

## **Bacteriophage contamination: is there a simple method to reduce its deleterious effects in laboratory cultures and biotechnological factories?**

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**Abstract.** Infection of bacterial cultures by bacteriophages as well as prophage induction in the host cells are serious problems in both research and biotechnological laboratories. Generally, prevention strategies (like good laboratory/factory hygiene, sterilisation, decontamination and disinfection) are necessary to avoid bacteriophage contamination. However, it is well known that no matter how good the laboratory/factory practice and hygiene are, bacteriophage infections occur from time to time. The use of immunised or resistant bacterial strains against specific phages may be helpful, but properties of the genetically modified strains resistant to phages are often worse (from the point of view of a researcher or a biotechnological company) than those of the parental, phage-sensitive strains. In this article we review recent results that may provide a simple way to minimise deleterious effects of bacteriophage infection and prophage induction. It appears that low bacterial growth rates result in a significant inhibition of lytic development of various bacteriophages. Moreover, spontaneous prophage induction is less frequent in slowly growing bacteria.

**Key words:** bacteriophages  $\lambda$  and T4, *Escherichia coli* cultures, lysogenization, phage development, prophage induction.

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### **Introduction: the problem of bacteriophage contamination in bacterial cultures**

Production of many biotechnologically important substances is based on cultivation of bacteria. Obviously, bacterial cells are also widely used model organisms in biochemical, genetic, and molecular biology studies. Therefore, the importance of these unicellular prokaryotic organisms in research and biotechnology is doubtless. However, serious problems occur in laboratories and factories when cultures, either in flasks or in large bioreactors, are contaminated by natural parasites of bacteria.

Bacteriophages are viruses infecting bacteria, so infection of bacterial cultures by bacteriophages leads to serious problems, including a complete loss of the desired bioproduct and spread of bacteriophages throughout the laboratory. This is fatal for each microbiological laboratory, in which the problems with lysis of cultures may reappear quite frequently even if it seems that all pieces of equipment had been decontaminated. However, the effects of phage infection may be even more dangerous when bacterial culture is performed on a large scale. Decontamination is very difficult in a large factory, and if a phage propagated in a bioreactor can spread throughout the plant, it may survive even for a long period in some places that have not been treated. Then, the problems caused by phages may reappear suddenly, even several months after the previous infection.

Although the deleterious effects caused by bacteriophages are known to those working with bacteria, there are relatively few published reports addressing this problem. However, this does not mean that bacteriophage infections are rare. On the contrary, the low number of articles describing problems with bacteriophage contamination seems to be due to the fact that loosing of the culture is a negative result, which is hardly publishable. Nevertheless, in recent literature it is possible to find descriptions of very serious problems caused by bacteriophages in industrial biotechnology (for a review, see JONES et al. 2000). An example of bacteriophage infection during fermentation, which occurred in one of our laboratories, is presented in Figure 1. This infection resulted in inhibition of bacterial growth and then in lysis of cells. If cultures like that are not carefully eliminated, phage contamination of the laboratories can easily occur, with long-lasting problems.

Apart from infections of bacterial cultures by bacteriophages, there is another problem caused by these viruses. Namely, upon infection of its host, some of them can insert their genetic material into the host chromosome, forming prophages, instead of lysing the cell. WU (1998) and JONES et al. (2000) reported that lysogenic strains (i.e. those bearing integrated prophages) may grow slower than their non-lysogenic counterparts, and the efficiency of synthesis of bioproducts in these strains can be decreased. Moreover, a number of commonly used strains of *Escherichia coli*, one of the most frequently employed host bacteria for overexpression

of recombinant genes, contain lambdoid prophages that often bear some regulatory genetic elements useful in the control of the expression of cloned genes. However, under certain conditions, prophage induction occurs and may have similar effects on a bacterial culture as phage infection has. Even under standard cultivation conditions, a spontaneous prophage induction occurs with a low frequency. This rare prophage induction results in the appearance of infecting phage particles in amounts ranging from  $10^{-8}$  to  $10^{-5}$  pfu (plaque forming units) per bacterial cell. These numbers seem to be low, but if cultivation is performed on a large scale, e.g. reaching  $10^{10}$  cells per ml, then from  $10^2$  to  $10^5$  phages may be present per ml. Considering even a very small bioreactor containing one litre of the culture,

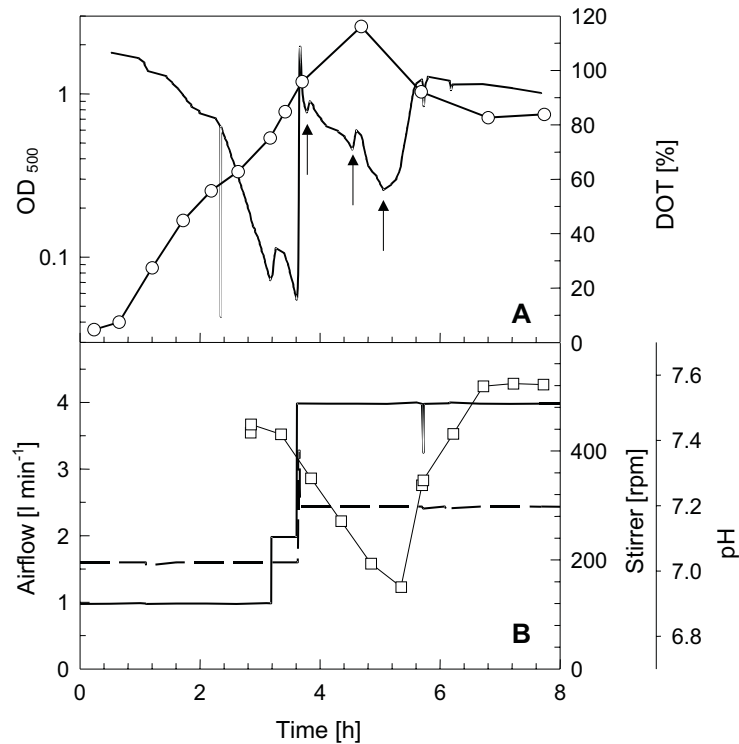


Figure 1. Example of a fermentation culture that was infected by an unidentified phage. *E. coli* RB791 strain containing the plasmids pUBS520 and pKK177glucC was cultivated as described earlier (LIN, NEUBAUER, 2000). Due to phage infection, which was visualized by the plaque test (data not shown), cell growth stopped after 5 hours ( $OD_{500}$ , —○—, A). Growth cessation was connected to a rapid increase in the dissolved oxygen tension (DOT, —, A) and to an increase in the culture pH value (—□—, B). The lower graph (B) also shows the airflow ( $l\ min^{-1}$ , —) and the stirrer speed (rpm, ---) to show which of the step changes of the DOT were caused by the process control scheme (at 3.2 and 3.7 hours). The other three step changes of the DOT (arrows) are possibly caused by cell lysis through phage release.

this adds up to  $10^8$  infecting phage particles. If we consider a 100-litre bioreactor, the number of phages in the medium may reach  $10^{10}$ .

Spontaneous prophage induction should not be dangerous for the culture of lysogenic bacteria due to the immunity phenomenon, i.e. resistance of lysogenic cells to infection by the same phage type. However, overlooking the presence of phages may be fatal for further cultures. Phage contamination may cause infection of non-lysogenic cells, and subsequent lytic development of the phage may destroy the culture and cause spreading of large amounts of phages throughout the laboratory.

Alternatively, phages can also provoke only a weak lysis of bacterial culture but strongly affect the metabolism of the host cells, thereby causing slow growth rates and low production. The continuous liberation of phages to the growth medium can be even more problematic than fast lysis of whole cultures, as it is often not recognised.

### **Standard procedures used to avoid bacteriophage contamination**

Some procedures may prevent phage infection of bacterial cultures. First of all, good laboratory hygiene is required. Some bacteriophages can survive even in dry places for many years, so careful microbiological work, especially during inoculation of cultures and sample withdrawal, is absolutely necessary. Nevertheless, it is worth mentioning that even in very clean laboratories, where people work carefully, phage infections occur from time to time, as it is impossible to keep sterile conditions during all cultivation procedures, especially on a large scale.

If a phage infection has already occurred, the most important thing to do is to clean and to sterilise everything that could have contact with the phage. The crucial point is the decontamination of laboratory equipment and rooms where cultivation is performed. Usually, UV irradiation is a powerful method for disinfection. Phage genetic material, covered only by capsid proteins, is very sensitive to UV. Overnight irradiation is often sufficient to remove most infectious phage particles. Therefore, UV lamps are highly recommended in all bacteriological laboratories. This may be a problem in large biotechnological plants, where installation of such lamps can be expensive or technologically difficult. Chemical disinfection remains an alternative.

### **What to do to minimise deleterious effects of phage infection?**

The general procedures described in the preceding paragraph are useful for avoiding phage infection or to prevent further infections if one has already occurred. However, as mentioned above, even the best laboratory hygiene cannot guarantee the absence of phage contamination. Therefore, a bacteriophage infection must

be considered anyway. In a small-scale cultivation, careful procedures of removing lysed cultures, in combination with chemical disinfection of the flask and any other equipment that could have contact with the infected culture, should be sufficient to avoid further extensive problems. Sometimes, phage decontamination by using overnight UV irradiation of the laboratory is recommended, especially if a leakage of small amounts of the culture was possible (for example during sample withdrawal at the time we were not aware of the infection). However, disinfection and phage decontamination is significantly more complicated and difficult in large laboratories and biotechnological factories. In such cases, it would be very important to inhibit phage development as efficiently as possible by using a simple and cheap method. One method of prevention of phage infection is the use of bacterial strains harbouring particular prophages. It is well-known that bacteria

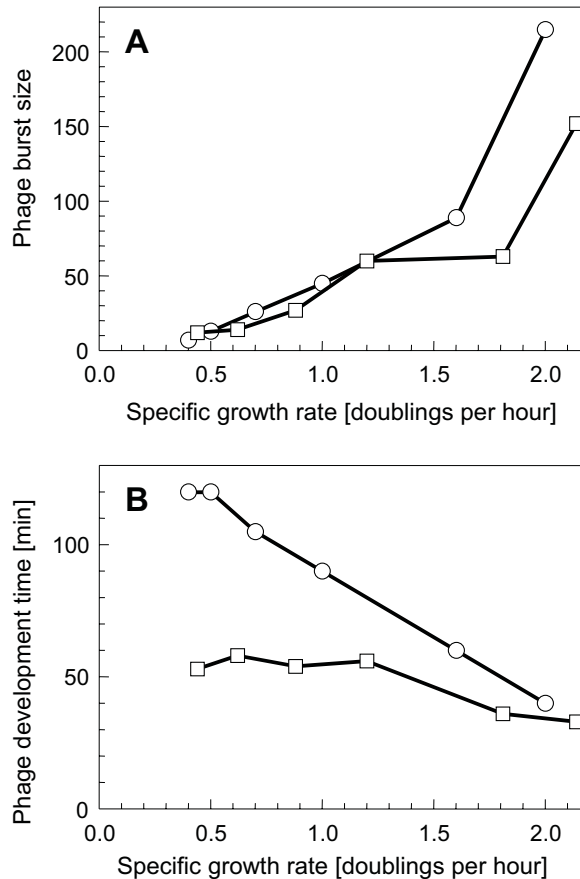


Figure 2. Effects of bacterial growth rate on the burst size (A) and the phage development time (B) of infecting phage T4 (—□—) or  $\lambda$ cIb2 (—○—). The values are from Table 1 of HADAS et al. (1997) for T4, and from Table 5 of WĘGRZYN et al. (2000) for  $\lambda$ .

lysogenic for one phage are resistant to infection by the same phage type. However, as mentioned above, lysogenic strains may grow less efficiently than non-lysogenic bacteria, and synthesis of bioproducts in such strains may be decreased (WU 1998, JONES et al. 2000). Moreover, the potential problems with prophage induction are significant and important (see above). Hence, one might ask if it is possible to find cultivation conditions of non-lysogenic bacteria under which effects of phage infection can be minimised. Recently published reports on bacterial physiology were very helpful in finding the answer.

Although bacteriophages have been considered as models in genetic and biochemical studies for over fifty years (GOTTESMAN 1999, TAYLOR, WĘGRZYN 1998, THOMAS 1993), many physiological aspects of bacteriophage growth have not been sufficiently investigated in relation to extensive molecular biology studies. Recent reports indicated that development of bacteriophages largely depends on the physiology of host cells (GABIG et al. 1998, HADAS et al. 1997, WĘGRZYN et al. 2000). Although the above-mentioned studies concerned basic research rather than applications, we found that some of those results may be very useful in developing a method for reducing the effects of bacteriophage infection on bacterial cultures.

It has been reported that phage burst sizes are significantly smaller when host cells grow slower (GABIG et al. 1998, HADAS et al. 1997, WĘGRZYN et al. 2000). In Figure 2, the most important results of those studies are summarised. Generally, the slower growth of host cells, the longer time of phage development and the lower burst size. Our subsequent studies indicated that at very low growth rates (below 0.1 doublings per hour), development of all tested phages ( $\lambda$ , P1 and T4) was almost completely inhibited (ŁOŚ, NEUBAUER, WĘGRZYN, unpublished data). Actually, complete inhibition of production of progeny phages in infected *E. coli* cultures was achieved by removing the carbon source from the synthetic medium (ŁOŚ, WĘGRZYN unpublished data). This was true for all tested phages ( $\lambda$ , P1 and T4), suggesting that it is a common phenomenon, which concerns most, if not all, bacteriophages.

We consider a possibility that cultivation of bacteria at low growth rates may be used as an efficient method for significant reduction of fatal effects on cells of lytically developing bacteriophages. In fact, while in small-scale cultures in most laboratories high growth rates (about 2 doublings per hour or even higher) are used, in fermentation processes effective growth to high cell densities may be achieved by controlling the growth at much lower rates (see, for example, ANDERSSON et al. 1996, LIN, NEUBAUER 2000). Therefore, the use of low growth rates of cultivated bacteria may be recommended to minimise the risk of deleterious effects of phage infection. Cultures can also be performed by the fed-batch mode from the beginning. Moreover, if symptoms of infection of a bacterial culture in a bio-reactor are observed, the feeding of bacteria should be stopped immediately, as phages should not develop efficiently in starved hosts. An additional

temperature downshift to 20°C, or even a lower temperature, and pH changes can be applied. However, it is worth mentioning that the method described above has been tested only in small-scale cultures and needs to be verified also in large bioreactors.

### What to do to minimise the frequency of spontaneous prophage induction?

As mentioned above, prophage induction may be as dangerous for a microbiological laboratory and a biotechnological factory as phage infection is. Therefore, finding conditions that are unfavourable for prophage induction and still acceptable for those who use bacterial cultures for production of particular substances would be helpful. In the case of lambdoid prophages, overexpression of the *cI* gene (or its homologue), coding for the main phage repressor, which is responsible for prophage maintenance, may be helpful (CZYŻ et al. 2001). However, in recombinant gene expression systems this may be a disadvantage because overproduction of the phage protein may indirectly decrease production of the desired recombinant protein.

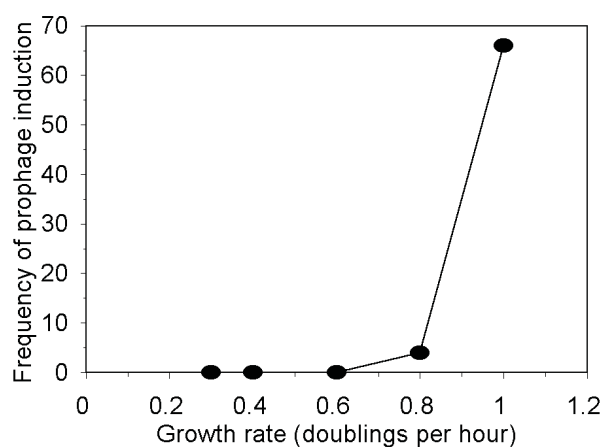


Figure 3. Effects of bacterial growth rate on the frequency of spontaneous prophage induction (per 10<sup>8</sup> cells). The values are from Table 1 of CZYŻ et al. (2001).

Recent studies indicated that low growth rates provide conditions for inefficient prophage induction (CZYŻ et al. 2001, 2002). Namely, the efficiency of spontaneous  $\lambda$  prophage induction is significantly decreased in slowly growing lysogenic bacteria, as compared to the same *E. coli* strain cultivated at high growth rates (Figure 3). Obviously, *recA* hosts are very poor in prophage induc-

tion, but some other properties of such mutants (e.g. high sensitivity to environmental stress factors) may be disadvantageous.

## Conclusions

Bacteriophage infection and prophage induction are serious problems in research laboratories and biotechnological factories. Therefore, it is crucial to use procedures preventing such events. Good laboratory/factory hygiene, sterilisation, decontamination, and disinfection are absolutely necessary to avoid fatal events caused by bacteriophages, although all these procedures cannot guarantee the absence of phage contamination. Lysogenic strains can be used to prevent infection by particular phages, but such strains may synthesise recombinant proteins less efficiently than non-lysogenic strains. Results of recent studies indicated that both phage lytic development and prophage induction are significantly less efficient in slowly growing bacteria, as compared to cells cultivated at high growth rates. Therefore, to minimise phage-mediated effects, it may be recommended to use as low growth rates of bacterial cultures as possible. Obviously, the growth rate must be sufficiently high to synthesise a desired product in cultivated cells effectively. Anyway, it is advisable to provoke starvation conditions in the culture as soon as phage infection is detected or even suspected.

Although unambiguous detection of phage contamination at early stages of infection or prophage induction may be difficult when using traditional methods, a newly developed technology of electric DNA chips enables an early detection of phage genetic material in bacterial cultures, long before they cause complete lysis of host cells (GABIG-CIMIŃSKA et al. 2004a, 2004b). A summary of problems with phage contamination and possible methods for avoiding serious problems caused by phages is presented below.

### Common sources of phages in laboratories

- Dirty pipettes
- Dirty or wet cotton props
- Spoiled shaking incubators
- Strain and plasmid collections (test all incoming strains!)
- Filters of the sterile hood
- Room aeration systems with connections to different laboratories
- Electroporation cuvettes, if repeatedly applied and not carefully treated
- Electrode membranes and buffers that have been in contact with phage-infected cultures

### Recognition of phage infection or prophage induction

- Lysis of cultures (unexpected decrease in optical density, low density after overnight cultivation)
- Visible clumps and threads in the culture
- Thick white foam on the culture (even when the culture is dense), which does not disappear if the flask is left for some time on the table



- Observation by light microscopy: clumps, fatty drops, dirty-like threads, no rod-shaped cells
- Plaque test
- DNA chip technologies (including electric DNA chips)

**Phage elimination methods**

- Temperature treatment at 180°C for several hours under dry conditions
- Basic pH (10 M KOH, final pH of 10),
- Formaldehyde treatment or addition to the culture (especially for pipettes and other plastic materials); increase in autoclaving time and/or temperature
- UV irradiation
- Attention: pore filter membranes are not able to exclude phages – use deep filters!

**Preventive measures**

- Sterile and clean working behaviour
- Dry boxes of pipette tips after autoclaving
- Treat all glass material covered by aluminium foil at 200°C overnight under dry conditions
- Use UV light regularly to disinfect the laboratory
- Use only clean pipettes (clean them regularly in formaldehyde)
- Use sterile cotton plugs for sterile cultivation (not aluminium foil to cover shake flasks); do not use wet or dirty plugs
- Do not open flasks during the cultivation if not necessary. If you must open them, do it only under sterile conditions
- Test your glycerol stocks for phages by the plaque test. Destroy spoiled cultures
- Autoclave old cultures! Treat them with strong disinfecting solutions
- Use basic washing solutions in your washing machine, as they inhibit phages
- Avoid moisture – dry all glass and plastic materials carefully
- Control regularly your sterile hood
- Clean your working tables regularly with disinfecting solutions and keep the laboratory dust-free

**Alleviation of effects of phage contamination**

- Stop or inhibit bacterial growth as soon as you detect phage contamination in the culture
- Remove the carbon source from the medium

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