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Comparative phylogenomics of pathogenic bacteria by microarray analysis
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DNA microarrays represent a powerful technology that enables whole-scale comparison of bacterial genomes. This, coupled with new methods to model DNA microarray data, is facilitating the development of robust comparative phylogenomics analyses. Such studies have dramatically increased our ability to differentiate between bacteria, highlighting previously undetected genetic differences and population structures and providing new insight into virulence and evolution of bacterial pathogens. Recent results from such studies have generated insights into the evolution of bacterial pathogens, the levels of diversity and plasticity in the genome of a species, as well as the differences in virulence amongst pathogenic bacteria.

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Introduction
Traditional phylogenetic classification of bacteria to study evolutionary relatedness is based on the characterisation of a limited number of genes, rRNA or signature sequences. However, owing to the acquisition of DNA through lateral gene transfer, the differences between closely related bacterial strains can be vast. By contrast, whole-genome sequencing comparisons allow a multitude of genes to be compared. Already, several bacterial species have had more than a single representative sequenced (e.g. Escherichia coli [1], Campylobacter jejuni [2], Helicobacter pylori [3], Yersinia pestis [4], Neisseria meningitides [5], Staphylococcus aureus [6] and several Chlamydia species [7]). Nevertheless, whole-scale genome sequencing remains an expensive endeavour and such comparisons are limited to only a handful of strains.

DNA microarrays represent an alternative technology for whole-genome comparisons, enabling a ‘bird’s-eye view’ of all the genes absent or present in a given genome compared to the reference genome on the microarray. Harnessing DNA microarray information through interrogative and robust algorithms has enabled a true ‘comparative phylogenomics’ approach to be developed. Recent comparative phylogenomics studies have been undertaken on increasingly large collections of strains of defined origin. One common feature of these studies is the unexpectedly large genetic diversity between strains of the same species, blurring our definition of species boundaries. Whole-genome comparisons typically identify sets of ‘core genes’, which are shared by all strains in a species, and ‘accessory genes’, which are present in one or more strains in a species and often result from gene acquisition. It is these differences that can often be used to identify genes and/or genetic islands related to ‘gain-of-function traits’ in pathogenic strains. Uncovering the mechanisms behind this variability is fundamental in understanding and ultimately counteracting the threats posed by our old adversaries.

This review will describe selected recent examples in which comparative phylogenomics using microarray data has provided new and often unexpected insights into the evolution, ecology, virulence and genome diversity of bacterial pathogens.

Comparative phylogenomics microarray methodology
Comparative phylogenomics studies involve analysis of the relative hybridisation capacity of sample bacterial DNA to reporter DNA elements that are bound to a microarray surface. DNA microarrays consist of minute samples of DNA (known as reporter elements) that are arrayed in a grid formation on a suitable medium. Each predicted coding sequence within a target genome or genomes is represented on the array by at least one specific reporter element. All DNA reporter elements are designed to minimise cross-hybridisation (hybridisation of DNA from one gene to reporter elements that represent other genes owing to sequence similarity) between spots.

Sample genomic DNA from a test strain is labelled with a fluorescent marker (usually Cy3 or Cy5) and is competitively hybridised with genomic DNA from a control strain that has been labelled with a different fluorescent marker (Figure 1). The control strain is usually DNA from the strain used to design the array, as this should hybridize to all reporter elements on the microarray. After washing to remove any unbound DNA, microarrays are scanned by a
high-resolution fluorescence scanner, and the image obtained is analysed by specific software packages that are not discussed in this review. Control spots on the array and DNA ‘spikes’ in the labelling reaction can be used to aid normalisation of array data. A comparison of the levels of test and control DNA bound to each reporter element on the array can be performed and statistical methods are utilized to determine if the coding sequence in question is present, absent or highly divergent in the test strain.

Which type of DNA microarray should be used?

Polymerase chain reaction product-based DNA microarrays

DNA microarrays often consist of gene fragments (reporter elements) amplified by polymerase chain reaction (PCR) that represent individual genes. Currently, up to 50,000 gene fragments can be spotted onto a single microscope slide using robotic technology. Advantages of this technology are: flexibility in the design of the array; the relative ease of production; and its relatively low cost. Multiple identical microarrays can be robotically printed in batches of over a hundred in a single run. Most of the cost in printing such arrays is caused by the synthesis of oligonucleotide primer pairs required for the amplification of target gene fragments. The use of clone libraries as the template for the amplification of PCR products can dramatically reduce this cost [8].

Oligonucleotide-based DNA microarrays

Oligonucleotide microarrays do not rely upon PCR amplification of gene targets, and areas of specificity within any gene sequence can be chosen for hybridisation analysis. However, the sequence of target DNA must be known before oligonucleotide synthesis. The two most frequently used formats are the Affymetrix and the Qiagen Operon systems. In the Affymetrix format (www.affymetrix.com), oligonucleotides are synthesized in situ on a derivatised glass surface using a combination of photolithography and combinatorial chemistry. The synthesized oligonucleotides are usually 20–25 bases in length. The GeneChip® E. coli Genome Array is the first Affymetrix microarray product for the analysis of gene expression in a prokaryotic organism. The Qiagen Operon format (www.operon.com) uses an optimised 70-mer oligonucleotide to represent each gene in a genome. Each 70-mer is designed to have optimal specificity for its target gene and is melting-temperature normalised.

Oligonucleotide-based microarrays offer many advantages over PCR-product-based microarrays including a reduction in cross-hybridisation and an increase in the differentiation of overlapping genes or highly homologous regions. For example, mutant alleles or single nucleotide polymorphisms can be detected using such oligonucleotide microarrays owing to the shorter probe size compared to PCR product-based microarrays.

Comparative phylogenomics to study the evolution of bacterial pathogens

Comparative phylogenomics using microarray data can be particularly useful for retrospective analyses of the recent (past hundred years) and the longer term (several thousand years) evolution of bacterial pathogens.

Recent evolution of bacterial pathogens usually involves lateral gene transfer and large-scale genomic deletions. Such genetic differences are more evident in the highly clonal species such as Vibrio cholerae, in which microarray studies showed that all strains tested were <1% divergent from each other over their entire genome [9]. These differences provide clues to the evolution of V. cholerae as a human pathogen and as an environmental organism. Historically, the first six V. cholerae pandemics between 1817 and 1923 were caused by classical biotype strains from the O1 serogroup. However, the emergence of the seventh pandemic El Tor biotype in 1961 eventually resulted in the global elimination of classical biotype strains as a cause of disease. Microarray analysis revealed chromosomal islands specific to all pandemic strains as well as two chromosomal islands that are uniquely present in the modern-day pandemic El Tor strain. This suggested that these regions might encode factors specifically associated with displacement of the pre-existing classical
strains in South Asia and might also promote the establishment of endemic disease in previously cholera-free locations [9].

A comprehensive comparative genomics study of pathogenic S. aureus revealed that ~22% of the genome is composed of accessory genes [10]. These are mainly grouped in large chromosomal regions of difference (RDs), and are often associated with phage genes, integrases or transposases. Of the 18 RDs identified, 10 contained putative virulence determinants (including enterotoxins and the methicillin-resistance gene mec) that appear to have been acquired independently into the chromosome background of several phylogenetically distinct strains. This demonstrated that methicillin-resistant strains have evolved multiple independent times, rather than from a single ancestral strain. It has also helped to resolve the long-standing controversy that the 1970s epidemic of toxic shock syndrome was caused by a change in the host environment (i.e. the use of certain tampons), rather than by the rapid geographic dissemination of a new hypervirulent S. aureus strain [10]. Interestingly, the epidemic methicillin-resistant S. aureus-15 (EMRSA-15) clone, one of the most prevalent MSRA strains in the UK, appears to have resulted from a large-scale recombination event [11]. Other microarray studies of this kind have recently identified specific RDs and coding sequences that might be responsible for hypervirulence of certain strains in animal models of muscoskeletal infections [12].

Yersinia pestis, the causative agent of plague, diverged from Yersinia pseudotuberculosis, an enteric pathogen, an estimated 1500–20 000 years ago [13,14]. Genetic characterization of these closely related organisms represents a useful model to study the rapid emergence of bacterial pathogens that threaten mankind. Studies using a Y. pestis-specific microarray identified 11 Y. pestis DNA loci that were absent or highly divergent in all 22 strains tested. Four were regions of phage origin, whereas the other seven included genes that encode a vitamin B12 receptor and the insect toxin SepC. These studies help predict the genome content of the single Y. pseudotuberculosis strain that evolved into the Y. pestis (sub)species [15]. Microarray-based studies to determine the evolution of Y. pestis have shown that recombination between the frequent insertion sequence elements scattered around the chromosome has resulted in large-scale deletions in most strains [15,16]. Recent studies on Y. pestis isolates from Brant’s vole plague foci in China have shown these strains (renamed Yersinia microtus) were attenuated in the human host. Microarray and sequencing analysis of these strains shows a specific pattern of loss of particular regions of the genome including five regions that are present in all virulent strains tested [15]. Additionally, several specific mutations were identified that are present in all Y. microtus strains but absent in human virulent strains. This study has therefore successfully identified regions of the Y. pestis genome responsible for Y. microtus niche-adaptation and for avirulence in humans, as well as mapping the accumulation of deletions in fully virulent Y. pestis strains as they spread among vole populations throughout China.

Comparison of the virulent Mycobacterium tuberculosis reference strain H37Rv with virulent Mycobacterium bovis strains and various Bacille Calmette-Guerin (BCG) daughter strains identified 11 regions (91 open reading frames [ORFs]) of H37Rv that were found to be absent from one or more M. bovis strains. Also, 5 additional regions (38 ORFs) were identified to be present in M. bovis strains but absent in some or all of the BCG strains [17]. This is indicative of ongoing genetic changes in BCG strains and offers a possible explanation for the loss of protective efficacy since their original derivation in 1921. Further studies have suggested that the common ancestor of the tubercle bacilli resembled M. tuberculosis or Mycobacterium canetti and might have been a human pathogen already, which contradicts the hypothesis that M. tuberculosis evolved from M. bovis [18].

Comparative phylogenomics to study genome diversity and plasticity

Many of the early comparative genomic studies investigated the presence or absence of genes in test strains compared to the sequenced strain represented on the array. Such studies have tended to be more informative for non-clonal populations of bacteria. Genome diversity refers to strains from the same species possessing many different accessory genes in different combinations, whereas genome plasticity refers to gain or loss of specific genes resulting in phenotypic changes.

Extensive genomic diversity between pathogenic E. coli and Shigella strains has been shown by way of microarray analysis [19,20]. The core genes for E. coli consist of about 2800 ORFs, and unique relationships between enteroinvasive E. coli strains and Shigella and between uropathogenic and enteropathogenic E. coli strains have been identified [19]. Similar studies for Salmonella species have also been reported, which suggests that there is a high level of gene gain and gene loss as well as rapid divergence along all lineages [21,22].

Analysis of genetic diversity amongst different clinical isolates of the human gastric pathogen H. pylori has demonstrated that ~22% of the H. pylori genome is composed of accessory genes [23]. Although the core genes encode most metabolic and cellular processes, the accessory genes include those that might be important for long-term infection of a genetically diverse host. Indeed H. pylori has been shown to exhibit genetic diversity within the stomach of a single human host [24]. Comparison of two clinical isolates that produced different levels of inflammation in the gerbil gastritis

Y. pestis/C15, 20% from Brant’s vole plague foci in China have shown these strains as they spread among vole populations throughout China.
model identified a large deletion in the cag pathogenicity island in the less proinflammatory strain [25].

The initial observation on genetic variability in the gastrointestinal pathogen *C. jejuni* [8] has been expanded by several recent studies [26–28], which suggest that *C. jejuni* exhibits high levels of genome diversity and low levels of genome plasticity [28]. Unfortunately, it has proved difficult to use microarray data to identify genetic markers that can predict the source or outcome of infection. Infection with *C. jejuni* can result in the development of the post-infectious neurological disorder Guillain-Barré syndrome (GBS). A comparison of isolates from patients who developed GBS with isolates from patients with uncomplicated gastrointestinal infection failed to identify specific GBS genes or regions [29].

A large chromosomal region (RD13), common to all strains of *S. aureus*, encodes a family of proteins designated staphylococcal exotoxin-like (SET) proteins. Extensive genetic diversity and variation has been shown in the set gene content [30]. This is indicative of multiple episodes of set gene deletion in different clonal lineages in parallel. There was evidence that horizontal gene transfer and recombination have also contributed to the diversification of RD13.

By contrast, not all pathogens exhibit extensive genetic diversity. Surprisingly, microarray analysis of serotype M18 group A *Streptococcus* (GAS) strains that are associated with acute rheumatic fever showed little or no variation in gene content [31]. In fact, the data indicated that M18 strains recovered during two acute rheumatic fever outbreaks that occurred 12 years apart were genetically identical.

Analysis of the genomes of 100 epidemiologically well-characterised *M. tuberculosis* clinical isolates showed that deletions were not distributed randomly, but were tightly characterised M. tuberculosis. Analysis of the genomes of 100 epidemiologically well-characterised M. tuberculosis strains recovered during two acute rheumatic fever outbreaks that occurred 12 years apart were geneti-

nonpathogenic bacteria within a species (or genus) are partic-
ularly useful for the identification of determinants that are likely to be important for virulence, transmission and host specificity. Whole-genome analysis of strains with documented clinical histories, or of those of known host origin, is crucial for deciphering genetic clues related to virulence or host specificity. In terms of identifying potential virulence factors, the comparison of isolates from patients with a spectrum of disease (including asymptomatic carriage) is particularly useful for pathogens in which a suitable animal model of disease is unavailable.

Comparison of clinical and commensal *Staphylococcus epidermidis* strains revealed high genetic variability in the genome and identified several markers of invasiveness for this nosocomial pathogen [34]. A proline-betaine transporter homolog gene was more frequent among invasive strains, which suggests a previously unexpected role for osmoprotection factors during infection. By contrast, a similar study demonstrated that *Pseudomonas aeruginosa* strains possess a highly conserved genome that encodes genes important for survival in numerous environments, thus allowing this opportunistic pathogen to cause a variety of human infections [35]. However, clusters of strain-specific genes were also identified, termed variable segments, which appear to highlight the presence of preferential sites for integration of novel genetic material into the *P. aeruginosa* genome.

*Neisseria gonorrhoeae* causes an infection that is generally localized to the genitourinary tract, whereas *Neisseria meningitidis* can enter the bloodstream and cross the blood–brain barrier to invade the meninges. Genomic comparisons of different clinical isolates of both pathogens and of *Neisseria lactamica*, a commensal of the nasopharynx, highlighted genes that are present in both pathogenic *Neisseria* species but are absent from *N. lactamica*, and also identified genes specific to *N. meningitidis* strains [36]. These pathogenic-specific sequences were of relatively small size and scattered throughout the genome rather than being clustered in pathogenicity islands.

Non-O1, non-O139 *V. cholerae* can cause gastroenteritis and extraintestinal infections, but, unlike O1 and O139 strains of *V. cholerae*, little is known about the virulence gene content of non-O1, non-O139 strains and their phylogenetic relationship to other pathogenic *V. cholerae*. In a recent comparative genomic microarray analysis of four pathogenic *V. cholerae* strains (non-O1/non-O139), it was shown that these strains are quite divergent from O1 and O139 strains. Genomic sequence analysis of a non-O1/non-O139 strain that appeared to be particularly pathogenic in experimental animals suggested that this strain carries a type III secretion system (TTSS). The genes for this *V. cholerae* TTSS system appear to be present in many clinical and environmental non-O1/
non-O139 strains, including at least one clone that is globally distributed. The presence of the TTSS in some pathogenic non-O1/non-O139 V. cholerae might be involved in the virulence and environmental fitness of these strains [37].

*Mycobacterium microti*, a member of the highly clonal *M. tuberculosis* complex, causes vole tuberculosis but is at least partially attenuated in other mammalian species including humans. Genomic comparisons of *M. microti* with *M. tuberculosis* identified a total of 13 deletions present in at least one of the twelve strains of *M. microti*, including ten deletions that were previously described for the human avirulent *M. bovis* [38]. Although the RD1 deletion is crucial for attenuation in *M. bovis* [39], only the RD3 deletion was present in all *M. microti* strains tested. The variation in the number of deletions in each strain might play a role in the different host ranges of the individual *M. microti* strains.

The molecular basis of the emergence of new virulent bacterial subclones is poorly understood and should prove a fruitful area for microarray-based research. For example, M3 GAS strains cultured from patients with invasive infection over an 11 year period that included two distinct infection peaks were analysed using genome-wide investigative methods including microarrays [40]. All variation in gene content could be attributed to the acquisition or loss of prophages, resulting in strains that had unique combinations of virulence genes. Distinct serotype M3 genotypes experienced rapid population expansion and caused infections that differed significantly in character and severity.

Limitations of microarray technology for comparative genome analyses

Despite the high throughput capability and potential applications of microarrays for comparative genome analysis, they have inherent problems as they are limited by the genetic information on the array. Most microarrays that are currently in use represent the genome of a single sequenced strain only. However, changes in the virulence potential of bacterial species are largely influenced by horizontally transferred DNA, therefore standard microarrays are unable to identify acquired DNA in diverse strains. As more genomes are sequenced, coupled with techniques such as suppression subtractive hybridisation, the interrogative capacity of arrays will be increased further and information on arrays could be used to investigate a given bacterial genus or mixture of genomes. The *S. aureus* pan-array currently produced by Bm@G@S (http://bugs.sgl.ac.uk/) represents the genome of seven sequenced strains.

Even with gene-specific microarrays, for some microbial species potential problems will occur with cross-hybridisation between gene fragments that represent different but very similar genes, meaning that genetic rearrangements, insertions, inversions and duplications are difficult to detect using spotted PCR products. The move towards using oligonucleotides as reporter elements rather than larger PCR products will increase the specificity of arrays for the individual target gene.

Further potential problems can occur when using mixed bacterial species for microarray analysis. Because of the range of G+C content in bacterial genes and genomes, hybridisation conditions have to be carefully considered as organisms with different G+C content will have differing hybridisation efficiencies to a given microarray. Additionally, microarrays that use amplified products will not identify point mutations or single nucleotide polymorphisms, although suitably designed high-density oligonucleotide arrays (eg: Affymetrix GeneChips®) are able to distinguish such mutations. However, oligonucleotide arrays remain expensive and, in the case of the Affymetrix system, the technology itself is proprietary. Currently, there are only a limited number of whole microbial genome arrays in this format. As the number of oligonucleotide arrays for different microorganisms increases and competition reduces the cost of these arrays, this technology platform should predominate.

Another stumbling block to reaping the benefits of DNA microarrays is the lack of globally accepted standardised bioinformatic methods to analyse the data generated. Standardised analyses of the data are crucial for interpretation and comparison across different studies. Simple concepts such as the presence or absence of coding sequences within a particular strain rely on how cut-offs are determined, a process that differs between laboratories. In the future, sustained improvements in software and computing speed, coupled with the application of improved mathematical models and Bayesian-based algorithms, will dramatically increase the scale of problems we tackle using microarray-based technology to understand the basic biology and evolution of bacterial pathogens.

Conclusions

Microarray analysis is a powerful enabling technology that allows global comparative analysis of the gene content between different strains in a given species and even between species in a genus. Comparative phylogenomics that use standard microarrays from a range of bacterial pathogens, such as *H. pylori, C. jejuni, E. coli* and *S. aureus*, clearly demonstrate the diversity and adaptability of these specialized groups of organisms. Studies have revealed much evidence of lateral gene transfer and recombination. This supports an evolutionary scenario involving vertical diversification by mutagenesis, punctuated by frequent lateral gene transfer, resulting in a global mosaic genome structure. By contrast, *M. tuberculosis*, *V. cholerae* and *Y. pestis* appear to exhibit limited genome diversity,
but nevertheless are adaptable enough for pandemic strains to emerge.

Recent advances in high-density oligonucleotide arrays have allowed for the development of high throughput resequencing techniques. Resequencing arrays are designed to cover the entire genome by overlapping oligonucleotides. Multiple versions of each oligonucleotide are spotted on the array to represent the four possible base combinations (A, T, G and C) for each nucleotide position. Competitive hybridisation of test and control strains to the array allows the identification of single nucleotide polymorphisms and small deletions or insertions that would be missed by standard microarrays. To date, this technique has only been applied to Bacillus anthracis, with 56 strains being resequenced using a custom-designed resequencing array [41**]. As this technique becomes more robust, many more bacterial species will be investigated in this way. The same technique has also been used to track the evolution of the severe acute respiratory syndrome coronavirus [42].

The development of a database of nucleotide differences among strains should allow the design of a universal microbial pathogen microarray that would have wide applications in the study of the epidemiology, population genetics, molecular phylogeny and evolution of microbial pathogens, as well as having diagnostic applications. Currently we are developing an ‘active surveillance pathogen’ microarray that consists of genes from mobile elements such as pathogenicity islands, phage and plasmid sequences; this will have multiple applications. The active surveillance pathogen array could be used as an ‘early warning system’ to allow identification of the emergence of highly transmissible or virulent strains that could, for example, be traced back to an individual, or a flock or herd, and then eliminated, thus averting the spread of an emerging virulent strain. No doubt the knowledge garnered from these and other microarray studies will be applied to well-designed intervention strategies to help reduce the burden of infectious disease.

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