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Transposon insertion sequencing: a new tool for systems-level analysis of microorganisms

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Abstract

Our knowledge of gene function has increasingly lagged behind gene discovery, hindering our understanding of the genetic basis of microbial phenotypes. Recently, however, massively parallel sequencing has been combined with traditional transposon mutagenesis in techniques referred to as transposon sequencing (Tn-seq), high-throughput insertion tracking by deep sequencing (HITS), insertion sequencing (INSeq) and transposon-directed insertion site sequencing (TraDIS), making it possible to identify putative gene functions in a high-throughput manner. Here, we describe the similarities and differences of these related techniques and discuss their application to the probing of gene function and higher-order genome organization.

In the mid 1970s, researchers surmised that transposons could be used as tools “to manipulate the genes of bacteria, phage and plasmids in ways which are otherwise difficult or impossible” (REF. 1). Transposons are genetic elements that can move within or between genomes by either replicative or ‘cut-and-paste’ mechanisms mediated by an enzyme called transposase. This enzyme recognizes the inverted repeats at the ends of the transposon and also recognizes the target sequence, in which it makes a double-strand break and inserts the transposon[2]. Transposons were originally discovered as “controlling elements” in maize by Barbara McClintock and have since been found in every kingdom of life[3]. They have important roles, such as in the evolution of speciation and antibiotic resistance in microorganisms[3,4]. The most frequently used application of transposons has been insertional mutagenesis, in which a library of bacterial strains, each containing a single randomly located transposon, is constructed. With the addition of transposons to the molecular toolbox, it became much easier to generate mutants and to identify their phenotypes by transductional crosses and complementation assays[5].

An enormous advance in the use of transposon mutagenesis was made after the first microbial genomes were sequenced[6–8]. For the first time, it became possible to directly link an observed phenotype to a genotype (for example, a gene disruption resulting from a transposon insertion) by sequencing of the genome. It is also now possible to sequence many transposon mutants simultaneously, thus allowing genome-wide analyses. At around the same time that this genome sequencing began, signature-tagged mutagenesis was invented[9],

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and together these methods established transposons as the most frequently used tool for genome-wide genotype–phenotype studies[10]. Such studies led to the identification of thousands of virulence genes in different bacteria, including *Salmonella enterica* subsp. *enterica* serovar Typhimurium[9], *Staphylococcus aureus*[11], *Vibrio cholerae*[12], *Streptococcus agalactiae*[13], *Listeria monocytogenes*[14] and *Streptococcus pneumoniae*[15–17].

With the advent of massively parallel sequencing (MPS; also referred to as second-generation sequencing)[18], microbial genomes continue to flood the public databases. Unfortunately there has been no corresponding explosion in our knowledge of gene function. Today, the situation is so challenging that if one were to sequence the genome of a new microorganism, the percentage of genes of unknown function would be similar to that obtained a decade ago (approximately 30–40%)[19–21]. To bridge this gap, we need high-throughput approaches that can reveal genotype–phenotype relationships and are applicable to a range of species. This void has now been filled by the development of a group of similar techniques, referred to here collectively as transposon sequencing, that combine transposon mutagenesis with MPS. The approach requires the construction of a transposon insertion library in which most or all non-essential genes contain insertions, followed by growth of the library in defined *in vitro* conditions or *in vivo* (infection of a host). The relative frequency of each mutant in the population is determined at the start and at the end of the experiment by means of MPS of the transposon junctions. From this data, the fitness contribution of each gene in each condition can be quantified.

Here, we describe the similarities and differences between the four main transposon sequencing approaches. We also discuss the emerging applications of this technology, such as the elucidation of higher-order genome organization, and the identification of small RNAs (sRNAs) and genes required for pathogenicity.

Transposon sequencing methods

With the recent advent of MPS technologies, it seemed only a matter of time before a genome-wide method that could accurately link genotypic changes to specific phenotypes became established. In 2009, four research groups independently published transposon sequencing methods for this purpose[22–26] (FIG. 1). MPS generates short sequence reads of millions of DNA molecules simultaneously, allowing whole genomes to be sequenced in a single experiment[27–29]. In addition, it is an effective technology for digital-counting applications, including RNA-seq (RNA sequencing)[30], sRNA-seq (sRNA sequencing) [31], ChIP–seq (chromatin immunoprecipitation followed by sequencing)[32], promoter assays[33], assessing histone occupancy[34] and, most recently, RNAi-target profiling[35]. The basic principle of all four transposon sequencing methods involves purification of genomic DNA from a pooled population of mutants, cleavage of the DNA (using either specific enzymes or random shearing), attachment of one or more adaptors to the DNA fragments to facilitate PCR amplification of the fragments containing transposon sequences, and finally, MPS of the amplified fragments to determine the location of the transposon and the relative abundance of mutants containing a transposon at this site.

The work described in each of the four original transposon sequencing papers was innovative and demonstrated the power and versatility of the technology by using different bacterial species to address different types of question. The high-throughput insertion tracking by deep sequencing (HITS) method was applied to a library of ~75,000 *Haemophilus influenzae* mutants[22]. In a mouse lung infection model, several virulence genes were identified, including those involved in lipopolysaccharide synthesis, metabolism and transport. Another group[23] generated a library of 370,000 *Salmonella enterica* subsp. *enterica* serovar Typhi mutants with, on average, an insertion every 13 bp in the genome.

Owing to this high coverage, the transposon-directed insertion site sequencing (TraDIS) method that was used was able to determine that ~8% of *S.* Typhi genes are essential for growth in a rich medium. In addition, this study identified genes with an advantageous or disadvantageous growth effect in rich media, as well as 169 genes that are involved in tolerance to bile, a substance that *S.* Typhi encounters when colonizing the human gall bladder. The insertion sequencing (INSeq) method[24] was used to determine whether the human symbiont *Bacteroides thetaiotaomicron* harbours specific genes that are necessary for survival in the colon. The data showed that colonization is partly influenced by microbial community composition and competition for nutrients such as vitamin B_{12}. The final method, termed Tn-seq (for transposon sequencing, following the terminology of RNA-seq, sRNA-seq and ChIP–seq), was applied to the pathogen *S. pneumoniae* to identify genes that are essential for growth in rich medium, to determine the fitness effect (advantageous or disadvantageous) of each non-essential gene and to identify 97 genetic interactions between five query genes and the rest of the genome[25].

Choice of transposon

Tn-seq and INSeq are nearly identical methods that make use of the Himar I *Mariner* transposon (FIG. 1). The teams that developed these two methods realized that a single nucleotide change in the terminal inverted repeats of *Mariner* introduced *Mme*I recognition sites[24,25]. *Mme*I is a type IIS restriction endonuclease that makes a 2 bp staggered cut 20 bases downstream of its recognition site[36]. Thus, when DNA from a library of transposon insertion mutants is digested with *Mme*I, fragments comprising the left and right transposon ends plus 16 bp of flanking genomic DNA are produced (the *Mme*I recognition site is located 4 bp before the end of each terminal repeat). This 16 bp is enough to accurately pinpoint the location of the transposon insertion in the bacterial genome. The 2-base overhangs facilitate the ligation of an adaptor; using a primer specific for this adaptor and another one specific for the transposon, the sequence is amplified by PCR, followed by agarose gel or PAGE purification to isolate the 120 bp product. Finally MPS is used to determine the flanking 16 bp sequences.

HITS[22] and TraDIS[23] are similar methods, and both use transposons that lack type IIS restriction sites within their inverted repeats. Therefore, to generate transposon end fragments, the DNA is sheared, after which several extra steps are necessary before an adaptor can be ligated (FIG. 1). In addition, affinity purification is carried out in the HITS method to remove extraneous DNA before sequencing. TraDIS uses a derivative of the Tn*5* transposon, which is commercially available and, similarly to the *Mariner* transposons used in the other three methods, is active in different species.

HITS and TraDIS have the advantage of being applicable to any transposon or insertional element. On the other hand, Tn-seq and INSeq have the advantages of a shorter sample preparation protocol and the generation of a final product of precise length. By contrast, the DNA shearing used in HITS and TraDIS results in a range of PCR product sizes, potentially allowing for PCR bias (shorter DNA templates are preferentially amplified over longer templates).

Data analysis

As well as the choice of transposon and the sample preparation method, data analysis is the other main difference between the four methods. All four are digital-counting methods, in which the number of sequence reads for a particular insertion corresponds to the frequency of that insertion mutant in the population. The change in insertion frequency after selection correlates with the fitness effect of the insertion mutation during the selection. Thus, if the frequency of a particular insertion does not change, this would indicate that the gene into

which the transposon is inserted is neutral (that is, it does not contribute to fitness (*W*)) in the condition tested. If a particular insertion disappears from the population, this would indicate that the corresponding gene is essential under the experimental conditions. Conversely, if a mutant increases in frequency, disruption of the gene is beneficial. There is a large gradient of fitness effects between neutral ($W = 1$) and essential ($W << 1$), so a precise and quantitative estimate of the fitness cost is crucial to capture such differences in fitness. Because of the digital and therefore highly accurate nature of MPS, a sensitive measure of fitness that correlates with the growth rate was developed[25]. By accounting for growth of the population during the selection, one can determine the effect of each disrupted gene on the growth rate (BOX 1). An example is shown in FIG. 2a of Tn-seq data for individual transposon insertions at three loci that are involved in pyrimidine biosynthesis in *S. pneumoniae*. By calculating the average fitness effect from all insertions within a gene (BOX 1; FIG. 2a), mutants that differ by as little as 5% in their growth rates can be accurately distinguished from each other. This means that it is possible to differentiate between, for instance, a wild-type strain and mutant strain that double every 35 and 37 minutes, respectively[25] (BOX 1). The other three methods estimate fitness from the change in the ratio of mutants over time. Although this provides a semiquantitative estimate of fitness, it is challenging to obtain robust statistical results and to compare fitness values across experiments and environments.

Applications of transposon sequencing

The development of transposon sequencing and its compatibility with non-model organisms mean that large-scale, quantitative phenotypic profiling is now possible for any microbial species to which transposon mutagenesis can be applied. In addition, with the amount of MPS data that can be obtained per experiment rapidly increasing (it currently stands at ~10^8 reads on the Illumina HiSeq 2000) and the ability to multiplex samples by incorporating a barcode within the adaptor[25], the cost of doing large-scale screens has decreased dramatically. Below, we highlight various biological questions that can be addressed using transposon sequencing.

Identification of new gene functions

Several groups have recently begun to sample the vast ‘genotype versus environment’ space with transposon sequencing. By screening in several *in vitro* conditions, phenotypes are revealed that help in identifying new gene functions. For example, transposon sequencing has been used in *Pseudomonas aeruginosa* to identify antibiotic resistance genes[37]. In this study, Tn-seq was modified slightly: after genomic shearing and adaptor ligation, the Tn*5* transposon was enzymatically cut and, together with the genomic DNA flanking the transposon, circularized in a ligation step. Non-circularized products were then removed by exonucleases, and the sequencing target was amplified by PCR. Tn-seq has also been used in *Mycobacterium tuberculosis*[38] to identify essential genes and pathways that are involved in the utilization of cholesterol (a crucial carbon source for *M. tuberculosis* during infection). Furthermore, a number of conditionally essential genes (genes that are essential for growth under specific conditions) have been identified in *S.* Typhimurium using Tn-seq[39]. Transposon sequencing was also used to identify an alternative citrate synthase and a novel dynamic branching pathway of the anaerobic tri carboxylic acid cycle in *Shewanella oneidensis*[40], and to identify essential genes in the periodontal pathogen *Porphyromonas gingivalis*[41]. Finally, by screening 17 different *in vitro* growth conditions for *S. pneumoniae*, more than 1,800 genotype–phenotype interactions were discovered[42]. From this data set, genes involved in differential carbon source utilization and in resistance against specific stresses were identified. The data from this study were used to carry out an overlap analysis, which involves combining condition-specific *in vitro* data with *in vivo* virulence data to reveal previously unknown selective pressures that *S. pneumoniae* encounters in specific

niches in the host[42]. At the gene level, the overlap analysis also served to provide leads on gene function for many hypothetical genes.

Identification of virulence genes

The original HITS[22] and INSeq[24] studies examined the fitness contribution of genes using infection and colonization models, respectively, and the application of these techniques for such studies has since grown. For example, a re-analysis of an *Escherichia coli* transposon library in cattle revealed that transposon sequencing is superior to the original signature-tagged mutagenesis analysis in terms of sensitivity, allowing for the identification of hundreds of additional virulence genes[43,44]. Another study[45] identified 17 genes that are essential for the growth of *Yersinia pseudotuberculosis* inside a mouse host. The authors of this study went on to show that MrtAB, a previously uncharacterized efflux pump, is essential for *Y. pseudotuberculosis* colonization of the mesenteric lymph nodes.

Such *in vivo* screens are extremely valuable for discovering previously unrecognized virulence mechanisms and for probing pathogen metabolism. However, a limitation to the use of transposon sequencing methods *in vivo* is that a substantial number of bacteria are often killed or removed stochastically during the establishment of infection. Such population bottlenecks are problematic because it is difficult to determine whether transposon mutants disappear by chance or because they are less fit. In such cases, it is necessary to adjust the number of different mutants present in the inoculum (that is, the complexity of the inoculated population) so that it is less than the bottleneck. For example, in an infection model that allows 10^4 bacteria to colonize the host regardless of inoculum size (thus, with a bottleneck equal to 10^4), an inoculum containing less than 10^4 unique transposon mutants should be used (from experience, we recommend a range of 10–100 cells per insertion mutant). By separately analysing a set of neutral insertions that should not disappear from the library (because the transposon has a neutral impact on fitness; for example, insertions in degenerate genes), it is possible to estimate such bottlenecks on a per animal basis and correct for them in the fitness calculations. This strategy, in combination with a method to calculate the bacterial generation time *in vivo*, facilitates a sensitive genome-wide analysis of fitness from *in vivo* experiments[42] (BOX 1).

Using transposon sequencing in several *in vivo* environments has the potential to reveal the requirement for specific genes in specific niches. For example, by applying Tn-seq to *S. pneumoniae*, it was found that the core biosynthetic genes for pyrimidines and purines have differential roles in a mouse lung infection model and in a nasopharynx model of asymptomatic colonization[42] (FIG. 2b). The genes in the inosine monophosphate (IMP) pathway, which are required for the synthesis of purines from glutamine, are important for survival in both niches, whereas the genes involved in synthesis of uridine monophosphate (UMP) and pyrimi-dines from glutamine are important only for survival in the nasopharynx. Further analysis showed that *pyrR* (*SP1278*), which is required for the alternative pathway of UMP synthesis from uracil, is important in the lung. This example illustrates two key points. First, *in vivo* niches present different challenges that the bacterium must cope with, and thus impose different selective pressures; in the nasopharynx, UMP is preferably synthesized from glutamine, whereas in the lung, it is synthesized through uracil by means of PyrR. Second, the essentiality of metabolic pathways differs according to the type of *in vivo* niche, and this in turn necessitates different treatment strategies. In the example described, although disabling the IMP pathway would reduce bacterial survival in both niches, inhibition of the core UMP pathway (synthesis from glutamine) would reduce the ability of the bacterium to colonize the nasopharynx, but would not compromise the ability of *S. pneumoniae* to cause pneumonia.

Genetic interactions uncover higher-order genome organization

The identification of a genetic mutation that confers a phenotype is only the first step towards understanding the function of the gene and its broader genetic context. Genes do not function in isolation and are typically integrated in highly interconnected networks. Such genetic networks can be deciphered by screening for genetic interactions between genes, wherein the combination of two mutations in a double mutant results in a strain with a fitness value that deviates from the expected fitness value given by the product of fitness values for each single mutant (that is, $W_{ij} \neq W_i \times W_j$). Such dependencies between genes have been extensively explored in *Saccharomyces cerevisiae* and have revealed the functional relevance, organization and transcriptional regulation of individual genes and pathways involved in diverse cellular processes[46–55]. Genetic interaction networks can also be explored using transposon mutagenesis by creating a transposon library in a strain that lacks a gene of interest (the so-called query gene). Previously, genetic interactions were successfully identified in *M. tuberculosis*[56] and *E. coli*[57] using DNA microarray-based tracking of transposon insertions. Recently, Tn-seq was used to elucidate genetic interactions between five query genes and the rest of the genome in *S. pneumoniae*, revealing both aggravating and alleviating genetic interactions[25]. One of the query genes, the catabolite control protein A gene (*ccpA*), was shown to interact with 64 other genes, suggesting that *ccpA* is a master regulator of complex carbohydrate metabolism in this pathogen.

Data from genetic interaction mapping can readily be combined with other types of data such as protein–protein interaction data or gene expression data to reveal the higher-order genome organization. For example, a genetic interaction screen was recently combined with gene expression data to determine the regulatory relationship between the response regulator SP2193 (a type of transcription factor) and the core pyrimidine biosynthesis genes[42]. Although mutations in any of the genes leading to UMP synthesis in the pyrimidine branch caused a severe fitness defect in glucose medium, these defects were fully alleviated by mutations in *SP2193* (FIG. 2a). Further analysis confirmed that SP2193 is a positive regulator of the genes in this pathway, leading to the hypothesis that mutations in any one of the genes in this pathway result in a loss of energy or in toxicity owing to the accumulation of toxic intermediates[42].

Examining the role of non-coding DNA regions

Transposon sequencing is not restricted to screening for the fitness effects of gene knockouts. In one study[58], this technology was used to construct a dense library of *Caulobacter crescentus* insertion mutants (with 8 bp resolution). In addition to the identification of essential genes, 402 regulatory sequences and 130 non-coding elements from intergenic regions were also shown to be important for growth. Intergenic regions required for optimal growth were identified in *M. tuberculosis*[59] using a similar approach. By combining RNA-seq with Tn-seq, 56 new putative sRNAs in non-coding regions of *S. pneumoniae*[60] were identified. RNA-seq was carried out to ascertain the complete transcriptional activity of the genome and to predict the presence of sRNAs in intergenic regions. Subsequently, Tn-seq was used in mouse models of colonization (infection of the nasopharynx), pneumonia (infection of the lung) and bacteraemia (intravenous infection) to reveal the contribution of the predicted sRNAs to fitness *in vivo*.

Transposon sequencing in mammalian cells

A method based on the same principle as transposon sequencing has been developed for loss-of-function screens in mammalian cells. Because mammals have two copies of each gene, inactivation of one gene copy rarely leads to a severe phenotype; thus, compared with haploid organisms, cell lines derived from mammals are relatively robust in their tolerance

to gene loss. In principle, transposon mutagenesis is therefore not an effective way to screen for genotype–phenotype relationships in diploid cells. However, using a derivative of the human KBM7 chronic myeloid leukaemia cell line, which is haploid for all chromosomes except chromosome 8, the phenotypic effects of insertion mutations are testable[61]. This so-called haploid genetic screen has been used to identify genes that are important for infection by influenza viruses, for the biosynthesis of diphth amide (which is required for the cytotoxic effects of diphtheria toxin and exotoxin A) and for the action of cytolethal distending toxin (including the gene encoding a cell surface protein that interacts with the toxin)[61]. This approach has also been used to screen for and identify other host factors, including the membrane receptor that mediates uptake of *Clostridium difficile* toxin[62], and several genes that are important for attachment of *Chlamydia trachomatis* to its target cell[63].

Conclusions and perspectives

Owing to the high-throughput nature of transposon sequencing and the sensitivity of this technique to even small fitness differences, this approach has emerged as the method of choice for examining genotype–phenotype interactions on a large scale. When used in a variety of environmental conditions, the technology has the potential to reveal putative functions for most non-essential genetic elements within an organism. It has been suggested that transposon sequencing methods lack the usefulness of ordered gene knockout arrays, the most important argument being that it is nearly impossible to fish out a particular gene knockout from a transposon insertion library. However, it has been shown that a transposon insertion library is conveniently turned into an ordered gene knockout array using robotic arraying of strains followed by MPS[24].

Despite the applicability of transposon sequencing, the technique has limitations. The gene function leads must be followed up with other, often lower-throughput approaches (for example, microscopy and biochemical studies) to determine the details of how the gene functions. In addition, although we have reached high sensitivity and can obtain a quantitative measure of fitness, there is room for improvement. Fitness differences of less than 5% are not detectable with the current methods, but they are important from the standpoint of population biology and evolution.

Transposon sequencing has the potential to be used for other purposes. For example, probing for differences in genotype–phenotype interactions between strains or closely related species has the potential to reveal divergent evolutionary trajectories for shared genes or pathways, such as functional pathways that have undergone reconfiguration either in terms of their regulation or to accomplish new tasks. Although transposon sequencing is ideally suited to bacterial strains for which the complete genome sequence is available, it should work nearly as well in strains with unfinished (draft) genomes.

Although it has been applied almost exclusively to bacterial species, transposon sequencing could be expanded to study other microorganisms. For example, it could be used to probe gene function and gene networks in viruses. All that is needed is a means of introducing transposon insertions into the viral genome. This could be accomplished by building an inducible transposition system within the host, such that insertions are introduced into the viral genome during viral replication. Alternatively, for viruses that are able to establish infection after transformation, transposon insertions could be introduced into a cloned copy of the complete or partial viral genome, such as on a bacterial artificial chromosome. It might also be possible to extend transposon sequencing to diploid mammalian cell lines using either transposons, or retroviral or lentiviral vectors, and to rely on haploinsufficiency to produce phenotypes. Haploinsufficiency generally results in mild phenotypes, but the sensitivity of transposon sequencing might allow the detection of such phenotypes.

One of the most difficult environments to probe is the infected mammalian host, wherein host–pathogen interactions are complex and dynamic. Although transposon sequencing has been used to identify genotype–phenotype interactions in the pathogen, little has been learned about the host environment per se, such as the host parameters that influence pathogen behaviour. However, it should be possible to use transposon sequencing to probe the host environment through the 'eyes' of the pathogen. For example, it would be possible to compare gene fitness data for a pathogen following infection of a wild-type host and an immune-deficient host. Because the pathogen would be the constant and the host the variable in this scenario, differences in pathogen gene fitness would help to identify host genes that are involved in pathogenesis. Analyses of this type could easily be expanded to other types of change in the host, such as diet, environment and chemical or drug treatment. In conclusion, the application of transposon sequencing has proved to be highly valuable for the probing of gene function, and further technological advances are likely to see it continue to be applied as a useful, high-throughput screening method for some time to come.

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Glossary

Bacterial artificial chromosome	A bacterial plasmid that contains a large eukaryotic DNA insertion (typically >150 kb) and can be used for cloning, genetic manipulation and transformation.
ChIP–seq	(Chromatin immunoprecipitation followed by sequencing). A method that uses crosslinking of a protein to DNA followed by immunoprecipitation of the complex and subsequent sequencing of the bound DNA to reveal the binding site of the protein.
Complementation assays	Assays in which a wild-type copy of a gene is reintroduced into a cell or organism that lacks the gene. This can confirm that the phenotype is caused by disruption or deletion of the gene in question, and that this phenotype can be reversed.
Degenerate genes	Genes that once had a function, but through the accumulation of mutations, became inactive.
Digital-counting applications	Methods that count the total number of reads obtained for a particular sequence after massively parallel sequencing (in contrast to hybridization-based methods of quantification).
DNA microarray	A glass slide (or other surface) on which oligonucleotides or PCR products of defined sequence are spotted. These microarrays are used to quantify the nucleic acids within a sample by hybridization.
Haploinsufficiency	The inability of a single functional copy of a gene to produce a wild-type phenotype in a diploid organism. This occurs when the second copy of the gene is inactivated by mutation.
Overlap analysis	An analysis that indicates a putative function for a hypothetical virulence gene using fitness data for the gene obtained during growth in defined *in vitro* conditions.

Promoter assays	Assays that measure the transcriptional activity of a gene promoter by converting the RNA transcripts to cDNAs and then using massively parallel sequencing to determine the number of cDNA molecules present.
Signature-tagged mutagenesis	A technique in which a transposon is tagged with a specific DNA sequence (a bar-code) that is used to determine the presence of the transposon in a DNA pool (as the amplified and labelled tag hybridizes to a probe on a membrane).
sRNA-seq	(Small-RNA sequencing). The discovery of non-coding sRNAs through direct sequencing of their cDNAs by massively parallel sequencing.
Transductional crosses	Experiments in which DNA is transferred from one bacterium to another by means of a bacteriophage.
Type IIS restriction endonuclease	An enzyme that cleaves DNA at a defined distance from an asymmetrical recognition site.

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Box 1 | Measuring fitness quantitatively

One approach that is commonly used to estimate the fitness cost of a mutation relies on calculating a competitive index (CI), which involves growing the mutant in competition with the wild-type strain. The CI is calculated by dividing the proportion of mutant cells at the end of the competition by the proportion at the start. In the depicted hypothetical example (see the figure, part **a**), the CI becomes smaller over time. The CI is thus a relative measure of growth, and its value depends on when the measurement is taken. In a quantitative sense, the CI is therefore not particularly informative; it can provide only a semiquantitative and time-dependent estimate of the fitness cost associated with a mutation. Growth curves for the same wild-type and mutant strains grown individually are shown (see the figure, part **b**), along with the exponential growth equations that define their growth (in which *wt* and *mt* are the number of wild-type and mutant cells, respectively; W is the growth rate; and t is time). The only difference between the equations is W; here, $W_{wt} = 1$ and $W_{mt} = 0.5$. In contrast to the CI, the growth rate is independent of when the measurement is taken and is therefore more meaningful; the mutant shown in this particular example grows half as fast as the wild type ($W_{wt} / W_{mt} = 2$). The most important assumption that is made here is that the measurements are taken during exponential growth; however, this is an assumption that holds for any calculation, including the CI (the carrying capacity, which refers to the maximum number of individuals that can be supported by the environment, is omitted from the equations, for simplicity).

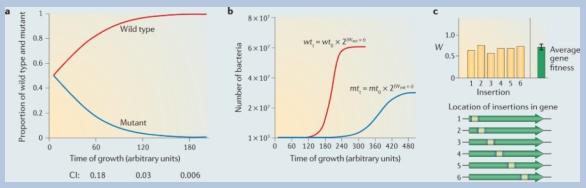
In order to extract this same value (that is, W) from a competition experiment, the following equation can be used[64]:

$$W_{mt} = \frac{\ln\left[N_{mt(t2)} \times d / N_{mt(t1)}\right]}{\ln\left[\left(1 - N_{mt(t2)}\right) \times d / \left(1 - N_{mt(t1)}\right)\right]}$$

$N_{mt(t1)}$ and $N_{mt(t2)}$ are the proportions of the mutant in the population at the start (t_1) and the end (t_2) of the competition, respectively, and d represents the growth of the entire population over the course of the competition (number of all bacteria at t_2 / number of all bacteria at t_1). Applying this equation to the competition experiment shown in the figure, part **a**, provides growth rate estimates that are consistent with those obtained from the growth curves in the figure, part **b**[25]. Extrapolating to a transposon sequencing experiment in which tens of thousands of mutants are competing against each other at the same time works in the same way. The frequency of each mutant in the population is determined by massively parallel sequencing at t_1 and after selection, at t_2. In addition, d is determined by simply dividing the number of bacteria in the entire population at t_2 by the number of bacteria at t_1.

To be able to apply the same analysis to infection experiments in which growth of the bacterial population is not easily determined, a plasmid segregation system was used to estimate net growth[42]. By transforming *Streptococcus pneumoniae* with a temperature-sensitive plasmid that does not replicate above 30 °C and thus can be lost by segregation during growth at physiological temperatures, the replication rate of the bacterium can be estimated from the proportion of bacteria that maintain the plasmid. The rate of plasmid loss from the bacterial population *in vivo* is affected by both growth of the bacterial population and clearance of bacteria from the host (clearance can occur through the host immune system and theoretically should be unaffected by the presence or absence of the plasmid), and thus bacterial population[65] and the population doubling time. d can then be determined from the following equations: infection time / doubling time = number of

generations (n), and $d = 2^n$). As an initial control, it was confirmed that *in vitro* estimates of the population growth rate could be accurately determined[42]. However, the complexity of *in vivo* environments might introduce unforeseen variation in the rate of plasmid loss, and this could make fitness estimates less accurate. *In vivo* validation of single knockout mutants indicated that fitness estimates were reasonably accurate, confirming that the approach was successful, at least under the tested conditions. This method was used to determine average gene fitness in a Tn-seq experiment (a type of transposon sequencing; see the figure, part **c**). To calculate fitness for a single gene, fitness must first be calculated for each transposon insertion, followed by averaging over all the insertions found within that gene to obtain a single fitness value (including a standard deviation). More than 150 genotype–phenotype relationships and genetic interactions obtained from *in vitro* and *in vivo* Tn-seq experiments have been validated using standard one-on-one competitions and growth curves[25,42]. Thus, this approach generates a highly meaningful quantitative fitness value that is reproducible across experiments and is a close approximation of the growth rate.



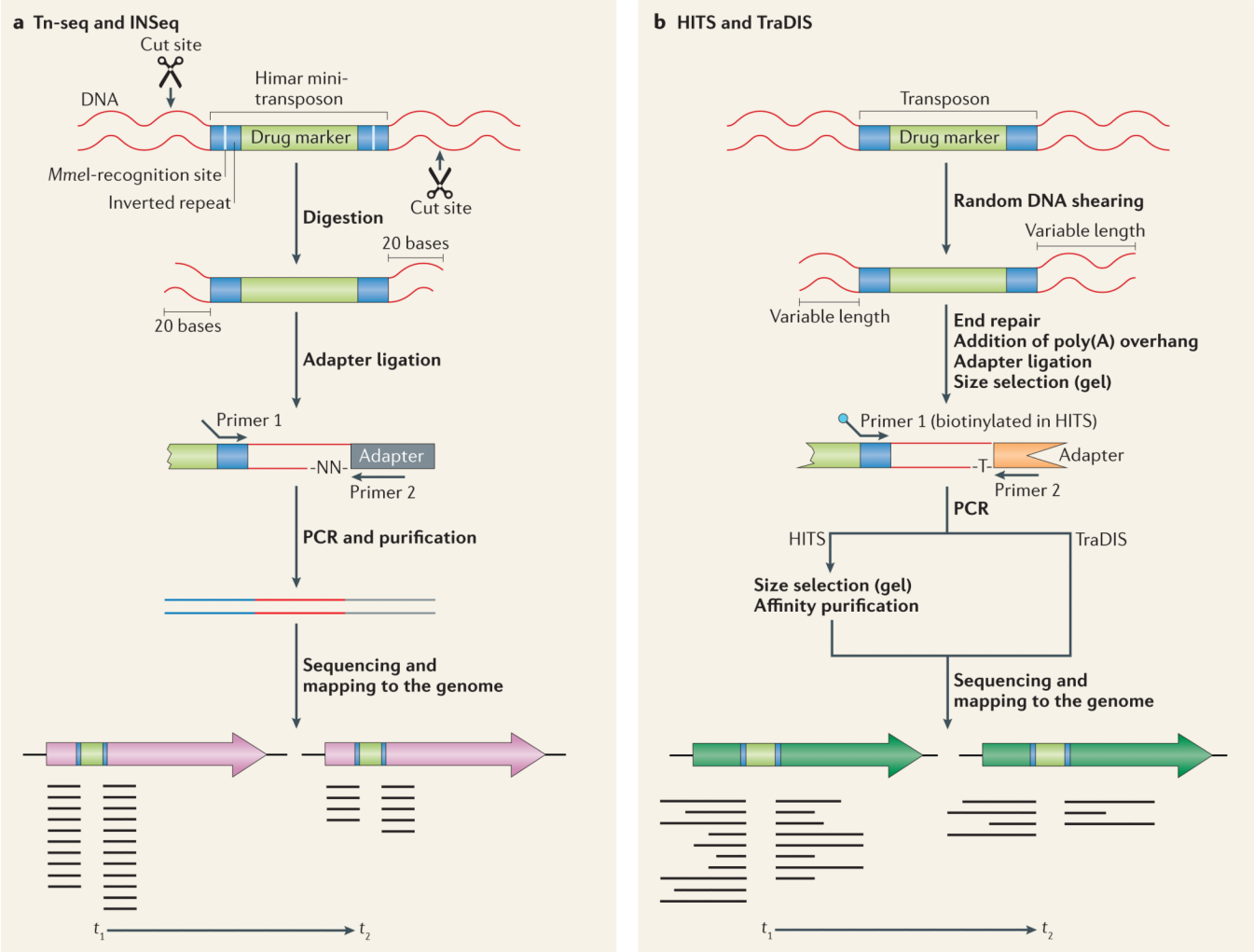


Figure 1. Four methods of massively parallel sequencing of transposon insertions

Each of four methods of transposon sequencing is illustrated, starting from pooled genomic DNA from the transposon insertion library and ending with sequencing of the left and right transposon junctions. The number of sequences (reads) for each junction can differ between the start of the experiment (t_1) and the end (t_2), after a selection has been carried out on the library of transposon insertion mutants. In both examples shown, the transposon insertion mutation decreases fitness during growth under the conditions tested, as indicated by there being fewer reads at the end of the experiment than at the start. **a** | The Tn-seq (named for transposon sequencing) and insertion sequencing (INSeq) methods are highly similar, but INSeq includes a PAGE gel purification step following adaptor ligation and PCR, whereas Tn-Seq includes an agarose gel purification at this point. A recent study[66] introduced additional steps to the original INSeq protocol: a linear-PCR step using a biotinylated primer and subsequent purification of the product with magnetic streptavidin beads were added following adapter ligation. These steps reduce both the amount of sample and the amount of enzymes needed. Although they make the protocol more laborious, the results suggest that these modifications increase the sensitivity of the technique. **b** | The high-throughput insertion tracking by deep sequencing (HITS) and transposon-directed insertion site sequencing (TraDIS) methods are more similar to each other than to Tn-seq and INSeq: after shearing of the DNA, the DNA ends are repaired, and a poly(A) tail is added. However, the methods diverge after the PCR step; in HITS, the PCR products undergo size selection (on a

gel) and affinity purification before sequencing, whereas in TraDIS, the PCR products are sequenced directly.

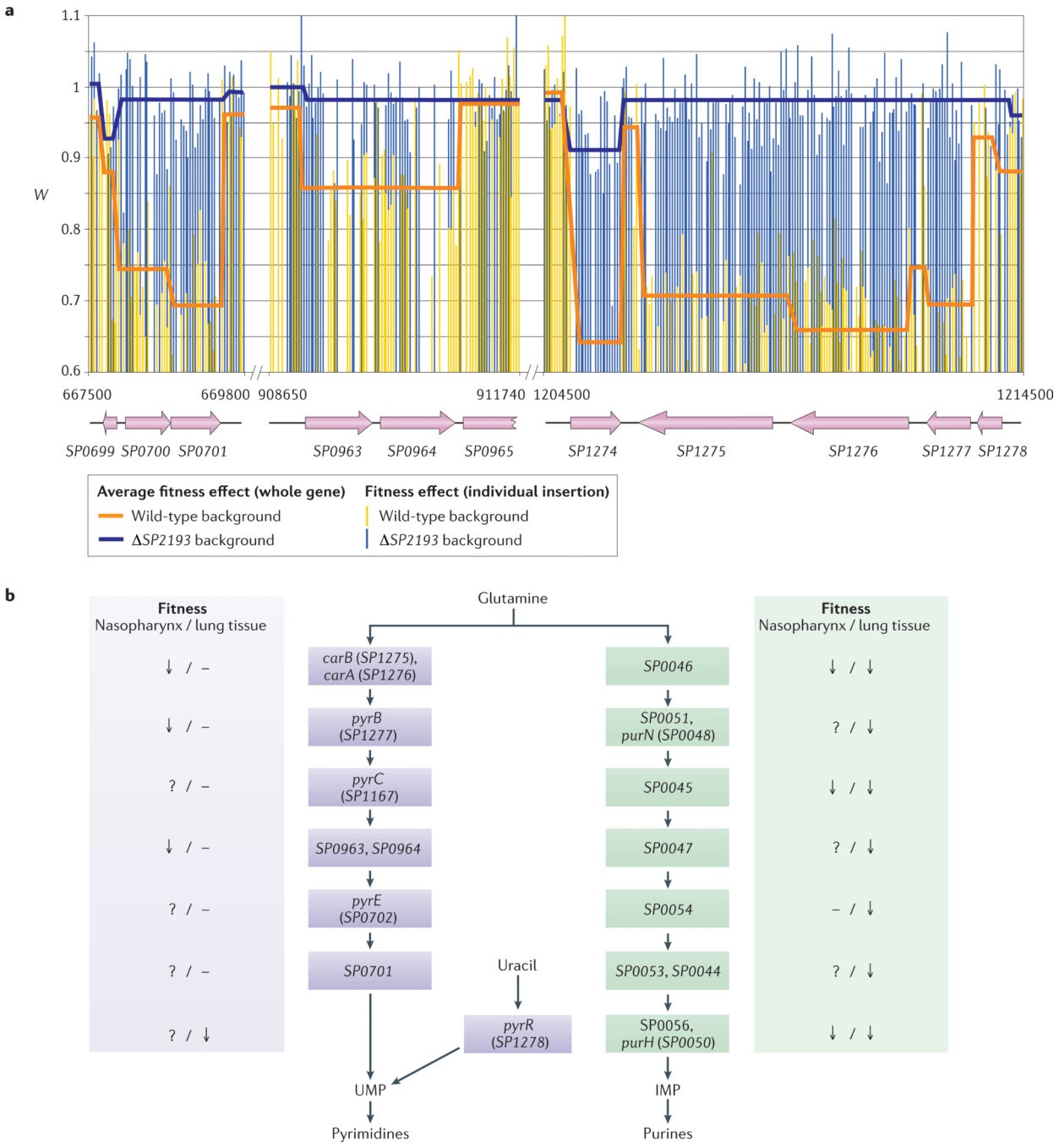


Figure 2. Pathway analysis *in vitro* and in different host niches

a | Fitness (*W*) of individual *Streptococcus pneumoniae* transposon insertion mutants with insertions in three loci involved in pyrimidine biosynthesis. The genes and genome coordinates of the insertions are indicated. *In vitro* fitness was calculated during growth in a semi-defined minimal glucose medium[42]. The data show that *SP2193*, which encodes a response regulator (a type of transcription factor) and positive regulator of pyrimidine biosynthesis genes, is a suppressor of the fitness defects that result from insertion mutations in each gene involved in the pyrimidine biosynthesis pathway, as determined by genetic interaction analysis. **b** | Three metabolic pathways in *S. pneumoniae* lead to the production of inosine monophosphate (IMP) and uridine monophosphate (UMP), which are essential

precursors for purine and pyrimidine synthesis, respectively. The effect of disrupting each gene in these three pathways, in terms of the change in bacterial fitness, is indicated for infections of the nasopharynx and of the lung. A question mark indicates that there is no data available. The pathway leading to the synthesis of IMP and purines is required for colonization of the nasopharynx and lung. For the synthesis of UMP and pyrimidines, the pathway that uses glutamine as a precursor is required only for colonization of the nasopharynx, whereas the alternative pathway, which uses uracil as a precursor, can be used for UMP synthesis in the lung.