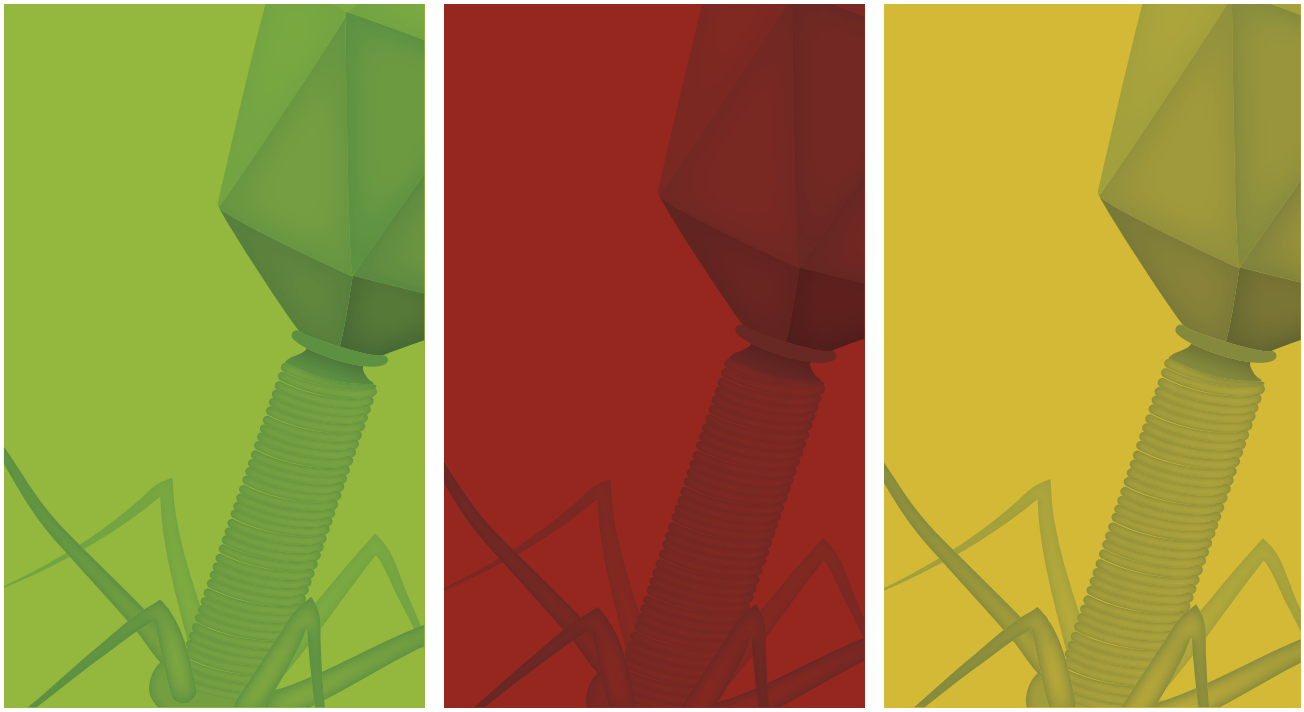

The Good, the Bad and the Ugly

Prophages are common and often uncontrable elements in bacterial strains, with effects ranging from increased host fitness to potentially fatal infections. These factors warrant careful consideration before selecting lysogenic bacteria as an expression system for protein manufacture

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Over the course of their evolution, viruses have developed several traits. Among these is virulence. This occurs after a cell is infected; the virus produces the maximum amount of progeny, usually killing the cell in the process. Another trait is the establishment of a provirus, the only difference being that a fraction of infected cells do not produce and release viruses, but instead viral genetic material is integrated with the cell chromosome, and may resume its development cycle later, usually triggered by some environmental signals. In the world of bacteria, both strategies are commonly used by bacteriophages – the viruses which attack them. Dormant bacteriophages are called prophages.

Are Prophages Common?

In the past, prophages were considered as a moderately frequent occurence in the natural environment, their abundance revealed by intensive sequencing of different bacterial strains. Many of them, especially pathogenic strains, appeared to have many different prophages in their genomes – some more than 20. Prophage content may form as much as 10-20 per cent of the whole genetic content of a given strain (1). Due to relative lack of knowledge about bacteriophages, many prophages may go unnoticed during sequencing projects and subsequent analysis of obtained results. Until now scientists have not fully understood their effect on the bacterial strain itself, on its environmental fitness and interactions with other bacteria and bacteriophages, or on its interaction with higher organisms. However, there are a lot of things we already know. The altered abilities of bacterial strains bearing prophages are important not only from the point of view of health hazards, but also from the point of view of production safety when bacteria are used to produce pharmaceutical or biotechnology products.

The Good

In general, the additional burden of prophage genetic material present in chromosomes should force bacteria to collect more resources before the chromosome can be replicated and so should prolong the process of chromosome replication, where the prophage is physically integrated with the chromosome. Contrary to these assumptions, some prophages have been observed to have a positive impact on host fitness. This is particularly apparent in conditions of carbon starvation, and these conditions are frequently used in the process of

biopharmaceutical molecule production (2). For a long time it was not understood how prophages compensated their host for the additional costs of their presence – essential to ensuring their survival and the success of this evolutionary strategy. The explanation is lysogenic conversion genes and, in some cases, modulation of the gluconeogenesis pathway (3). Lysogenic conversion genes usually bring some entirely new characteristics to the host strain, which can help the host cell to be more competitive in certain conditions. Modulation of the gluconeogenesis pathway allows for a more economic use of carbon sources – crucial when carbon source is a growth-limiting factor. On this principle, bacteria lysogenic with phage may show better growth and production characteristsics in all types of biotechnology processes, as conversion of various carbon sources, most common of which is glucose, into the final product, is the basis of this business.

Another positive factor resulting from the presence of prophage(s) in a given strain is that they often encode powerful phage exclusion systems, which may prevent infection of the strain with the much more dangerous phages. From the point of view of production safety, this defence is very important, as infecting bacteriophages can contaminate a facility, paralysing productiveness. Prophages are also a useful tool for molecular biology and biotechnology. They allow for inserting a gene of interest into a bacterial chromosome, which enables strict control of the gene copy number, stable gene maintenance and, when inserted into the right place, may also provide control over gene expression triggering. The most frequently used prophage in biotechnology is DE3, which allows for controlled expression of T7 RNA polymerase. This is a very convenient system enabling high protein overexpression with minimal burden for a cell before the expression of T7 RNA polymerase gene is triggered (4).

Prophages also provide effective and easy-to-use molecular tools, such as Red recombination system from phage lambda, which is commonly used for bacterial strain engineering.

The Bad

Unfortunately, the presence of prophage in bacterial cells also has drawbacks. One of the most important is that the same mechanism that allows prophages to increase their host fitness also help them to convert harmless bacteria into lethal pathogens. The association of some prophages with pathogenicity of different bacterial strains was first noticed some time ago: since then, a growing number of diseases has been discovered to be associated with the strains which were lysogenised by phages bearing major pathogenicity genes (5). One example is diphtheria, caused by *Corynebacterium diphteriae* being lysogenised by prophage β. The strain bearing prophage is able to cause disease, while prophage triggers extremely effective toxin production and can kill an infected person. Another well known example is cholera, caused by saprofitic *Vibrio cholerae* – quite common bacterium found in water-associated environments. It is lysogenisation by CTXϕ, which turn this harmless bacteria into a real killer.

Prophages also play an important role in emerging diseases. One such example is enterohaemorragic *Escherichia coli*, causing food-borne outbreaks with potential fatalities. Thus, the presence of lysogenic conversion genes in a production host, especially when biopharmaceuticals are to be produced, should raise questions regarding their impact on product safety and possible cross reactions in patients organisms, since few purification processes can deliver a truly homogenous protein solution. A good example is bacteriophage lambda and its derivatives, which carry the immune evasion gene *bor*. The gene product modifies the complement system disabling the classical complement activation pathway, and is associated with the cell wall, leading to manufactures attempting to minimise the risk of co-purification of *bor* with expressed protein in most circumstances.

Prophages are also dangerous in biotechnology. When induced, they may cause a lysis of the bacterial cells. Synchronised induction can result in unexpected and very rapid destruction of the whole bacterial culture, regardless of size. In many cases the massive induction is caused by factors triggering an SOS response, including diverse DNA-damaging factors and conditions. The problem is that overexpression of some proteins may also trigger an SOS response, so there is a possibility that after beginning protein production one will obtain a phage lysate instead of the protein of interest. This should of course be revealed by an initial study; however, since fermentation conditions may differ slightly from batch to batch, the risk of inducing prophage may still exist, even if well designed preliminary tests excluded such possibility. To make the situation worse, there is a large group of prophages whose induction is SOS-independent, which means that we usually do not know which stimuli can efficiently induce them. An example of such phage is P2-like phage Wϕ, which is present in *E coli W*. Due to the fact that no one knows what condition may trigger massive prophage induction in such cases, it is much harder to prevent it and, when it does occur, it is also harder to understand the root cause of fermentation failure.

The Ugly

Some characteristics of prophages don't necessarily have a negative impact on all biopharmaceutical production, but can still be problematic in some cases. They quite often go unnoticed and due to their sneaky nature may cause long-term problems in facility environment. The first of these is spontaneous induction. Even when conditions which trigger prophage induction are avoided, in the case of many prophages, a small fraction of a cell's prophage will be induced anyway. The degree of induction strongly depends on the prophage itself and cultivation conditions used, but in many cases it is possible to detect using standard methods. Due to homoimmunity of lysogenised cells to the same type of phage, this is not a significant problem, at least as long as various strains are not used for production in the same facility. When a facility runs multiple projects, a higher degree of care must be used in order to avoid cross-contamination and phage-caused outbreak or lysogenisation of other strains. There are several documented cases of horizontal spread of prophage in laboratory environment (6).

Due to bacteriophages' abilities to perform similar processes as the host cell, but usually in a different and effective way, there are some potential problems encountered when lysogenic strains are used for production. One of the characteristics that may affect the strain itself is the very potent recombination system found in the majority of bacteriophages. This system allows them to evolve very quickly and to adapt to the changing environment. One side effect of its activation may be the decreased stability of host cells. To prevent problems with host stability, proper methods of cell bank storage and propagation as well as suitable tests should be employed. Another possible effect is decreased stability of plasmids used for product expression, especially during over-expression of a gene from the afore-mentioned plasmid. This may be due to several factors. The most evident will be improved growth rate of host cells, which minimise the effort necessary to express the gene by mutating or deleting the gene or regulatory elements responsible for the product formation. This enables them to grow much faster, and in effect, they may constitute an important fraction of bacterial culture.

Another factor may be the fluctuation of repressor concentration in cells with strong over-expression of cloned gene. Repressor in prophage prevents its induction, and thus prevents expression of the vast majority of genes, excluding lysogenic conversion genes and repressor gene itself. When the protein synthesis system is saturated with the protein of interest, the production of repressor may be less effective, which may cause the repression to become 'leaky'. This can lead to expression of small quantities of prophage genes, including recombination genes, which in turn may increase the probability of recombination occurrence in the host. One effect may be an increased frequency of loss of ability to overproduce the given protein.

Conclusion

Should prophages be eliminated from production strains? As in many similar cases, the answer is 'it depends'. In general, using a lysogenic strain can be a good idea when high process efficiency, especially for the production of secondary metabolites, is required. In the case of unusual hosts, finding a prophage even after strain sequencing may be quite tricky, and the removal may not be possible or economically justifiable (1). Additional protection against some virulent phages, which some prophages provide for the host strain, may be very valuable, especially in large scale fermentation where a proper degree of sterility is much harder to obtain than a smaller volume fermentation usually used for protein production. In protein production strains of *E coli*, prophage DE3 provides an efficient, well known expresion system successfully used for decades and tested with many different products. Despite its well documented contribution to protein expression, it is highly recommended to test this with the protein of interest in the early stages of a project under real fermentation conditions in order to avoid problems, which may be caused by extensive induction of defective DE3 prophage, that still carries full repertoire of functions required for cell lysis.

Another aspect is the presence of lysogenic conversion genes, especially when they encode toxins or immune system modulating proteins. They may be problematic, especially in the pharmaceutical production of proteins. This does not usually constitute a great risk as purification procedures should be efficient enough to eliminate them; however, when constructing a new protein production process, one should consider removal or inactivation of such genes.

These potential problems do not exclude lysogenic bacteria as proper hosts for the production of either proteins or secondary metabolites. What is required when using such hosts is a higher degree of care during construction of the production process, well developed control tests and procedures, and last but not least, proper training of the personnel involved in production.

References

1. Srividhya KV, Alaguraj V, Poornima G, Kumar D, Singh GP, Raghavenderan L, Mohan Katta AVSK, Mehta P and Krishnaswamy S, Identification of Prophages in Bacterial Genomes by Dinucleotide Relative Abundance Difference, *PLoS ONE* 2(11): e1193, 2007
2. Dykhuizen D, Campbell JH and Rolfe BG, The influences of a lambda prophage on the growth rate of *Escherichia coli*, *Microbios* 23(92): pp99-113, 1978
3. Chen Y, Golding I, Sawai S, Guo L and Cox EC, Population Fitness and the Regulation of *Escherichia coli* Genes by Bacterial Viruses, *PLoS Biol* 3(7): e229, 2005
4. Studier FW and Moffatt BA, Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes, *J Mol Biol*, 189(1): pp113-130, 1986
5. Los M, Kuzio J, McConnell MR, Kropinski AM, Wegrzyn G and Christie GE, Lysogenic conversion in bacteria of importance to the food industry, in Sabour PM and Griffiths MW (eds) *Bacteriophages In the Control of Food- and Waterborne Pathogens*, pp157-198, 2010
6. Rotman E, Amado L and Kuzminov A, Unauthorized Horizontal Spread in the Laboratory Environment: The Tactics of Lula, a Temperate Lambdoid Bacteriophage of *Escherichia coli*, *PLoS ONE* 5(6): e11106, 2010

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