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Sex to the Rescue: A classical solution to a cutting-edge problem

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A comprehensive understanding of the cellular functions of every gene and gene network is a primary goal of the post-genomic era. Analysis of double-mutant phenotypes has long been appreciated as a powerful approach for realizing this goal. In this issue of Nature Methods, two studies present new approaches for applying the power of whole genome double-mutant analysis to the bacterium *E. coli*[1,2].

Genetic interactions such as synthetic lethality (a term coined by Theodosius Dobzhansky in 1946 [3]) can reveal related or redundant function of two gene products in an essential cellular process, and tests of epistasis (a term coined by William Bateson in 1909[4]) can reveal order in signal transduction or metabolic pathways. More recently, quantitative phenotypic analysis has been used to reveal both positive or negative genetic interactions that are more subtle than lethality[5], and technical advances, first pioneered in yeast, enabled the power of double-mutant analysis to be extended to the whole-genome scale [6]. By grouping genes of unknown function with genes of known function, genome-wide studies connect genes to processes, thereby improving our understanding of both the functions of specific genes and the molecular underpinnings of specific processes. Since the information to be gleaned from such genome-wide genetic interactions depends on having an existing understanding of a significant subset of genes, there is a particular benefit to applying this approach to a well-characterized model system such as *E. coli*.

Although a collection of single-gene knockout *Escherichia coli* mutants has been available for some time (the Keio collection [7]), there seemed no obvious way to construct double mutants on a genome-wide scale. *E. coli* geneticists typically transfer genes from one strain to another by transduction, a rather inefficient phage-mediated process that did not seem suitable for semi-automated use with thousands of strains. Large-scale construction of double mutants for genome-scale genetic interaction analysis was originally pioneered in yeast by exploiting their ability to mate [6]; two haploid cells of different mating types can fuse to form a diploid zygote. Bacterial mating, known as conjugation, was discovered in *E. coli* 62 years ago by Lederberg and Tatum [8]. However, bacterial mating is different from yeast mating in that the two bacterial mating types (Hfr and F^-) have fundamentally different roles[9] and there is no stable diploid state. The Hfr strain behaves solely as a donor, transferring its circular genome as single-stranded DNA into the F^- cell, which behaves solely as a recipient (Figure 1). Linear transfer of DNA occurs at a rate of about 45,000 base pairs per minute, and it takes roughly 100 minutes to transfer the entire Hfr chromosome into the F^- [10]. In other words, the conjugal act can last four to five times longer than the lifetime of a typical individual. Although titillating, this fact, coupled with the fragility of mating pairs, means that complete zygotes are almost never formed unless great care is taken; “coitus interruptus” is a very common occurrence. Nonetheless, in the new approaches presented in this issue, both research groups have worked out high-throughput protocols that use conjugation for the efficient construction of double mutants on a genome-wide scale. They both solve the coitus interruptus problem by allowing mating to occur on a solid agar surface, where mating pairs can be left in peace long enough to complete the conjugal union.

Typas et al. term their method GIANT-coli (Genetic Interaction Analysis Technology for *E. coli*) and Butland et al. term their method eSGA (*E. coli* Synthetic Genetic Array). The two approaches are conceptually similar. They both use an Hfr strain carrying a disruption mutation in the gene of interest. This Hfr strain is then mated with the Keio collection, an isogenic (genetically identical) set of F^- strains carrying single-gene disruptions of all nonessential genes. The mating is allowed to occur on non-selective media for approximately a day, which is plenty of time to produce a significant number of complete zygotes.

Diploid zygotes produced by bacterial mating are unstable, and stable haploid daughter cells arise from them by homologous recombination. This allows inheritance of genetic markers from both Hfr and F^- parents (Figure 1) at more or less equal probability so long as the genetic markers are not positioned too close to each other on the chromosome. Each parental mutant is marked with an antibiotic resistance cassette, allowing antibiotic selection to be used to identify the desired double mutant. Both groups find empirically that genetic interactions between genes closer than 30,000 base pairs cannot be reliably analyzed without further effort. Despite their similarity, the two groups use somewhat different specific protocols to generate double mutants efficiently, with low rates of false positives or negatives. Time and experience will determine which protocol is preferred by practitioners.

What is critical is that a relatively uniform number of doubly mutant, recombinant, daughter cells is pinned onto selective media containing both antibiotics in the final step. The size of the resulting colony is proportional to the growth rate of the double mutant, and this is what allows meaningful comparisons of double mutant growth. Software similar to that used to analyze colony size of yeast double mutants is then used to analyze the data [5]. Subsequent normalization of colony size for variations in growth between different plates and different parent strains helps to identify meaningful genetic interactions.

To avoid complications that can arise from mating different strain backgrounds, Typas et al. custom-engineered Hfr strains that are isogenic to the genomic deletion library. As proof of principle, they performed all possible pair wise crosses with 12 mutant genes and examined their growth in both rich and minimal media. The genes chosen all specify cell envelope proteins, increasing the probability of finding genetic interactions. Some of the genes chosen were known to interact, and these served as positive controls. Even in this small set, several new interactions were identified and different interactions were observed in the two media, establishing the importance of analyzing interactions in different contexts. Data obtained by extending this approach to the entire non-essential knockout collection allow Typas et al. to propose an unexpected role for the *pal* gene product as a central organizer of cell envelope functionality.

Butland et al. focused on crosses between mutants in two well-characterized, functionally-redundant Fe-S cluster biosynthetic pathways for their proof-of-principle experiments. The expected genetic interactions were readily apparent. In addition to the Keio knockout collection, this group also added 149 strains in which an essential gene is marked with an affinity tag, reasoning that they might represent partially-compromised (hypomorphic) alleles. Indeed, a high proportion of the detected genetic interactions involved a tagged essential gene. By performing two-dimensional hierarchical clustering on the data from 39 genomic interaction screens, the two known Fe-S cluster biosynthetic pathways could be separately identified. This analysis also grouped the known pathway members with a number of new genes. Further experiments suggested that at least one such additional gene, *ydhD*, indeed represents a novel member of the Suf pathway.

Solving the problem of generating double mutants in high throughput in *E. coli* arms the modern microbiologist with a powerful new tool for connecting genes to functions and networks. In

addition to the analysis of synthetic interactions, Typas et al. provide a pilot example of how this same strategy can be used to screen for enhancers and suppressors of conditional mutants. Since conjugation works in many bacterial species, including most Gram-negative bacteria, these methods also pave the way for applying genetic interaction analysis to additional bacteria. In all model systems, the results of double-mutant analysis are highly dependent on the architecture of the specific pathways examined, especially when dealing with potentially hypomorphic alleles, in which gene function is not completely eliminated. Technical improvements in the ability to assay growth and other phenotypes will improve the utility of these approaches. However, the real remaining challenge is to improve the experimental and computational methods that are necessary for extracting and validating meaningful insights from the extreme amount of data generated by this approach. Perhaps the solutions to these new challenges could also benefit from rejuvenating classical technologies.

Joshua Lederberg passed away this year. We wish he was still with us to witness how the use of his Nobel-Prize winning discovery opened the door for a comprehensive analysis of genetic interactions in *E. coli*.

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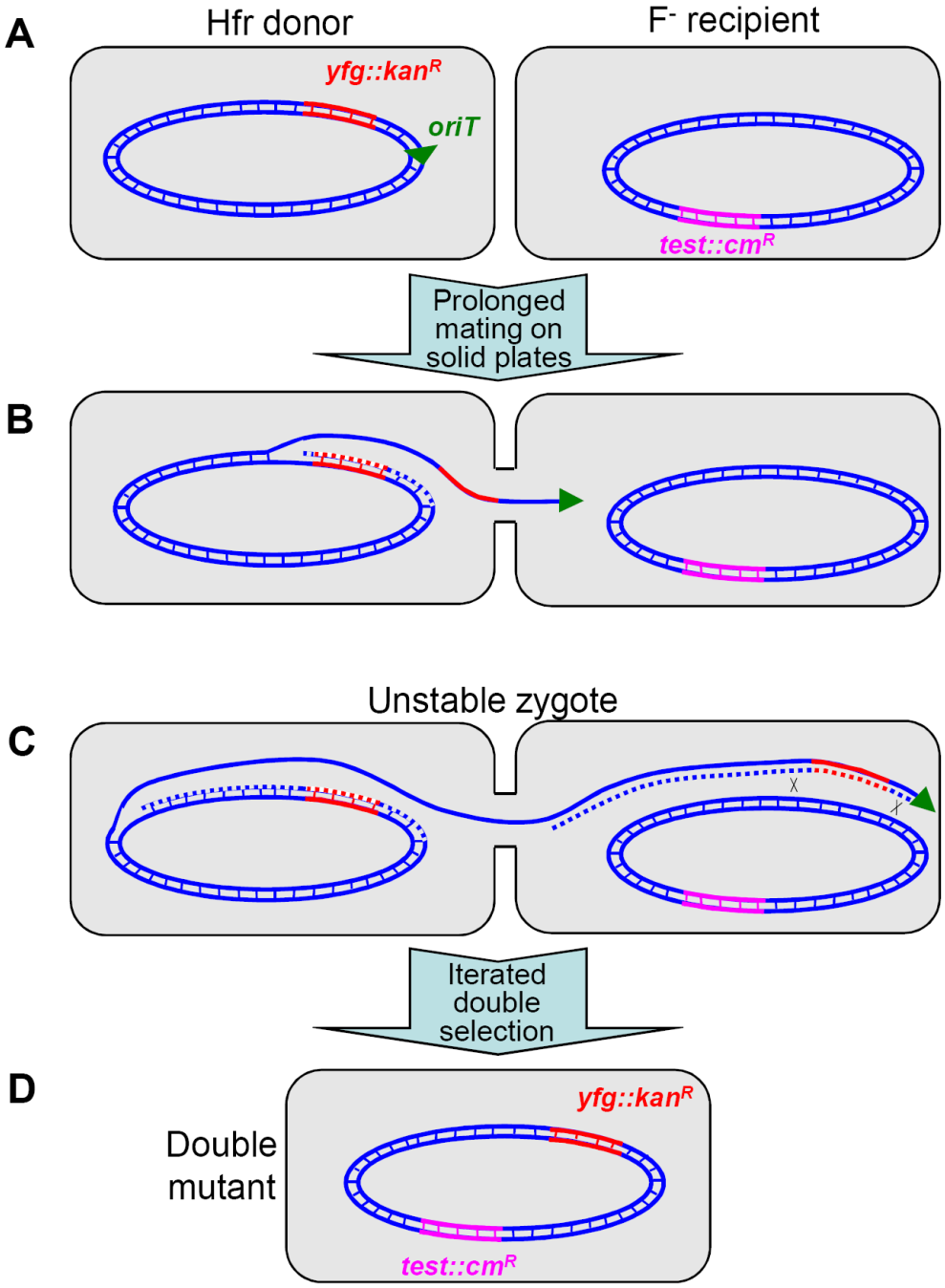

Figure 1. Creating double mutants in *E. coli* by Hfr conjugation

A. The Hfr strain genome (blue) carries the origin of transfer from the F plasmid (*oriT*, green) and a disruption of your favorite gene that is marked with an antibiotic resistance cassette (*yfg::kan^R, red)*. The F$^-$ strain has a disruption in a second test gene that is marked by a different antibiotic resistance cassette (*test::cm^R, purple)*. B and C. During conjugation, the Hfr chromosome is nicked at *oriT* and single-stranded DNA is transferred into the F$^-$ cytoplasm. DNA replication in both parents (newly-synthesized DNA is denoted with dashed lines) generates an unstable zygote. Both groups use prolonged mating on solid plates to prevent "coitus interruptus", thereby reducing complications associated with the distance between

oriT and *yfg::kan*^{R}. D. Two rounds of selection yields a stable population of the desired double mutant strain.