates of *P. aeruginosa* showed low sequence diversity (Kiewitz & Tummeler, 2000; Spangenberg *et al.*, 1998).

The causes of diversity

Microbiologists are used to dealing with diversity. Most are aware of the phylogenetic tree of life that reveals vastly more diversity among the bacteria than among the eukaryotes (Doolittle, 1999). Many papers, and not solely those concerned with *Pseudomonas*, document this diversity at the whole-organism level; others describe diversity at the molecular level, in terms of DNA

sequence or variation in specific proteins or macromolecules, such as lipopolysaccharide. Only rarely are the causes and ecological significance of this diversity considered, yet the evolutionary causes of biological diversity and the ecological significance of this diversity are two of the most fundamental and pressing issues in biology (Haldane, 1932).

Diversity arises and is maintained through interplay between ecological and genetic factors. The ultimate cause is mutation, but the variation generated by mutation and recombination is rapidly sorted and shaped by selection and subject to the vagaries of genetic drift. While acknowledging the role of drift in maintaining molecular variation, our emphasis throughout this article is on ecologically significant variation, and particularly on its emergence. We briefly consider ecological factors that affect patterns of diversity, but we avoid detailed discussion of the mechanisms maintaining diversity, which are considered elsewhere (Bell, 1997; Rainey *et al.*, 2000).

Ecological causes of diversity

A variety of ecological factors are known to determine patterns of diversity. Darwin was among the first to consider the major causes and suggested two candidates: ecological opportunity (vacant niche space) and competition (Darwin, 1859). At its simplest, Darwin envisaged a single species entering a pristine environment that afforded a multitude of unoccupied niches. In time, variation would increase and new species would evolve to occupy vacant niche space. He saw competition among species as the engine of divergence.

Despite the small size of bacteria and the reluctance of many microbiologists to accept that bacterial populations are subject to the same forces of evolution as higher organisms, there is *a priori* no reason that diversification in bacteria could not occur by similar processes. In fact, experimental bacterial populations have proved powerful tools in ecology and evolution, and the ecological and evolutionary forces believed to operate on populations of higher organisms have been observed directly in experimental bacterial populations (Lenski *et al.*, 1998; Rainey *et al.*, 2000).

Ecological opportunity

As envisaged by Darwin, ecological opportunity is indeed an essential factor and primary cause of diversity, and it is clear that bacteria have a remarkable capacity to exploit the subtlest of opportunities. In a series of now classic experiments using test tube populations of *Paramecium*, Gause showed that two species competing for the same niche could not coexist – one would drive the other to extinction (Gause, 1934). This led to formulation of the niche-exclusion principle, which states that no two organisms can occupy the same niche. If an environment lacks spatial or temporal heterogeneity (i.e. is homogeneous) and affords just a single niche, then in principle, it will house just a single species (or single bacterial genotype) (Hardin, 1960). As the popu-

lation evolves, more successful species (or asexual genotypes) will simply replace the previously most common form when they arise. Precisely this step-wise mode of evolution occurs during propagation of an asexual bacterial genotype in a homogeneous chemostat environment (Atwood *et al.*, 1951). Evolution occurs by a series of clonal replacements (periodic selection) and diversity is purged each time a beneficial mutant arises and ‘sweeps’ through the population.

Remarkably though, even in the chemostat environment, diversity can evolve and be stably maintained. Julian Adams and colleagues showed that a single *Escherichia coli* genotype propagated in a glucose-limited chemostat partitions the primary resource (glucose) through its own metabolic activities (Rosenzweig *et al.*, 1994). Excreted metabolites, such as acetate, provide new ecological opportunities for the emergence of mutants with an enhanced capacity to grow on acetate. When these niche-specialist mutants arise [and they readily do (Treves *et al.*, 1998)], they are stably maintained through the operation of density-dependent processes (Rainey *et al.*, 2000). This simple and elegant work shows in a very profound way how the activities of one bacterial genotype can provide opportunities for the emergence of new types. A number of similar studies have also shown the emergence of diversity from single bacterial genotypes during the course of evolution in simple unstructured environments (Rozen & Lenski, 2000; Turner *et al.*, 1996).

To test more explicitly the effects of ecological opportunity on the emergence of diversity, Rainey & Travisano (1998) propagated experimental populations of *P. fluorescens* in two environments identical in every way except the degree of ecological opportunity they afforded the evolving populations. Diversity, in the form of niche-specialist genotypes, emerged rapidly in the spatially heterogeneous, but not in the spatially homogeneous, environment.

Competition

Competition is frequently thought of as the driving force, or engine, of diversification (Abrams, 1987). Its operation is easily envisaged. Imagine a single bacterial genotype growing in a simple structured environment. As the density of the population increases, growth eventually becomes limited due to depletion of resources such as oxygen and nutrients. At this stage competition becomes intense and selection will favour any mutant that can gain access to limiting resources. The most favoured genotypes will be those that occupy niches that are different to that occupied by the dominant genotype, because resources will be most abundant in unoccupied niches. Having diverged, selection will continue to favour mutants that show increasing degrees of niche specialisation so that eventually there will be minimal niche overlap among genotypes. The moment-by-moment effects of competition are notoriously difficult to detect; nevertheless, direct evidence of its operation has been obtained in studies of both bacterial (Rainey &

Travisano, 1998) and eukaryotic (Schluter, 1994) populations.

Other ecological factors

While ecological opportunity and competition are thought to be the most significant ecological causes of diversity, a number of other ecological factors affect niche availability and the outcome of competition. The effect of productivity (energy input) and disturbance (periodic mortality events), for example, have received wide attention in the ecological literature (Rosenzweig, 1995). Using laboratory populations of *P. fluorescens*, effects of productivity and disturbance on bacteria have been studied, and consistent with theory, unimodal relationships between diversity and both productivity (Kassen *et al.*, 2000) and disturbance (A. Buckling, R. Kassen, G. Bell and P. B. Rainey, unpublished) have been observed [a unimodal relationship is one where diversity peaks at intermediate levels (of both diversity and productivity)]. Significantly, these relationships were dependent on spatial heterogeneity and were not detected in homogeneous environments.

Parasites are another important factor affecting diversity, and bacterial populations are particularly prone to parasitism by phages. Again, using experimental populations of *P. fluorescens*, their importance has been explicitly tested (A. Buckling & P. B. Rainey, unpublished). When phage and *P. fluorescens* populations are co-cultivated, strong selection for host resistance leads to genetic bottlenecks that greatly increases diversity among spatially distinct populations. Diversification can further escalate when interactions between host and parasite lead to an arms race of infectivity and resistance, sending populations along different evolutionary trajectories. As previously observed, the effects of environmental heterogeneity can be profound – persistent coevolution is maintained in heterogeneous environments, but not in environments lacking spatial structure.

Genetic causes of diversity

Diversification isn't solely an ecological process. Genetic factors are equally significant (although ecological forces are the ultimate determinants of patterns of diversity). Without variation there can be no evolution and no divergence. Variation arises by mutation and recombination, and both processes operate in bacterial populations. In terms of the extraordinary diversification within *Pseudomonas*, a key issue is whether the *Pseudomonas* genome has an enhanced capacity for evolutionary divergence (evolvability). Is there something in the organization of the *Pseudomonas* genome that facilitates the generation of heritable variation; that might even influence the kinds of variation and thus increase the capacity of these bacteria to exploit new niches? There need not be. Classical 'spontaneous' mutation and selection alone are powerful evolutionary forces capable of bringing about radical changes in bacterial populations (Levin & Bergstrom, 2000). Nevertheless, the ubiquitous distribution of *Pseudomonas* and

remarkable adaptability does lead many to wonder whether *Pseudomonas* are inherently more evolvable than other bacteria. Here, we briefly discuss genetic causes of diversity and evidence for enhanced evolvability of *Pseudomonas*.

Mutation

Variation is essential for adaptive divergence, and mutation is the ultimate source. One way that evolvability may be enhanced is by modulation of mutation rate. It is therefore tempting to postulate that selection may have led to the evolution of mechanisms by which *Pseudomonas* increase their mutation rate under stress, thus generating extra variation when necessary and facilitating adaptation to novel environments. Although an attractive possibility, there is no evidence to support this hypothesis. Nevertheless, strains with elevated mutation rates (mutators) have been found. For example, mutator strains of *P. aeruginosa* (containing defective mismatch-repair genes) were recently found in long-term infections of the lungs of cystic fibrosis patients, but not in a comparable sample from patients suffering acute *P. aeruginosa* infections (Oliver *et al.*, 2000). Such strains may facilitate rapid adaptive diversification in the heterogeneous lung environment (despite the costs associated with a globally elevated mutation rate), which may be essential for long-term persistence in the respiratory tract. However, there is no evidence that the mutator strains are themselves adaptive, that is, that they have evolved because they are inherently more fit than non-mutator strains (Rainey & Moxon, 2000; Sniegowski *et al.*, 2000). They may simply increase in frequency, particularly in novel or heterogeneous environments, because of genetic linkage to beneficial mutations that they cause (Leigh, 1973). Moreover, mutators have been found in other bacterial genera that are less diverse than *Pseudomonas*. Mutation rates in bacteria are known to generally increase under stress because of the SOS response and reduced ability to deal with DNA-damaging free radicals generated by metabolism (Friedberg *et al.*, 1995).

The best examples of evolvable DNA sequences are the contingency loci found in certain pathogenic bacteria (Moxon *et al.*, 1994). These loci, characterized by short repeated DNA sequences that are slippage prone and therefore inherently unstable, enable bacterial populations to generate substantial phenotypic diversity in a short number of generations. In the case of bacterial pathogens, this diversity is thought to be crucial for avoidance of the host immune response. Perhaps *Pseudomonas* genomes are rich in contingency loci?

Polymorphisms within single clones, akin to the phase or antigenic variation of pathogenic bacteria, are well known in *Pseudomonas*. The best-studied example is the mucoid to non-mucoid colony transformation observed in some strains of *P. aeruginosa*. Despite rapid loss of the mucoid form on agar plates, suggestive of control by a contingency locus, no such mutational mechanism has been discovered. In fact, detailed investi-

gations have revealed a number of point mutations at different chromosomal loci (particularly regulatory genes) that cause the transition between colony types (Govan & Deretic, 1996). Further searches for contingency loci by scanning the genome sequence of *P. aeruginosa* PAO1 show few significant tracts of repeated DNA sequence. Assuming that the PAO1 sequence is typical of other *Pseudomonas* species, it would appear that *Pseudomonas* has no specific mutational mechanisms that enhance evolutionary divergence.

Recombination

The most striking finding from the analysis of whole-genome sequences is the extent to which bacterial genomes are chimeras. The acquisition of genes and accessory genetic elements (plasmids, transposons, integrons and phages) by lateral gene transfer is a pervasive and important source of innovation for bacterial evolution (Ochman *et al.*, 2000). In *Pseudomonas*, population-genetic studies have shown that recombination occurs (Haubold & Rainey, 1996; Kiewitz & Tummeler, 2000), but overall there is a pervasive clonal framework as evidenced by the repeated sampling of identical clones from different environments – often years apart. Nevertheless, accessory genetic elements play an important role in the lateral movement and integration of novel DNA sequences in *Pseudomonas*. Plasmids are commonplace (Sesma *et al.*, 2000; Thomas, 2000): they are often large, many have a broad host range (beyond *Pseudomonas*) and they frequently encode complete pathways (often catabolic or virulence associated) plus associated regulatory machinery. Large mobile islands that integrate into tRNA genes and use phage-like integrases for movement have been reported (Jackson *et al.*, 1999; Ravatn *et al.*, 1998), and the presence of tracts of DNA with atypical codon bias indicative of recent acquisition are also present (Alfano *et al.*, 2000). No doubt further acquisition and analysis of whole-genome sequences will reveal substantial mosaicism among *Pseudomonas* genomes.

Intragenomic recombination also appears to be an important factor in *Pseudomonas*, possibly accounting for much of the diversity in (macro)restriction fragment patterns observed among closely related isolates. Kiewitz *et al.* (2000) show that spontaneous integration and insertion of plasmids at tRNA^{lys} loci within the *P. aeruginosa* genome explain macrorestriction fragment differences among sequentially isolated strains. These authors also suggest that use of phage attachment sites by conjugative genetic elements may contribute to mosaic genome structure in *Pseudomonas*.

Despite the undoubted significance of lateral gene transfer for *Pseudomonas* evolution, there is no evidence that *Pseudomonas* has evolved any special mechanisms to enhance its ability to acquire novel DNA. With the exception of *P. stutzeri*, *Pseudomonas* are not known to be naturally competent [competency itself may not be an adaptation for acquiring novel sequence – see for example, Redfield (1993) and Levin & Bergstrom (2000)]

and the genome of *P. aeruginosa* does not contain any of the machinery typically found in competent bacteria, such as *Neisseria gonorrhoeae*, and necessary for the uptake of naked DNA.

Genome size, physiological flexibility and modularity

Strictly speaking, genome size, physiological flexibility and modularity are not in themselves ‘genetic mechanisms’ for adaptability; however, the idea that selection may have favoured certain genome architectures because they provide adaptive flexibility is appealing (Jablonka *et al.*, 1998). There is growing evidence that supports this general view and it has even been argued that the success of the metazoans is largely attributable to features of genome architecture (Kirschner & Gerhart, 1998). A central idea is that regulatory pathways controlling the spatial and temporal expression of genes have special properties relevant to evolutionary change. These properties include versatile protein elements and weak linkage among components. Versatility among protein elements is particularly common in protein kinases, where minor mutational modification can alter the specific target of activity or affect the timing of activation (Hoch, 1995). Weak linkage is also a feature of signal-transduction pathways; components have switch-like properties and signals act to release the activity, but do not act instructively. Such regulatory organization facilitates a component’s accommodation to novelty and reduces the cost of generating variation (for more information see Kirschner & Gerhart, 1998). The fact that *Pseudomonas* genomes are so rich in regulatory genes, particularly the so-called ‘two-component’ type may be of special significance (*P. aeruginosa* has ~9 histidine protein kinase sensors per Mbp, compared to ~5 per Mbp in *E. coli*).

Bacterial genomes are highly dynamic and streamlined, accumulating ecologically useful sequences by lateral gene transfer and losing genes by deletion if they no longer provide a useful function (Lawrence, 1999). The large genome size of *Pseudomonas* therefore presumably reflects the fact that most strains encounter a broad range of ecological niches on a regular basis. To ensure optimal performance in such a range of niches, the cell must not only possess a diverse range of metabolic and nutrient-scavenging pathways, but also be able to co-ordinate these systems so as to optimize nutritional and reproductive potential. But there is a further crucial aspect. If genomes are to exploit potentially useful gene clusters acquired by lateral transfer, then they must be rapidly integrated within the regulatory networks of the cell (unless they arrive as a complete unit, e.g. on a plasmid – but even these will require some degree of regulatory integration). Precisely how this is achieved is unclear; however, the flexibility inherent within signal-transduction pathways – both at the level of domains within proteins and among separate components of phosphorylation cascades – may play a role. The fact that *Pseudomonas* genomes are rich in these regulatory components could well be a crucial factor affecting the ability of these bacteria to take advantage of novel

ecological and genetic opportunities presented by lateral gene-transfer events.

Whether regulatory pathways have specifically evolved for the purposes of further enhancing evolutionary change, or whether adaptability is a coincidental property of modular systems is unclear. One intriguing possibility is that the driving force behind the evolution of flexible regulatory pathways may be the ability to acquire genes from other organisms as a source of variation for adaptive evolution.

Conclusion

On balance, with the possible exception of the extensive collection of regulatory genes, it appears that there are no particularly notable features of *Pseudomonas* that make the genus inherently more evolvable than other bacteria. We therefore favour a mundane (but not trivial) explanation for the diversity – in fact one that invokes nothing more than a long evolutionary history (and concomitant opportunities for lateral gene transfer) and continual exposure to a range of spatially and temporally complex environments. Evidence that this might indeed be sufficient is revealed by simple selection experiments in which single *Pseudomonas* genotypes are propagated in a spatially heterogeneous environment (a static broth culture). Within the space of a single week these populations rapidly diversify, producing a range of niche-specialist genotypes (Rainey & Travisano, 1998). Moreover, work on one *P. fluorescens* population shows that diversification is caused, in part, by simple mutations in regulatory pathways that affect the spatial and temporal expression of a surface polymer (A. Spiers, P. Goymer, E. Bantinaki, J. Bohannon & P. B. Rainey, unpublished). If diversification on this scale can occur in a single week, then the diversity of *Pseudomonas* comes as little surprise given the ancient evolutionary history of the genus and the vast complexity of natural environments.

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