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Research Article

An enhanced bioindicator for calorimetric monitoring of prophage-activating chemicals in the trace concentration range

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Viruses that infect bacteria (bacteriophages) can either lyse bacteria directly or integrate their genome into the bacterial genome. In the latter case, the viral genome (called prophage) remains dormant, and both phages and bacteria are able to survive in this state. But the silent prophages can be reactivated by, e.g., chemicals, accompanied by the release of substantial quantities of phage particles that further infect other phage-sensitive bacteria, thus harming ecosystems or technical systems by way of a viral bloom. Recently, a calorimetric method was developed to monitor the prophage-activating properties of chemicals. The method evaluates the difference in the metabolic heat of the *Escherichia coli* bioindicator with ($\lambda+$) and without ($\lambda-$) lambda prophages under the influence of the test substances. Simulations and experiments clearly demonstrate that the sensitivity of the test can be significantly improved, when a customized mixture of $\lambda+$ and $\lambda-$ *E. coli* strains is used for enhanced bioindication. Hence, the new method mirrors a common situation in nature, where bacteria with and without prophages coexist. In summary, a monitoring method is suggested that provides quick results (after few hours) and offers both the option for automation with low workload (requires only a few minutes) and usage of commercially available instruments.

Keywords: Bacteriophage / Bioindicator / Biosensor / Calorimetry / Lysogenic cycle / Toxicity test



Additional supporting information may be found online in the Supporting Information section at the end of the article.

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1 Introduction

Bacteriophages (phages) are viruses that infect bacterial hosts and are ubiquitous and abundant in every ecosystem so far investigated on Earth [1]. They are divided into two main categories: lytic (or virulent) phages and temperate phages with two different strategies of phage multiplication. During the lytic infection, the phages kill and lyse their hosts resulting in a release of up to hundreds of progeny virions in a short period of time [2]. In nature, phages' ability to reach hosts is the critical factor of the lytic

lifestyle, as the free phage particles have a finite lifetime. A lytic strategy seems to be mainly sustainable for fast growing hosts. Thus, any changes in the environment affecting the growth rate of bacteria will likely impact the success of the lytic strategy [3]. In contrast, the temperate phages integrate their genomes into the host chromosome. The cells survive and replicate the phage DNA along with their own chromosome. The future fate of the incorporated prophage is hence aligned with its host bacterium. Furthermore, the lysogenic lifestyle makes the host immune against a second infection with one and the same phage and improves

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Abbreviations: *E. coli*, *Escherichia coli*; LB, Luria–Bertani medium; TEM, transmission electron microscopy

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Compiled in honour of the 80th birthday of Professor Wolfgang Babel.

competitiveness by affecting cell metabolism, bacterial adhesion, colonization, immunity, destruction of competing bacteria, and antibiotic resistance [4, 5]. Yet, evidence is accumulating that the percentage of bacteria with prophages can reach up to 80% of a natural bacterial community [6]. The prophages can “live in peace” with the host as long as the environmental conditions do not change. Such changes not only include UV irradiation or the pH shifts but also include man-made chemicals, such as methotrexate, antibiotics, norepinephrine, H_2O_2 , and many more [5, 7, 8]. This is also the point where both propagation cycles become connected. Chemicals causing damage to the DNA are known to trigger on the associated repair mechanisms leading to a prophage activation and phage multiplication. Finally, the triggered host is killed and the phages are released into the ecosystem. They can infect further bacteria in an autocatalytic and self-amplifying manner. Such a viral bloom can threaten the functioning of ecosystems or technical systems (for waste water treatment, biogas formation, etc.) by killing the catalytically active bacteria. The number and kinetics of the released phage particles depend on the lysis time (30–90 min) and the burst size (some tens up to a few hundred phages per bacteria) [2]. Hence, silent prophages in bacteria could be designated as a “dangerous molecular time bomb” [9]. Indeed, the discharges of pollutants containing organic agents (mitomycin C and methotrexate) or metal complex (Pt (II) agents) into the soil have already been reported to activate prophages and to release virions [10, 11].

For screening these prophage-activating properties of chemicals, either the phage number itself or the infection process in its entirety, has to be monitored and quantified. For the quantification of phage particles, many methods have been developed in the last decades. The most obvious method is the counting of bacteriophages under different types of microscopes, which is very laborious and may also involve the inactivated phage particles [12, 13]. The double layer agar method is the most widely used approach considered as the gold standard for phage quantification [14]. But even the gold standard has disadvantages as its result is heavily dependent on the physiological state of the bacterial indicator strains. Furthermore, at a high virus to bacteria ratio, more than one phage can adsorb to a single host cell, leading to an underestimation of the real phage titer. It has been reported that the observed plaques represent between 49 and 100% of the real phage titer [15]. Alternatively, phages can also be quantified using specific marker genes [16], spectrophotometry of the major coat protein VIII [17], Raman spectroscopy [18], and real-time quantitative PCR [19]. For such methods, the samples must often be thoroughly purified and treated prior to analysis. Many of these techniques are unsuited for high-throughput screening and need a highly qualified staff.

Therefore, other options for directly monitoring the process of phage replication through online signals are under investigation. In particular, the metabolic heat production rate is a suitable online parameter, as the infection process is reflected in real time [20]. Microcalorimetric multichannel instruments combine the ability for high-sample throughput with the sensitivity to thermal trace signals [21]. Another potential calorimetric approach is chip calorimetry [22, 23], which in principle allows for a high-sample throughput using magnetic beads [24, 25] or applying segmented fluid technology [26]. However, this lab-on-chip technology is still in its infancy. For these reasons, we

successfully developed and tested a simple and reliable method to assess the influence of chemicals on prophage activation that is based on microcalorimetry. This method was not only shown to be feasible with the established commercially available instruments, but also suitable for automation and high-throughput measurements [27].

2 Materials and methods

2.1 Bacterial strains and culture conditions

Two *Escherichia coli* DSM4230 strains with ($\lambda+$) and without ($\lambda-$) lambda prophages were applied. The prophage-free *E. coli* DSM4230 ($\lambda-$) host strain was obtained from the DSMZ (Braunschweig, Germany). The construction and characterization of the prophage wearing strain as well as the applied media are described in [27]. All induction experiments were conducted at 37°C. The cultures were maintained on Luria–Bertani medium (LB) medium. The pH was nearly constant during the measurement (7.2 ± 0.2) due to the high-buffer capacity of the medium.

2.2 Calorimetric measurements

The measurement principle of the suggested method is explained in the results section. All experiments were conducted using a Thermal Activity Monitor III (TA Instruments, New Castle, DE, USA). The ampoules and caps were autoclaved (for 30 min at 121°C) prior to the experiments. Two types of calorimetric experiments were carried out (A: an anaerobic in liquid culture; and B: an aerobic on solid agar). The first experimental set up was chosen because under such conditions the highest heat effect per mole of generated ATP is to be expected (for the calculation see [27]), whereas the second experimental set up was selected because under its conditions any oxygen diffusion effects on the measurement can mainly be excluded [28]. In the anaerobic experiments (A), 1.9 mL LB media, 0.1 mL bacterial suspension (containing 0.09 mL *E. coli* ($\lambda-$), and 0.01 mL *E. coli* ($\lambda+$)) in LB medium (both with OD₆₀₀ = 0.1) and different concentrations of the test chemicals were transferred to the calorimetric vessel. Thereafter, overlaying 0.2 mL liquid paraffin was added to keep the oxygen away from the media. In aerobic experiments (B), 1 mL agar was transferred to the calorimetric ampoules, and then 10 μ L bacterial suspensions with different concentrations of the test chemicals were dropped onto the solid agar. In both cases, the ampoules were sealed air tight prior to calorimetric monitoring. As test chemicals, mitomycin C (aerobe: 0–30 μ M; anaerobe: 0–6 μ M), Cr (VI) (aerobe: 0–1 mM; anaerobe: 0–80 μ M), and Cis-Pt (aerobe: 0–80 μ M; anaerobe: 0–100 μ M) were considered. These test chemicals were selected for their ability to activate prophages, as has already been established [11, 29].

2.3 Quantification of biomass and induced augmentable phages

To get a better understanding of the observed heat signal, the formation of biomass and intact phage particles were measured offline. Due to its simplicity, the OD was used to record growth.

For the calculation of the cell number, the OD was correlated with the cell number. To ensure comparability, induction experiments were conducted under the same conditions as in the respective calorimetric experiments, and samples were taken hourly and measured immediately at 600 nm using a double beam spectrophotometer (UV-Vis-100PC, Hitachi High-Tech, Tokyo, Japan). For that purpose, the calorimeter ampoules were used, filled as described in the calorimetry section, placed into a tempered chamber (at 37°C), and regularly sampled.

The numbers of the induced phages were quantified by means of the double layer technique according to Adams [14]. In this technique, an augmentable phage particle formed plaque in a phage-sensitive bacterial layer. The results are provided as PFU. The basic layer contained an LB medium in 1.4% agar; whereas the cover layer, in addition to 0.7% agar, contained an exponentially growing indicator bacteria (*E. coli* (λ -); OD600 = 0.3). For PFU quantification, 1 mL of the induced samples were mixed with 100 μ L chloroform to circumvent potential biases that might arise by growing as well as phage-releasing lysogenic bacteria. The samples were centrifuged at $4500 \times g$ for 20 min at 4°C. The chloroform-free supernatant was collected and stored in a fridge at 4°C prior to the final PFU quantification. Ten-microliter portions of serial dilutions of cell-free supernatants were dropped onto the double layer plates with the prophage-free indicator strain. The plates were incubated overnight at 37°C and the plaques were counted on the following day as PFU in each drop area.

2.4 Transmission electron microscopy

Apart from the energetic aspects and the numerical changes in the number of cells and phage particles, variations in the shape and electron beam transparency of infected cells were considered. For that purpose, transmission electron microscopy (TEM) using a TECNAI G2 (ThermoFisher Scientific, Oregon, WA, USA) was carried out. Bacterial cells were aseptically harvested at the end of the anaerobic experiment after 8 h, centrifuged at $5000 \times g$ for 5 min, washed six times in phosphate buffer saline (pH 7.4), and resuspended in 1 mL 2.5% glutaraldehyde. For TEM examination, 1 μ L aliquot of the treated and untreated *E. coli* cells was transferred onto a carbon-coated copper grid [6].

2.5 Modeling and statistical data analysis

Berkley Madonna Version 8.1, developed by R.I. Macey and G.F. Oster at the University of California (Oakland, CA, USA), was applied in order to model the growth pattern under the influence of prophage activation and the resulting heat signals. The differential equations describing the model were integrated using a Runge–Kutta algorithm with an integration interval of $dt = 2 \times 10^{-3}$ h. A reduction in the integration interval was proved to not influence the simulation results significantly. For all kinds of statistics and data fitting, OriginPro 2015 (OriginLab Corporation, Northampton, MA, USA) was used.

3 Results and discussion

3.1 Measuring principle

The goal in using the suggested sensor was to monitor prophage activation in real time as metabolic heat. The measuring principle is shown in Fig. 1. The measured heat signal is based on the voltage difference between the Peltier elements on the measuring and the reference side. With most commercially available instruments, inert material is feasible and implemented on the reference side with approximately the same heat capacity as the sample on the measurement side. Potential problems with the bioindicator response arise from the overlap of prophage activation and toxicity effects (Table 1). The problems can be circumvented, when the signal differences between λ + or the mixture of (λ + and λ -) and λ - are evaluated.

To intensify the bioindicator reaction, λ + is mixed with λ - strains for the reason that the lysogenic lifestyle should make the host immune against further phage infections. Thus, in the case of using a λ + strain alone, only the signals from the cells with chemically induced prophage activation are monitored. In contrast, when a mixture of λ + and λ - is used simultaneously, intensification is to be expected as the released phages from the λ + strain can infect the λ - cells. Provided the infected λ - strain follows the lytic pathway, further bacteria could be infected, and an autocatalytically self-amplifying infection process is to be expected (Fig. 1B). A real solution (for future instruments) could be achieved, when the metabolic heat of a strain without prophages

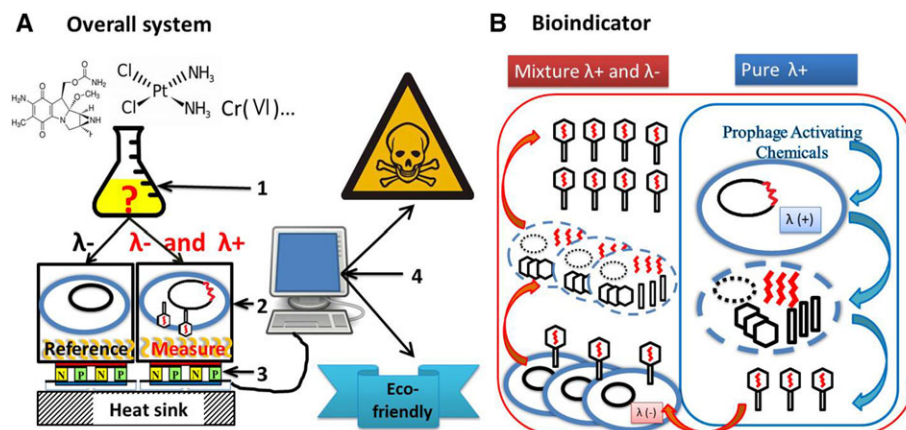


Figure 1. Measuring principle: (A) overall system: (1), preparation of the chemical or the mixture to be examined; (2), λ - on the reference side and the mixture of λ - and λ + strains as the bioindicators on the measuring side; (3), thermal transducers (Peltier elements); (4), electronic evaluation unit; (B) bioindicator: the test chemical activates the prophages in λ + and lead to a phage release and cell burst (blue box, traditional bioindicator). In the mixture (λ - and λ + strain, new bioindicator) the released phages infect more bacteria (λ -).

Table 1. Advantages and weaknesses of different thermal sensor assignments (RS, reference side; MS, measuring side; IM, inert material)

RS	MS	Property of the chemical that affects the heat signal	Tested/feasible with commercially available multichannel instruments
IM	$\lambda-$	Toxicity	In vitro/yes
IM	$\lambda+$	Toxicity + prophage activation	In vitro/yes
IM	$\lambda+$ and $\lambda-$	Toxicity + amplified prophage activation	In vitro/yes
$\lambda-$	$\lambda+$	Prophage activation	In silico/not yet
$\lambda-$	$\lambda+$ and $\lambda-$	Amplified prophage activation	In silico/not yet

is directly (physically) subtracted from the heat of the mixed cultures (Fig. 1A) by placing the $\lambda-$ strain on the reference side. This offers the additional advantage that the influence of random environmental temperature fluctuations on the signal is also reduced. In the following, the data for the effect of mitomycin C (a well-known prophage-activating chemical [29]) is exemplarily shown and discussed. Nonetheless, in the Supporting Information, it is demonstrated that the measuring principle works also with other known prophage-activating chemicals, such as Cr(VI) and cis-Pt.

3.2 Simulations of the heat signal and growth pattern

For finding out the optimum bioindicator composition (ratio of $\lambda-$ and $\lambda+$) and understanding the potential signal, a thermokinetic model has to be developed that considers all the main effects (prophage activation in $\lambda+$, lytic infection of $\lambda-$, growth of both strains, and the carrying capacity of the medium) and combines them with the heat signal. This model uses an established bacteriophage infection model [30] and expands this model by assuming a cell-specific heat [31, 32] ($[7.8 \pm 1.5] \times 10^{-9}$ J/cell) and an elevated heat for the cells in the lytic cycle [21, 23, 33]. Whereas the details of the model are given in the Supporting Information, the results of the modeling are shown in Fig. 2.

In the following, the results of the simulation are discussed. The left side (Fig. 2A and C) shows the expected changes in the thermal traces as a function of the chemical concentration when *E. coli* $\lambda+$ is used as a bioindicator, whereas the right side (Fig. 2B and D) shows the signals, when a mixture ($\lambda+$ and $\lambda-$) is used as a bioindicator. In the case of $\lambda+$ and using inert material as a reference (Fig. 2A), the prophage activation by the chemical leads to a concentration-dependent signal shift at later times and an increase in the signal maximum. The first is calculated due to the inhibition of the growth of the $\lambda+$ strain by prophage activation and the second is due to the enhanced heat production rate of bacteria during the shift from the lysogenic to the lytic infection cycle [21, 23, 33]. Figure 2C represents the expected signal, when $\lambda-$ is used as a reference and subjected to the same conditions as the bioindicator. In this case, prophage activation is at its minimum at hour 10 and maximum at hour 15, where both are concentration dependent. The influence of the chemical on prophage activation is more easily detected, when the $\lambda-$ strain is used as a reference. A prerequisite is, of course, that the conditions on the measurement and reference side are absolutely identical and that $\lambda+$ and $\lambda-$ behaves equally with the exception of the prophage activation.

Figure 2B demonstrates the behavior, when a mixed bioindicator ($\lambda-/\lambda+ = 9$) is exposed to a relatively low-chemical concentration (0.2 au). Here, a biphasic heat signal is predicted when inert material is applied as a reference. The first phase is dominated by exponential growth of the $\lambda-$ strain (as a result of the superiority of this strain) and the activation of the prophages. The phase is finished by an avalanche-like increase in the infection of the $\lambda-$ strain and the related phage number, which finally leads to the extinction of the $\lambda-$ strain. The $\lambda+$ strain cannot become extinct for the reason that the lysogenic lifestyle makes the host immune to any further phage infections [34]. This also explains the second peak, which is dominated by the growth of the $\lambda+$ strain. Obviously, even during this phase, a certain portion of the $\lambda+$ strain shifts to the lytic cycle and dies. However, this process is not autocatalytic and therefore only a linear increase in the phage concentration during this phase is predictable.

Figure 2D illustrates the influence of the bioindicator composition on the heat signal using the $\lambda-$ strain as a reference for a small concentration of the test chemical (0.2 au). The smaller the initial proportion of $\lambda+$ to $\lambda-$ is, the stronger the influence of the chemical will be. The response to the prophage-activating chemical is much stronger for the mixed bioindicator than for the $\lambda+$ strain as a bioindicator. For testing the simulation results, the prophage-free strain $\lambda-$, the prophage wearing strain $\lambda+$, as well as a mixture of 9:1 ($\lambda-:\lambda+$) were treated with the three different test chemicals; the heat production was monitored online; and the numbers of bacteria and phages were measured offline.

3.3 The defined metabolic energy flows

The metabolism and the related heat production rate of the applied enhanced bioindicator to a large extent depend on the bioavailability of oxygen. The price for the simplicity of the measuring principle presented here is the use of a closed measuring ampoule without a homogenization option of the content. Therefore, oxygen gradients are to be expected. Such gradients have already been proven to control the metabolism of *E. coli* [28]; and shifts from the respiratory to the fermentative-respiratory metabolism are reported. Two different methods were used to reduce the influence of the oxygen diffusion.

In the first option, the enhanced bioindicator and the reference were cultivated and exposed to different concentrations of the test chemical (mitomycin C) on the surface of LB agar to keep the oxygen diffusion ways short. Figure 3A clearly demonstrates

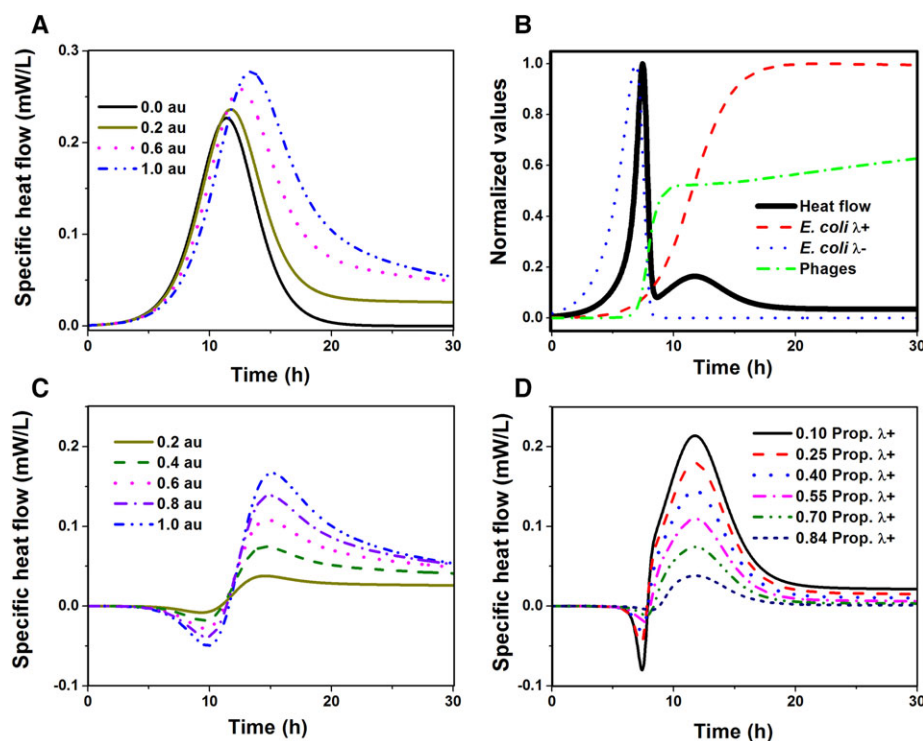


Figure 2. Simulation results: (A) shows the influence of the chemical on heat production of a pure $\lambda+$ strain using inert material on the reference side, (B) represents the influence of the chemical (0.2 au) on a mixed culture (1 part $\lambda+$ and 9 part $\lambda-$) using inert material on the reference side, (C) shows the influence of the chemical on heat production of a pure $\lambda+$ strain using the $\lambda-$ strain on the reference side, (D) shows the influence of the bioindicator composition using the $\lambda-$ strain on the reference side (chemical concentration = 0.2 au).

that up to a concentration of $30 \mu\text{M}$ the test chemical has no significant toxic effect on the $\lambda-$ strain. In case the $\lambda+$ strain is used as a bioindicator, a prophage-activating property required a concentration $>22.5 \mu\text{M}$ to have a thermally measurable influence (Fig. 3B). But when a mixture of $\lambda+$ and $\lambda-$ strains is used as an enhanced bioindicator, a clear effect has already been observed at concentrations $>7.5 \mu\text{M}$ (Fig. 3C). As predicted from our model, the bioindicator mixture shows a biphasic behavior. The significantly stronger heat signal for the mixed bioindicator as compared to the pure bioindicator strain was confirmed by a higher number of phages measured as PFU after growth (Fig. 3). The amount of phages achieved when the mixed bioindicator was used was between 3.5 and 6 times higher as that when a bioindicator with a single strain was applied. The advantage of employing a mixed bioindicator has also been proven by employing further chemicals, such as cis-platinum and Cr (VI), as shown in the Supporting Information.

Another option for preventing a shift in the metabolic pathway was the exclusion of oxygen by covering the growth medium with a thin liquid paraffin film. Potential traces of oxygen in the medium were consumed within a few minutes before measurement had begun due to the highly concentrated and active biomass. Typical results for this approach are illustrated in Fig. 4. Similar to the results obtained under aerobic conditions, no toxic effects for the reference strain (Fig. 4A), slight effects for the pure bioindicator strain (Fig. 4B), and strong effects for the enhanced (mixed) bioindicator (Fig. 4B) were observed in the investigated concentration range. The result indicated that the anaerobic method is also well suited for monitoring the prophage-activating properties of chemicals.

Advantageously, the data confirmed a higher sensitivity of the anaerobic bioindicator in comparison with the aerobic bioindicator. This was likely associated with the fact that the chemical was diluted after the bacterial/agent mixture was added to the agar, whereas in the liquid culture, the agent directly interacted with the bioindicator mixture. Agar has structures [35] that allow small molecules like mitomycin C to easily diffuse into the matrix. Larger entities, such as bacteria, cannot penetrate and grow for this reason on the surface of the agar. The effective concentration of mitomycin C in the vicinity of the bacteria was reduced in this matter. Another explanation could simply be that the applied bioindicator is more sensitive under anaerobic conditions.

In the anaerobic measurements, similar effects on the number of bacteria (Fig. 5A–C) and phage particles (Fig. 5D and E) were observed. The phage number was again 3.7–6 times higher in the tests with the mixed bioindicator compared to those with the pure bioindicator. Notably, mitomycin C had already activated the prophages at a concentration of $0.5 \mu\text{M}$, being not toxic to *E. coli* $\lambda-$.

TEM was used to observe changes in the morphology of the enhanced (mixed) bioindicator in dependency on different mitomycin C concentrations (Fig. 6). The ratio of longer (in the lytic cycle) to shorter (in the lysogenic cycle or the uninfected pathway) cells increased with increasing mitomycin C concentration (Fig. 6, top row). Furthermore, the electron transparency of the cytoplasm rose with increasing mitomycin C concentration. The electron beam transparency might be seen as a measure of cellular degradation, confirming the explanation of the calorimetric results due to prophage activation.

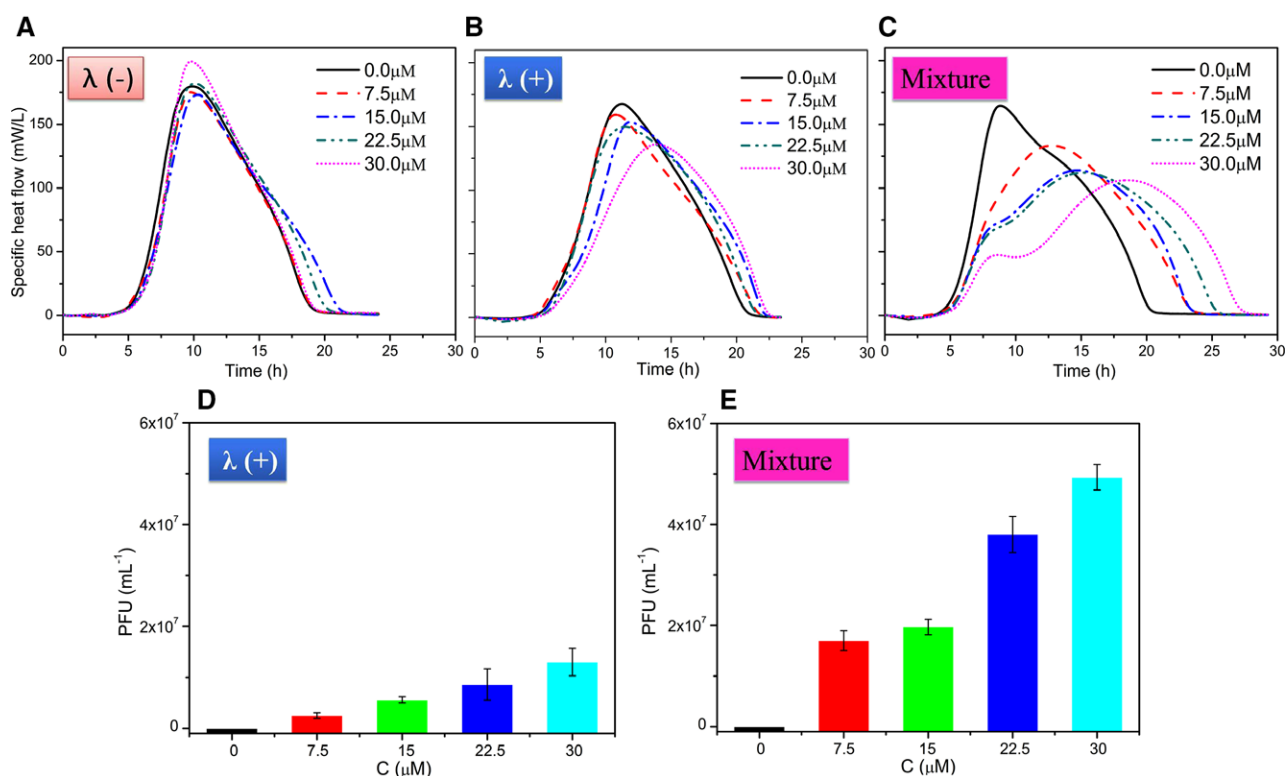


Figure 3. Metabolic heat production rate and plaque forming units (PFU) of *Escherichia coli* growing on 1 mL LB agar in the presence of different mitomycin C concentrations. (A) *E. coli* without prophage ($\lambda -$), (B) with prophage ($\lambda +$), (C) mixture ($\lambda - : \lambda +$, 9:1), (D) PFU: ($\lambda +$), and (E) mixture ($\lambda - : \lambda +$, 9:1).

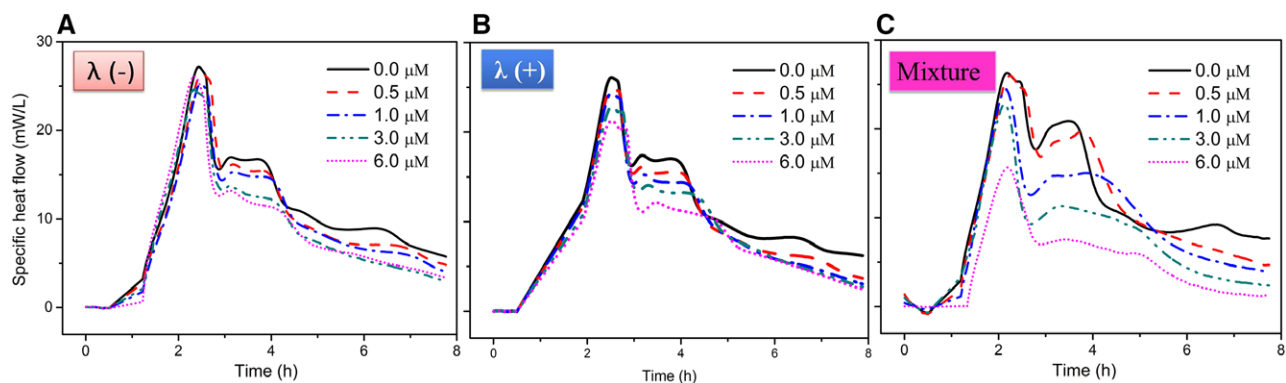


Figure 4. Metabolic heat production rate of anaerobically growing *E. coli* in the presence of different mitomycin C concentrations. (A) *E. coli* without prophage ($\lambda -$), (B) with prophage ($\lambda +$), and (C) mixture ($\lambda - : \lambda +$, 9:1).

3.4 Key figure for describing prophage activation

Based on this level, it is important to find a simple key figure for the strength of the prophage activation. As the heat production rate $P(t)$ is often proportional to the growth rate, and heat $Q(t)$ (Eq. (1)) is proportional to the cell concentration, logistic models (describing the evolution of the cell concentration) promise a good description of the heat

time curve $Q(t)$ and deliver characteristic parameters for growth [32].

$$Q(t) = \int_{t=0}^t P(\bar{t}) d\bar{t}. \quad (1)$$

In our case, the following simple logistic Eq. (2) was found to be most suited to describe the experimental data:

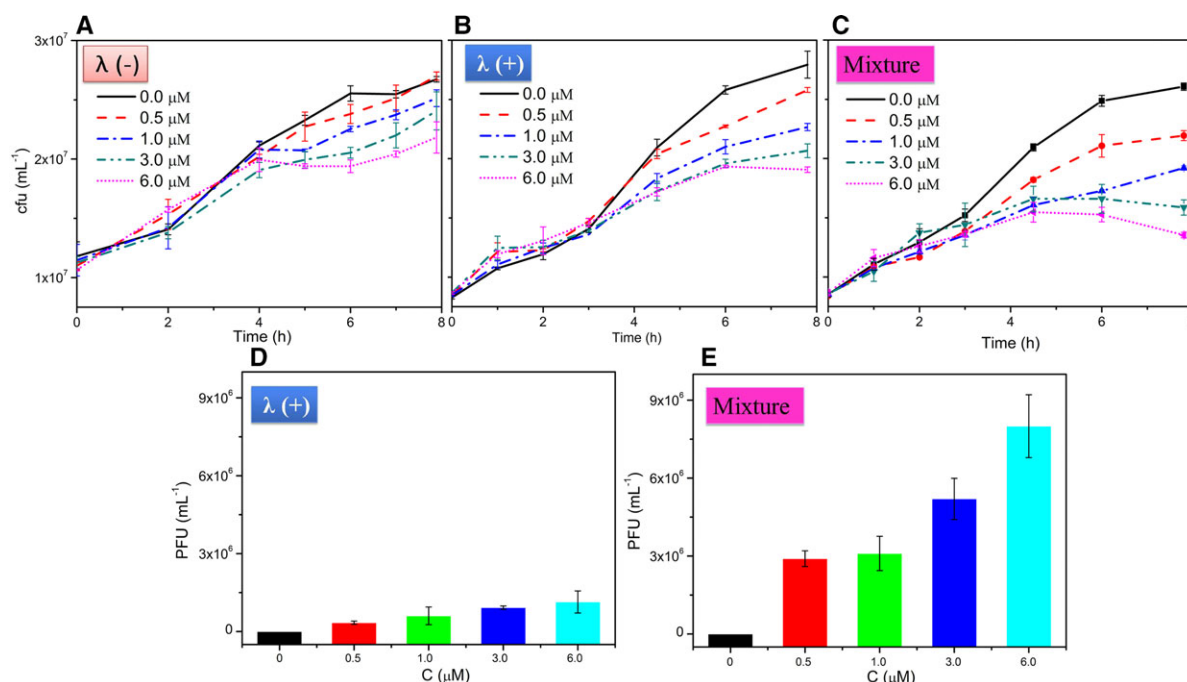


Figure 5. Offline measures of anaerobically growing *E. coli* in the presence of different mitomycin C concentrations. Cell number: (A) *E. coli* without prophage ($\lambda-$), (B) with prophage ($\lambda+$), (C) mixture ($\lambda-:\lambda+, 9:1$), (D) plaque forming unit (PFU): ($\lambda+$), and (E) mixture ($\lambda-:\lambda+, 9:1$).

$$Q(t) = \frac{Q^\infty}{(1 + b \cdot e^{(-k \cdot t)})}. \quad (2)$$

Q^∞ , b , and k are adjustable parameters. The results of the parameter fitting are given in the Supporting Information (Table S1 for aerobic growth on agar and in Table S2 for anaerobic growth). Q^∞ is the total metabolic heat output from the beginning to the end of the growth. It represents the part of the Gibbs energy of all assimilated

nutrients, which is dissipated as heat and not used for biosynthesis. Therefore, Q^∞ could be considered as an indirect measure of the growth efficiency and k as a kinetic parameter describing the growth rate. It should not be confused with the specific growth rate of the conventional Monod model, although it is of the same magnitude.

In search for the most suitable characteristic number, the k parameter (as a measure of the growth rate), the Q^∞ parameter

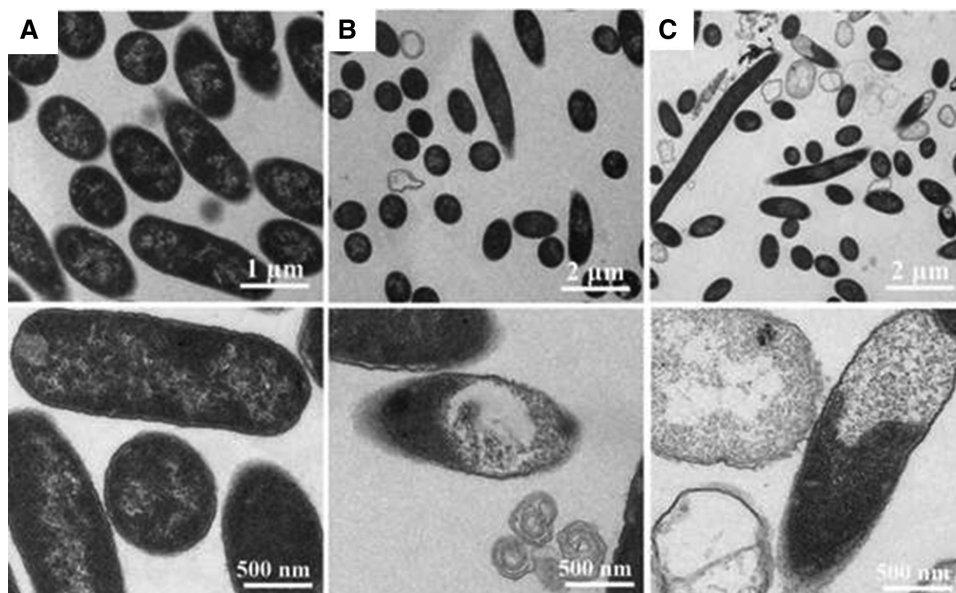


Figure 6. TEM image of *E. coli* (mixture) cells under anaerobic conditions (A) control (0 μM mitomycin C), (B) 1.0 μM mitomycin C, and (C) 6.0 μM mitomycin C.

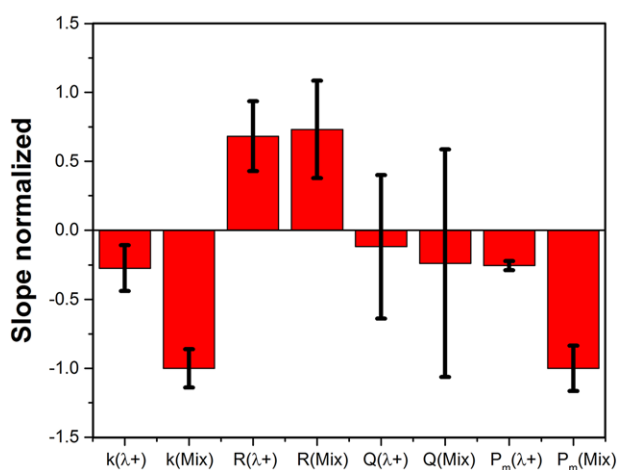


Figure 7. Sensitivity of the growth kinetics (k), the regression coefficient (R), the total metabolic heat (Q), and the maximum heat production rate (P_m) against the prophage-activating chemical.

(as an indirect measure of the growth efficiency), the correlation coefficient R of the nonlinear data fitting (as a measure of the dispersion of the data around the model), and the maximum heat production rate P_m (as a second measure of the growth rate) were correlated with the concentration of the test chemical. The normalized slope of an assumed linear correlation and the respective standard deviation are compared in Fig. 7. The comparison identified both kinetic parameters k and P_m as mainly affected by the prophage activation and therefore as most suited to be used as key parameters. Both parameters confirm again the better suitability of the mixed bioindicator in comparison with the $\lambda+$ bioindicator.

In summary, calorimetry is well-suited as a basic principle for screening chemical agents with regard to their potential prophage-activating properties in the lower micromolar concentration range, when the difference of the heat signal of a prophage wearing bacterial mixture is compared with the signal of prophage-free bacteria. The mixture of $\lambda+$ and $\lambda-$ as a bioindicator has been proved to be more sensitive against the test chemical as the prophage wearing strain alone. The anaerobic growth conditions allow for providing the quickest results and responses to still traces of the test chemical. These findings are in accord with previous reports dealing with a simpler $\lambda+$ bioindicator [27]. Practical advantages of the proposed method are the potential for automation, prevention of sampling errors, ease of data evaluation, and consumption of still fewer and less expensive chemicals for the media. The potential of the method for application in future high-throughput measurements is supported by the recent progress in the development of (1) multichannel calorimetric instruments [36], (2) arrays of chip calorimeters [37, 38], and (3) the rapid sample transport by segmented fluid technology for a single channel lab-on-chip calorimeter [39, 40].

Practical application

Bacteriophages in a “dormant” state (prophages) can be reactivated by chemicals. As a result, the phages are released into the environment and can further infect other phage-sensitive bacteria, which leads to disturbances in technical and environmental processes. A simple and reliable method to assess the risk for chemical prophage activation is therefore crucial. Our new method exploits the metabolic heat difference between bacteria, which switch from lysogenic to the lytic infection pathway, and uninfected bacteria. The usage of a bacterial mixture (with and without prophages) as an enhancer was proven to make the method highly sensitive, even in the concentration range of a few micromoles per liter. The practical advantages of the method are the real-time monitoring of the prophage-activating effect, the suitability for automation and high-throughput measurement, the low-working load, the usage of low-cost chemicals, and inexpensive Peltier elements as thermal transducers.

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The authors have declared no conflict of interest.

4 References

- [1] Roux, S., Enault, F., Hurwitz, B. L., Sullivan, M. B., VirSorter: Mining viral signal from microbial genomic data. *Peer J.* 2015, 3, e395. <https://doi.org/10.7717/peerj.985>. eCollection 2015
- [2] Wang, I. N., Lysis timing and bacteriophage fitness. *Genetics* 2006, 172, 17–26.
- [3] Campbell, A., Conditions for the existence of bacteriophage. *Evolution* 1961, 15, 153–165.
- [4] Richter, C., Chang, J. T., Fineran, P. C., Function and regulation of clustered regularly interspaced short palindromic repeats (CPISPR)/CRISPR associated (Cas) systems. *Viruses* 2012, 4, 2291–2311.
- [5] Wagner, P. I., Waldor, M. K., Bacteriophage control of bacterial virulence. *Infect. Immun.* 2002, 70, 3985–3993.
- [6] Choi, J., Kotay, S. M., Goel, R., Various physico-chemical stress factors cause prophage induction in *Nitrosospira multififormis* 25196—An ammonia oxidizing bacteria. *Water Res.* 2010, 44, 4550–4558.

- [7] Schmidt, H., Shiga-toxin-converting bacteriophages. *Res. Microbiol.* 2001, 152, 687–695.
- [8] Herold, S., Karch, H., Schmidt, H., Shiga toxin-encoding bacteriophages—genomes in motion. *Inter. J. Med. Microbiol.* 2004, 294, 115–121.
- [9] Paul, J. H., Prophages in marine bacteria: Dangerous molecular time bombs or the key to survival in the seas? *ISME J.* 2008, 2, 579–589.
- [10] McDaniel, L., Paul, J. H., Effect of nutrient addition and environmental factors on prophage induction in natural populations of marine *Synechococcus* species. *Appl. Environ. Microbiol.* 2005, 71, 842–850.
- [11] Johnstone, T. C., Alexander, S. M., Linand, W., Lippard, S. J., Effects of monofunctional platinum agents on bacterial growth: A retrospective study. *J. Am. Chem. Soc.* 2014, 136, 116–118.
- [12] Alsteens, D., Trabelsi, H., Soumillion, P., Dufrene, Y. F., Multiparametric atomic force microscopy imaging of single bacteriophages extruding from living bacteria. *Nat. Commun.* 2013, 4, 2926.
- [13] Zago, M., Scaltriti, E., Fornasari, M. E., Rivetti, C. et al., Epifluorescence and atomic force microscopy: Two innovative applications for studying phage-host interactions in *Lactobacillus helveticus*. *J. Microbiol. Meth.* 2012, 88, 41–46.
- [14] Adams, M. H., *Methods of study of bacterial viruses*, The Yearbook Publishers, Chicago, IL 1950.
- [15] Kay, B., Winter, J., McCafferty, J. *Phage display of peptides and proteins: a laboratory manual*, Academic Press, San Diego, CA 1996.
- [16] Waller, A. S., Yamada, T., Kristensen, D. M., Kulima, J. R., Sunagawa, S., Koonin, E. V., Bork, P., Classification and quantification of bacteriophage taxa in human gut metagenomes. *ISME J.* 2014, 8, 1391–1402.
- [17] Smith, G. P., Phage display. *Chem. Rev.* 1997, 97, 391–410.
- [18] Goeller, L. J., Riley, M. R., Discrimination of bacteria and bacteriophages by Raman spectroscopy and surface-enhanced Raman spectroscopy. *Appl. Spectrosc.* 2007, 61, 679–685.
- [19] Refardt, D., Real-time quantitative PCR to discriminate and quantify lambda bacteriophages of *Escherichia coli* K-12. *Bacteriophage* 2012, 2, 98–104.
- [20] Maskow, T., Kiesel, B., Schubert, T., Yong, Z. et al., Calorimetric real time monitoring of lambda prophage induction. *J. Virol.* 2010, 168, 126–132.
- [21] Liu, G.S., Liu, Y., Chen, X.D., Liu, P. et al., Study on interaction between T4 phage and *Escherichia coli* B by microcalorimetric method. *J. Virol. Methods* 2003, 112, 137–143.
- [22] Lerchner, J., Mueller-Hagen, D., Roehr, H., Wolf, A. et al., Chip-calorimetric evaluation of the efficacy of antibiotics and bacteriophages against bacteria on a minute-timescale. *J. Therm. Anal. Calorim.* 2011, 104, 31–36.
- [23] Mariana Morais, F., Buchholz, F., Hartmann, T., Lerchner, J. et al., Chip-calorimetric monitoring of biofilm eradication with bacteriophages reveals an unexpected infection-related heat profile. *J. Therm. Anal. Calorim.* 2014, 115, 2203–2210.
- [24] Lerchner, J., Schulz, A., Poeschel, T., Wolf, A. et al., Chip calorimetry and biomagnetic separation: Fast detection of bacterial contamination at low cell titers. *Eng. Life Sci.* 2012, 12, 615–620.
- [25] Hartmann, T., Mühling, M., Wolf, A., Mariana, F. et al., A chip-calorimetric approach to the analysis of Ag nanoparticle caused inhibition and inactivation of beads-grown bacterial biofilms. *J. Microbiol. Meth.* 2013, 95, 129–137.
- [26] Lerchner, J., David, K., Unger, F., Lemke, K. et al., Continuous monitoring of drug effects on complex biological samples by segmented flow chip calorimetry. *J. Therm. Anal. Calorim.* 2017, 127, 1307–1317.
- [27] Xu, J., Kiesel, B., Kallies, R., Jiang, F. J. et al., A fast and reliable method for monitoring of prophage activating chemicals. *Microbial Biotech.* 2018. <https://doi.org/10.1111/1751-7915.13042>
- [28] Maskow, T., Mariana Morais, F., Rosa, L. F., Qian, Y. G., Harnisch, F., Insufficient oxygen diffusion leads to distortions of microbial growth parameters assessed by isothermal microcalorimetry. *RSC Advances* 2014, 4, 32730–32737.
- [29] Otsuji, N., Sekiguchi, M., Takagia, T., Takagi, Y., Induction of phage formation in the lysogenic *Escherichia coli* K-12 by mitomycin. *Nature* 1959, 184, 1079–1080.
- [30] Beretta, E., Kuang, Y., Modeling and analysis of a marine bacteriophage infection. *Math. Biosci.* 1998, 199, 57–76.
- [31] Beezer, A. E., Bettelheim, K. A., Newell, A. D., Stevens, J., Diagnosis of bacteriuria by flow microcalorimetry: Preliminary report. *Sci. Tools* 1974, 21, 13–16.
- [32] Braissant, O., Bonkat, G., Wirz, D., Bachmann, A., Microbial growth and isothermal microcalorimetry: Growth models and their application to microcalorimetric data. *Thermochim. Acta* 2013, 555, 64–71.
- [33] Liu, Y. G., Li, M. J., Chen, X. D., Liu, Y. et al., Calorimetric study of the metabolic activity of *Escherichia coli* B infected by T4 phage in restricted medium. *Thermochim. Acta* 2005, 435, 34–37.
- [34] Dy, R. L., Richter, C., Salmond, G. P., Fineran, P. C., Remarkable mechanisms in microbes to resist phage infections. *Annu. Rev. Virol.* 2014, 1, 307–331.
- [35] Lahaye, M., Rochas, C., Chemical structure and physico-chemical properties of agar. *Hydrobiologia* 1991, 221, 137–148.
- [36] Wadsö, I., Hallen, D., Jansson, M., Suurkuusk, J. et al., A well-plate format isothermal multi-channel microcalorimeter for monitoring the activity of living cells and tissues. *Thermochim. Acta* 2017, 652, 141–149.
- [37] Torres, F. E., Recht, M. I., Coyle, J. E., Bruce, R. H. et al., Higher throughput calorimetry: Opportunities, approaches and challenges. *Curr. Opin. Struct. Biol.* 2010, 20, 598–605.
- [38] Huynh, T. P., Zhang, Y., Yehuda, C., Fabrication and characterization of a multichannel 3D thermopile for chip calorimeter applications. *Sensors (Basel)* 2015, 15, 3351–3361.
- [39] Maskow, T., Schubert, T., Wolf, A., Buchholz, F. et al., Potentials and limitations of miniaturized calorimeters for bioprocess monitoring. *Appl. Microbiol. Biotechnol.* 2011, 92, 55–66.
- [40] Wolf, A., Hartmann, T., Bertolini, M., Schemberg, J. et al., Toward high-throughput chip calorimetry by use of segmented-flow technology. *Thermochim. Acta* 2015, 603, 172–183.