

Perspectives

Anecdotal, Historical and Critical Commentaries on Genetics

Edited by James F. Crow and William F. Dove

REPLICA PLATING AND INDIRECT SELECTION OF BACTERIAL MUTANTS: ISOLATION OF PREADAPTIVE MUTANTS IN BACTERIA BY SIB SELECTION

HISTORICALLY and conceptually, the two themes in these titles (LEDERBERG and LEDERBERG 1952; CAVALLI-SFORZA and LEDERBERG 1956) are complexly intertwined. *Replica plating* is a homely methodology to ease the technical burdens of screening large numbers of bacterial colonies for mutants to use in genetic analysis. This was sometimes easy. For example, phage or drug resistance could be readily obtained with positive selection using phage or a drug, respectively. But many other kinds of mutants were desired, and replica plating facilitated their discovery. Furthermore, the broader philosophical question remained open, whether the resistance was a preadaptive or a postadaptive change: did it emerge by natural selection of rare preexisting mutants or did the agent somehow induce the heritable change? I elaborate first on this second broader question and its resolution by *indirect (sib) selection*.

LURIA and DELBRÜCK (1943) introduced a carefully thought out quantitative kinetics into the study of bacterial mutation. The jackpot theory (LURIA 1984) was a consequence of clonal expansion. Spontaneous mutations, in a culture started from a small inoculum, would occur rarely among the few cells present at early stages of exponential growth. But when this happened they would have a disproportionate number of mutant offspring. Hence, while the distribution of mutational *events* should follow a Poisson distribution, over a series of similar cultures, the distribution of mutant *cells* would show occasional jackpots. Uncited by and perhaps unknown to LURIA and DELBRÜCK, YANG and BRUCE WHITE (1934) had previously noted such fluctuations and remarked that they signified the spontaneous occurrence of "rough" mutants in *Shigella*.

Nevertheless, the theoretical rigor of LURIA and DELBRÜCK's analysis and its concurrence with the quantitative data were for many the decisive stimulus to look seriously at mutation in bacteria, to think about a bacterial genetics. In my own development, this paper was one of the key impulses to inquire

about genetic recombination in bacteria (LEDERBERG 1987).

By 1950 it was no longer controversial among geneticists. *Pace* periodic selection, the monotonic increase of mutant number with time in long-term chemostat cultures observed by NOVICK and SZILARD (1950) sealed lacunae in the 1943 experiments. (Otherwise, one could have entertained some labored hypothetical alternatives like uncontrolled environmental variation from tube to tube, or a subset of that: temporal fluctuations within a culture in its overall propensity to turn resistant on exposure to a selective agent.) Nevertheless, many, perhaps most, readers of the 1943 article did not understand its abstruse mathematical argument and would respond either with uncritical acceptance or uncritical rejection. Notable for the latter, the last holdout, was the prestigious Sir CYRIL HINSHELWOOD, President of the Royal Society of London, who denounced every assertion of genes in bacteria in favor of an extended reaction network model of biological continuity and adaptivity (HINSHELWOOD 1946; DEAN and HINSHELWOOD 1957). As inappropriate as these network models have proven to be for the fundamental elements of genetic structure, they have been revived for the explanation of developmental switches, where one gene product reinforces its own synthesis and represses an alternative gene's and *vice versa* (DELBRÜCK 1949; PTASHNE 1986).

Meanwhile, bacterial genetics had grown to the point of needing ever larger libraries of mutants, most of which bore biochemical defects and were not readily amenable to positive selection. Today we apply many ingenious tricks to this game (VINOPAL 1987). In 1948, the penicillin method (LEDERBERG and ZINDER 1948; DAVIS 1948) partly answered this need by selectively killing cells actively growing in minimal medium. Penicillin could exert positive selection in favor of auxotrophic mutants. But many bacterial strains were relatively recalcitrant to the feeble mutagens than available, and even after penicillin selec-

tion there was still the tedious task of screening thousands of colonies for the 1% or so that might be growth-factor dependent. In addition, the escalation of recombination studies imposed the equally tedious task of classifying vast numbers of individual recombinant colonies to score them on a series of growth factors, sugars, drugs and bacteriophages. For the first several years of my work at the University of Wisconsin, starting in the fall of 1947, I was deeply preoccupied with these technical and doctrinal issues and eager to follow other leads.

L. SZILARD and A. NOVICK, at the University of Chicago, faced similar problems in scoring the phenotypes of an abundance of colonies. In February 1951, at one of the monthly phage seminars that SZILARD had organized, they remarked that they had been using multipronged inoculators, even a wire brush, for a primitive kind of what I later called replica plating (NOVICK 1972). This was not very satisfactory owing to the poor resolution available with that material.

For a couple of years prior to that point, possibly as the result of a serendipitous accident, it had been common practice in our laboratory to make impressions of the dark and light colonies on eosin-methylene blue agar plates simply by pressing a piece of paper onto the agar surface and then mounting this under cellulose tape in our laboratory notebooks. This was far more convenient and cheaper than photography for retaining a permanent record of the actual colony distribution when this was manifest in a pigment difference. We had the idea of trying to use such prints as inocula for fresh plates, but there was far too much smearing on the paper to allow this to be useful (as was also found by N. VISCONTI at the Cold Spring Harbor Laboratory). (Have no fear about the biological hazard in these notebooks: we have never been able, alas, to recover viable organisms from these impressions, and we do not advocate such a procedure for spore formers or pathogens. Even so, this practice is probably disallowed under present-day regulations.)

Meanwhile, HOWARD NEWCOMBE (1949) evoked a still more graphic image of the clonal expansion of spontaneous mutants. Allowing a lawn of bacterial growth on an agar surface, he would count the number of mutational events by directly spraying the mature plate with phage. Each individual resistant cell, or the clone clustered around it, would be scored as a single colony. However, if the plate were respread prior to selection, there was always a substantial increase in the number of mutant colonies: this was a direct translation of the clonal expansion of the individual mutational stem cells. Sometime thereafter, I elaborated on his experiment by making a single streak of growth rather than a two-dimensional lawn. I would then move a spreader perpendicular to the

stroke, and each clone would then be represented by a line of resistant colonies along the direction of the second stroke. These findings engendered still more intense preoccupation with the imagery of what was happening to mutant clones buried within the bacterial population. If only there were some constructive method to sample those clones prior to exposing them to the selective agent!

Perhaps the multipoint sampling technology of NOVICK and SZILARD could be applied to this broader problem as well as to the tedium of colony scoring! But: how to improve upon the poor resolution and handling properties of the wire brush? ED TATUM had taught me to use a beakerful of sterilized tooth picks, one by one, for colony picking; that saved the time needed to flame a platinum loop between picks. The brush was conceptually an ordered array of tooth-picks. What might be a functional equivalent?

Paper was unsatisfactory: its lateral capillarity and its compression of the colonies distorted and broke up the original growth pattern. It occurred to me that some fabric with a vertical pile would be an analog of the paper on one hand and the wire brush on the other, and I soon collected a wide variety of remnants from the local dry goods shops to put them to empirical tests. (The predictable myth that I invaded my wife's wardrobe for this purpose is pure fantasy.) Also helpful were books on fabric structure (like STRONG 1947) which helped me to focus on cotton velveteen as the most desirable material. (Nylon velvets were then far more expensive and their stiffer fibers caused some problems.) The cotton velveteen has become quite standardized and the material can, if necessary, be conveniently laundered and reesterilized for repeated use. The experimental trials soon confirmed that one could rapidly enrich the proportion of resistant mutants by fishing the growth at the point on the original plate where a replica demonstrated the presence of a resistant clone. Without difficulty, one can exclude about 99% of the irrelevant growth on a bacterial lawn, which offers the prospect of a 100-fold enrichment at every cycle. This means that one can achieve a pure culture of resistant organisms in three or four cycles based on selection applied to the sibling clones that have been exposed to the selective agent. This procedure thus accomplished a constructive proof that had been so long elusive, namely, the preadaptive initiation of these rare resistant mutant clones. One can argue that this need never have been in doubt, but here for the first time was a general procedure that could be applied to any such problematical situation.

JAMES CROW, my colleague in the Wisconsin Genetics Department, enjoyed being able to analogize bacterial indirect selection with sibling selection as practiced in dairy and poultry husbandry. Bulls and

roosters are selected by indices of milk and egg production by their sisters and daughters (LUSH 1945). At a time when the genetic basis of altruism is in question (MICHOD 1982), we can reflex on a system whereby replica-platefuls of clones, sensitive and resistant alike, were relegated to the autoclave once they had given the locational cues for their indirectly selected cousins.

As satisfying as this demonstration was, I lamented that it depended on a new technology (that of replica plating). An Euclidean bent has always led me to seek for proofs that came closer to the use of nothing more than a straight edge and a compass. Furthermore, it was difficult to quantitate the progress of indirect selection on agar plates. It was difficult to maintain rigorous control over the proportion of cells that were transferred during replica plating or the proportion that could be plucked by going back to the source plate. There was no reason, I thought, that the same methodology could not be used with an array of test tubes taking the place of the agar plate, with pipettes transferring well defined volumes from tube to tube in place of the velvet transfer. This was the burden of CAVALLI-SFORZA and LEDERBERG (1956): during one of several visits to our laboratory, CAVALLI took an interest in following the quantitative aspects of selection using that methodology. If one starts, say, with 100 tubes so adjusted that a mutational event will have occurred in only one of them, one can find out which tube that was by testing a sample of each for drug resistance. Discarding the other 99 tubes would in principle then give a 100-fold enrichment of the proportion of mutant cells within that tube compared to the overall population. Then one knows what dilution to make of the culture in that tube for a second cycle of inocula and can recycle from there with progressive enrichment. CAVALLI found that this procedure worked very well, although this progress of selection was often not as rapid as predicted by the first-order theory. This could be explained by the observed growth lags of the resistant mutants and thus offers no great problem.

Replica plating has grown into a major industry, its progeny including the various blots around the compass—Northern, Southern, etc.—as well as its direct application to a number of microbiological problems. Sib selection by serial dilution has been used in several genetic engineering enterprises where a special producing clone is a needle in the haystack but its products can be sensitively detected (KEDES *et al.* 1975; NAGATA *et al.* 1980).

A 1989 perspective on postadaptive mutation: The concept of the gene as immutable in the course of hereditary transmission (MORGAN 1926) was an idealization that played a constructive role in the emergence of Mendelian genetics. As HALDANE (1949)

pointed out, this view taken to an extreme contradicts the understanding of the gene as a material substance (we would now say as DNA). We must have an open mind about evolutionary specializations where metabolic alterations can target the DNA itself. This might sometimes lead to postadaptations, that is, adaptive genetic changes specifically induced by an environmental stress. As specialized evolutionary developments, one does not expect them to be a routine occurrence. They have been hard to find and authenticate, with one generic exception: lysogenizing viruses typically confer immunity to the lytic function of the virus, a subset of lysogenic inductions (HERSHEY 1971; PTASHNE 1986). The frequency of “processed pseudogenes” in eukaryotic genomes is particular testimony to a history of RNA insertions (see pp. 448ff. in DARNELL, LODISH and BALTIMORE 1986). With site-directed mutagenesis we know today how to expose, or even directly use, DNA sequences so as to achieve mutation by intelligent design.

To turn to less specific responses, mutational storms hypothetically related to insertional transposons may also be mediated by RNA in train of environmental stress (MCDONALD 1983). In addition, contemporary with the development of replica plating, my own laboratory had concluded that UV mutagenesis was probably a secondary physiological response during recovery and DNA repair (LEDERBERG *et al.* 1951). We know now how environmental DNA damage can evoke the “SOS” response (WALKER 1987) leading to several categories of genetic instability. One could argue that UV resistance was a postadaptive mutation, but this is to ignore the broad-ranging nonspecificity of the mutations that occur during the SOS response.

It is too early to answer many questions that have been raised during the past few months about postadaptive mutational responses claimed to occur in glycosidase-deficient *Escherichia coli* mutants incubated in the presence of lactose or salicin (CAIRNS, OVERBAUGH and MILLER 1988; CAIRNS *et al.* 1988; HALL 1988). There are many pitfalls in the exclusion of artefacts in mutagenesis experiments (LEDERBERG 1948) and each has entrapped unwary investigators in the course of microbial genetics. Such artefacts aside, cells starved for carbon but receiving a trickle of nutrient through spontaneous or allospecific enzymatic hydrolysis of the substrate are in a metabolic state that requires critical examination. As a reducing sugar, unmetabolized lactose might well be expected to react with the DNA of such mutants (LEE 1987). Even if it should be verified that lactose is a mutagen for *lac*⁻ mutants, its specificity, *i.e.*, lactose at the *lac* locus *vs.* salicin at the *bgl* locus in a common genetic background, should be corroborated before further evolutionary speculation.

Specific DNA alterations are achieving higher cred-

ibility in a role in epigenesis, in reaction against long-held dogmas of the uniformity of the genome of somatic cells (LEDERBERG 1958). Segmental DNA excisions responding to an environmentally induced, site-specific DNA recombinase are associated with terminal heterocyst differentiation in *Anabaena* (HASELKORN *et al.* 1987). A similar story has just been reported for the terminal differentiation of the mother cell during sporulation in *Bacillus subtilis* (STRAGIER *et al.* 1989).

In microbial genetics, two other phenomena also fit the paradigm of directed mutation. Bacteria can be cured of many plasmids under the influence of acridine dyes (HIROTA 1960) or other chemical and physical agents (TREVORS 1986). Acridine dyes alter mitochondria in yeast and also remove kinetoplasts from trypanosomes, and streptomycin ablates chloroplasts from *Euglena* and other green plants (reviewed in LEDERBERG 1952). Acridine sensitivity of bacterial plasmids depends on the presence of specific DNA sequences (WECHSLER and KLINE 1980). LEDERBERG and ST. CLAIR (1958) showed that *E. coli* cells could be converted *en masse* into spheroplasts with penicillin in hypertonic media, and that these spheroplasts could be propagated as wall-deficient clones in agar but promptly reverted in the absence of penicillin. LANDMAN (1968) has reported, however, that wall-deficient "L forms" of *B. subtilis* induced by lysozyme were clonally propagated as L-forms in the absence of lysozyme, although they would revert in solid media. This is evidently an extranucleic event and deserves further study as a unique instance in bacteria of morphogenetic continuity of a cytoplasmic organelle, so long the focus of study in the genetics of *Paramecium* (SAPP 1987).

These exceptions notwithstanding, reinforcing the Darwinian model of adaptive resistance mutation in bacteria bolstered the eventual discard of instructional theories of induced enzyme formation (MONOD 1956, 1966) and antibody formation (LEDERBERG 1989). Any heuristic can be treacherous, but a Darwinian explanation is the first I would seek in explaining a biological enigma. I do not insist that it will always last, but it has had enormous power in bringing us to our present understanding.

JOSHUA LEDERBERG
The Rockefeller University
New York, New York 10021

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