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What Have Restriction Enzymes Ever Done For Us?

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WHAT HAVE RESTRICTION ENZYMES EVER DONE FOR US?

Restriction enzymes, along with their counterparts the DNA methyltransferases, make up restriction-modification systems in almost all bacteria. The restriction enzymes have the ability to cut DNA into fragments. This has allowed experimenters to join such fragments together to make novel arrangements of DNA which had never before existed in nature. This ability to perform genetic engineering, developed from the 1970s onwards, has had an enormous influence on the modern world – establishing the biotechnology industry and transforming our understanding of biology and of medicine. Furthermore, the restriction enzymes have had an enormous impact on the rate of evolution of life on earth. They were almost certainly present in the earliest forms of bacterial cells some four billion years ago to control their uptake of DNA from their environment. If the restriction systems had had too much or too little control of this process then the rate of evolution of bacteria, and hence the time for the appearance of higher organisms such as ourselves, would likely have been greatly delayed – such that we might not yet exist on earth.

Introduction

In this paper I wish to provide two answers to the question posed by the title. The first answer is that modern genetic engineering, biotechnology and medicine would have been almost impossible. The second is that the restriction enzymes, ubiquitous in humble bacteria, have shaped the evolution of life on earth since the first cells arose.

The bacterial phenomenon of restriction and its counterpart of modification were discovered in the early 1950s and published in two papers (Luria and Human, 1952; Bertani and Weigle, 1953). Before I explain the nature of the restriction-modification (RM) effect in bacteria, I will have to introduce the other player in this effect, namely bacteriophage (Salmond and Fineran, 2015). Bacteriophage, literally 'bacteria eaters', are the viruses infecting bacteria and they were first discovered by Alexander Twort (Twort, 1915) and independently by Felix d'Herelle (d'Herelle, 1917). They found that the phage, although too small to be observed with a light microscope and too small to be trapped on a filter, would, when mixed with a solution of bacteria, cause the death of the bacteria. This could be observed in a solution of bacteria, which is normally turbid, by the rapid clearing and loss of turbidity, or on a Petri dish by the appearance of holes or 'plaques' on a layer of growing bacteria. Phage were actually rapidly developed in the 1920s for therapeutic use to cure people and animals of bacterial infections but they fell out of use in the Western world as soon as antibiotics were discovered (they were still used in the former USSR and the Phage Institute in Georgia was particularly advanced in this area). The increasing prevalence of antibiotic-resistant bacteria is now driving a new interest in 'phage therapy'. The scientific study of phage and their interaction with bacteria continued through the 1920s and 1930s but their physical nature and chemical composition did not become clear until the electron microscope was invented in the 1940s. Eduard Kellenberger and Jean Weigle at the University of Geneva in Switzerland showed that phage were objects of ~50nm in size with polyhedral 'heads', a tail and often some fibres projecting from the tail (see Bradley, 1967, for a review). To infect bacteria, they would attach themselves to the outside of the bacterial cell. The material inside the head would enter the bacterial cell and eventually more copies

of the phage would be assembled inside the bacterial cell and the cell would eventually burst or 'lyse' to release new phage particles. The nature of the material inside the head was shown by Martha Chase and Alfred Hershey to be DNA in their famous 'Waring blender' experiment (Hershey and Chase, 1952). It was this DNA which was injected into the unfortunate bacterial cell. The study of phage continued for many years (Salmond and Fineran, 2015) and many tremendous advances in our understanding of molecular biology were made.

However, it is now time to return to the RM effect (Luria and Human, 1952; Bertani and Weigle, 1953). The concentration of a phage solution or the number of phage particles in a defined volume such as 1 ml could be easily determined by mixing a small aliquot of the phage solution with bacteria and plating the mix on agar in a Petri dish. The number of plaques appearing would give the number of phage in the small aliquot as each plaque was the result of a single phage particle infecting a bacterium. It was noticed that the number of plaques observed on certain strains of *E. coli* was much less than expected. For instance, if 1000 plaques appeared on one strain (I am going to call it *E. coli* C), only one or two might appear on the other strain (which I will call *E. coli* K12) even though the same number of phage and bacteria had been put on each Petri dish. This was the restriction phenomenon and was presumed to be due to the destruction of the phage DNA by enzymes when entering a restricting strain such as *E. coli* K12. The plaques on these Petri dishes actually contain a large number of phage particles produced by the replication of the single original particle and these phage can be recovered by simply sticking a sterile toothpick into the plaque. Let us for the sake of argument say that 2000 phage were recovered from a plaque on the *E. coli* K12 Petri dish. If we mixed 1000 of them with *E. coli* C and plated them, then we would get 1000 plaques. If we mixed the other 1000 phage with *E. coli* K12, which would have given us only one or two plaques in our first experiment, then rather surprisingly 1000 plaques appear on the *E. coli* K12 dish. It was realised that the phage surviving on *E. coli* K12 had acquired a modification that allowed them to propagate on this strain. If the phage from the *E. coli* K12 plate were passed by growth through the *E. coli* C strain again and then put back on the K12 strain once more, the number of phage recovered was again very small (restricted) and the modification had been lost.

What was the nature of the modification acquired by the phage which had survived on the *E. coli* K12 plate? In the 1960s, Werner Arber and Daisy Dussoix working at the University of Geneva suspected that the modification was occurring on the phage DNA and was caused by an enzyme in the *E. coli* (Arber and Dussoix, 1962). They found that the DNA had become methylated by an enzyme transferring the methyl group from the abundant small molecule S-adenosyl methionine to the DNA. This modification of DNA is actually found in almost all organisms and its study in humans and other higher organisms is called 'epigenetics' and is a very vigorous area of research in the present day.

Answer 1: Restriction Enzymes Enabled Modern Genetic Engineering, Biotechnology and Advances in Medicine

The hunt was now on for the restriction and modification enzymes (Loenen et al., 2014a; Roberts, 2005) and this was successful in 1968 when two groups (Mat Meselson with Bob Yuan and Werner Arber with Stu Linn) purified the RM activities from two strains of *E. coli* named strain K12 and strain B. The assays they used to follow each step of the purification were, by modern standards, very time-consuming and difficult but they were successful in purifying the minute amounts of these enzymes found in the bacteria. Over the next few years, these enzymes were found to combine restriction and modification activities in a single multifunctional enzyme. The restriction phenomenon was shown to be the cleavage of DNA into

fragments but this cleavage activity also required the hydrolysis of considerable amounts of another abundant small molecule, adenosine triphosphate (ATP). The enzymes became known as the Type I restriction or restriction-modification enzymes (Loenen et al., 2014).

It was found that the Type I restriction enzymes cut DNA into fragments of random lengths and this raised the question of whether there were other restriction enzymes capable of cutting DNA into precise fragments. Fragments of defined lengths would be useful for the mapping of genes on DNA chromosomes, so the discovery of the Type I restriction enzymes prompted researchers to look for the Type II restriction enzymes – the ones that would cut DNA in a defined manner (Pingoud et al., 2014). It did not take long to isolate these enzymes from other bacteria. The first ones were again detected using complex experimental techniques but the arrival of agarose gel electrophoresis greatly simplified the detection of the enzymes and also gave a direct visualisation of the different sizes of DNA fragments produced by each Type II restriction enzyme (Roberts, 2005). As further Type II restriction enzymes were discovered in the early 1970s, the application of new sequencing methods revealed the specific DNA target sequence which each enzyme recognised and how the enzyme cut the DNA at the target sequence. The cutting sites were found not all to be the same: some restriction enzymes left short single-stranded overhangs either on the 'top' strand or on the 'bottom' strand while others left no overhangs and gave a 'blunt' cut. Pieces of DNA cut with the same restriction enzyme would therefore all have the same ends and this meant that they could be joined to create hybrid DNA molecules. The actual joins could be made perfect again by using a second enzyme called DNA ligase, also discovered in the late 1960s (Shuman, 2009).

These discoveries set the scene for genetic engineering via the construction of hybrid DNA molecules with new properties. Several companies such as New England Biolabs and Promega in the United States and Fermentas in Lithuania were rapidly established to discover, purify and supply restriction enzymes with an ever-increasing range of target sites. Several companies were founded to exploit this new technology and to produce therapeutic proteins of high value. For instance Biogen, founded in 1978, produced the first 'recombinant' vaccine for hepatitis B and Genentech, founded in 1976, was able to produce human insulin in *E. coli* thus greatly increasing the availability of these important medicines. Such was the astounding speed of discovery and exploitation of restriction enzymes that the Nobel Prize was awarded to Werner Arber, Hamilton 'Ham' Smith and Daniel Nathans in 1978, only 10 years after the purification of the Type I restriction enzymes. It is worth noting that as the power of the new technology became apparent to the relatively small number of scientists who could use it, they became worried about the possibility of creating 'monsters'. Thus a strict set of rules and conditions were established after a meeting at Asilomar in 1975 for the manipulation of DNA until such time as possible dangers could be assessed. After a couple of years of investigation, it was concluded that genetic engineering was not dangerous (unless one set out deliberately to create a problem) and the technology then became much more widely available.

For the next two decades, the restriction enzymes transformed biology and medicine, making a vast number of experiments possible and greatly increasing our understanding of all forms of life from bacterial to human. They also enabled the biotechnology industry to flourish.

One of the goals of genetic manipulation has been to alter or correct the genetic code of humans and in particular those with a genetic disease. It was hoped that the faulty gene could be targeted by a restriction enzyme and then repaired. The problem with this approach is that restriction enzymes recognise fairly short DNA sequences – in over-simplified terms, about 10 base pairs is the maximum length of target. Moreover, the shorter the sequence, the more frequently it occurs in chromosomes. Ideally one would want an enzyme that had only a single

target on a chromosome. Thus there was a drive from the mid 1980s through to the 2000s to alter the restriction enzymes by genetic engineering to make artificial enzymes with much longer target sequences (Stoddard, 2011; Chandrasegaran and Carroll, 2015). Several types of such enzymes were indeed made: zinc-finger, TALE and meganucleases. The first two groups were thought to hold the most promise, as the construction of an enzyme to recognise a desired target sequence was fairly straightforward. Unfortunately, these enzymes did not show enough discrimination between the desired target and other sequences containing slight differences from the desired target. Thus errors would be made. Meganucleases, on the other hand, did have enough discrimination but had a very limited set of possible targets, so it was unlikely that a particular human gene could be targeted in the manner originally hoped. Nevertheless, these enzymes have found research and commercial uses.

The desire to find enzymes with very long target sequences continued to push research and it was eventually realised only a few years ago that an obscure and perplexing discovery made in the 1980s, again using phage and bacteria, was actually the result of an enzyme with the desired properties! This new system has been given the odd name of CRISPR-Cas (Chandrasegaran and Carroll, 2015). The CRISPR-Cas enzyme has the ability to cut DNA very precisely, just like a Type II restriction enzyme, within a very long (~30 base pairs) target sequence, and it can be programmed to recognise new target sequences very easily. This is because the enzyme actually binds a short piece of RNA and uses it as a 'reader' to recognise the DNA target sequence. If the sequence of the RNA reader is changed then a complementary change occurs in the sequence targeted for DNA cutting by the enzyme. Much effort has been made in the last five years to make the CRISPR-Cas system usable for targeting individual genes. The system works in a test tube but the effort is now being made to edit genes in living germ-line human cells. This of course raises enormous ethical questions just like those raised when genetic engineering started and there is much debate going on at the present time. In September 2015, scientists at the Crick Institute in London were given permission to use CRISPR-Cas for gene editing in human embryos formed via *in vitro* fertilisation procedures which produce many more embryos than can be used in IVF. What the future holds for CRISPR-Cas and gene editing is unknown but as long as the scientists keep debate about the process and their results open, as was done after the Asilomar meeting, then progress in positive directions should result.

Answer 2: Restriction Enzymes Have Shaped the Evolution of Life on Earth

Restriction enzymes have made a clear and immediate impact on human society but what are they doing in nature? It is worth considering their role in bacteria and the scale upon which they operate (Oliveira et al., 2014). The RM systems have been found in over 95% of sequenced bacterial genomes and are generally absent only from bacteria whose lifestyle is that of an obligate intracellular pathogen. They have also been found in almost all sequenced archaea, the other kingdom of prokaryotes and which were originally mistaken for bacteria. Many prokaryotes contain more than one RM system and numerous types and subtypes have been identified using biochemistry and structural biology (Loenen et al., 2014, 2014a, Loenen and Raleigh, 2014; Mruk and Kobayashi, 2014; Pingoud et al., 2014; Rao et al., 2014). They all however appear to have the major role of controlling the influx of foreign DNA into the cell via a process called Horizontal Gene Transfer (HGT), and there appears to be an optimum density of RM systems on a genome (Oliveira et al., 2014).

The main processes of HGT are invasion of phage DNA (a process called transduction), uptake of naked DNA from solution (transformation) and direct transfer of DNA from one bacterium

to another (conjugation). If HGT is not done carefully, then the foreign DNA can replicate and take over or even kill the cell as seen originally by Twort and d'Herelle (Twort, 1915; d'Herelle, 1917). The benefit of HGT is that the cell may acquire useful new genes enabling it to be fitter in its environment. Thus HGT drives the evolution of prokaryotes.

The scale of HGT in the environment is astonishing. It has been estimated that there are ~10^{31} phage and ~10^{30} bacterial cells in the oceans and there are ~10^{25} phage attacks on these bacterial cells every second (Bergh et al., 1989). This means that the entire biomass turns over every three days. These numbers refer specifically to the lytic phage where bacterial cell death results, however the lysogenic phage are probably just as numerous and active. Furthermore, similar numbers of phage and bacteria are expected on land in the soil. Estimates of the amount of transformation and conjugation are not as clear but still the number of HGT events will be large. If one considers each HGT event as a genetic experiment resulting in a possible evolutionary increase in the fitness of the bacterium, then one can appreciate how important HGT is for prokaryotic evolution.

The genes for RM enzymes have been found in bacteria recovered from halite (salt) crystals that are at least 120 million years old, indicating an ancient origin for these systems and an ancient origin for HGT (Jaakkola et al., 2016). Indeed, it is strongly believed that phage would have been established in the environment at about the same time as the first bacteria some four billion years ago (Koonin and Wolf, 2012). If HGT has been occurring at the rate found in the oceans today, then the effect of HGT and the control of HGT by RM systems must have been enormous in the four billion years since the first cells evolved.

So, one can ask what would have happened to the rate of evolution of the earliest cells if there had been too much RM in the cells (too strong a barrier to HGT) or too little RM (no barrier to HGT). If no HGT were possible then the primitive bacterial cells would only have been able to evolve by the accumulation of mutations in their genome. This is a much slower process than evolution by HGT, where entire genes can be acquired easily and, generally, safely. If too much HGT occurred, then it would be very difficult for an early cell to maintain any degree of genome integrity and to become stable and increase in population. In both of these cases (too much or too little RM) the result would be the drastic slowing down of the evolution of the prokaryotes. This would almost inevitably delay the appearance of the eukaryotes, and hence of *homo sapiens*, on the modern day earth, which would instead be covered only by bacterial slime.

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Insights

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