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PAPER

Titanium dioxide-assisted photocatalytic induction of prophages to lytic cycle

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The investigations on the kinetics of photocatalytic inactivation of bacteriophages, lactic bacteria and lysogenic lactic bacteria have shown that the rate of bacterial inactivation is *ca.* 10 times less than the inactivation of bacteriophages. Titania-assisted photorelease of bacteriophages from lysogenic bacteria proves that photogenerated reactive oxygen species affect the deoxyribonucleic acid (DNA) of bacteria before their deactivation. On this basis a novel photocatalytic method of a prophage induction to the lytic cycle and detection of lysogenic bacteria is proposed.

Introduction

In the last decades, TiO_2 photocatalysis has attracted great attention as a promising method for water and air cleaning.[1–4] Highly-active radical species produced at TiO_2 surfaces under UV irradiation can participate in a series of oxidation reactions. In particular, hydroxyl radicals are generated by photoholes from the TiO_2 valence band and superoxide ions are formed due to interaction of photoelectrons from the conduction band with molecular oxygen. Thus, the destruction of organic contaminants and their mineralization is expected.[5–8] As strong oxidants, the reactive oxygen species generated by TiO_2 photocatalytic reactions cause a variety of damage in microorganisms, leading to their rapid inactivation. Since the pioneering work of Matsunaga *et al.*[9] reported the microbiocidal effects of platinised TiO_2, the interest towards TiO_2-assisted photocatalytic inactivation has risen intensely.

These disinfection studies established the basic photokilling mechanisms and identified the effective disinfection factors.[9–14] It is also obvious that accurate kinetic data of photoinactivation is a prerequisite for the design of an effective photocatalytic disinfection system.[14] Inaccurate estimation of a system's performance may lead to inadequate use and wasted financial investment. Therefore, a robust mechanistic model is needed to determine the most efficient combination of contact time, catalyst quantity and light exposure.[14] Understanding the fundamental reactions which occur during the photocatalytic process will be important in improving and modelling the process for different microorganisms (bacteria, viruses, fungi, algae, protozoa).[15] In contrast to bacteria [9–15] the mechanism of inactivation of simple biological structures (viruses, phages) still remain unclear. We focus here on bacteriophage (*Lactococcus phage*, *Siphoviridae* 936) and Gram-positive bacteria (*Lactococcus lactis* (*L. lactis*)). The bacteriophage was chosen as a microorganism as it has a simple organization when compared to bacteria, and phages are more resistant to UV-inactivation. Recently it was shown, that titania-assisted inactivation of Gram-negative bacteria occurs faster than that of Gram-positive bacteria due to their different cell wall morphology.[13–15] Therefore, the UV exposure ensuring the inactivation of Gram-positive bacteria should be enough to provide complete inactivation of Gram-negative bacteria. In the presence of photocatalysts capable of the efficient production of superoxide ions (*e.g.*, TiO_2 modified by bimetallic Ag–Ni particles) the rate of inactivation of Gram-positive bacteria increases dramatically.[13] Here, we use the unmodified photocatalyst to ensure a relatively low rate of inactivation of model microorganisms, allowing us to trace the kinetics of this process.

Along with inactivation kinetic studies, fundamental research is needed in order to fill the gaps in the existing knowledge of the application of photocatalysis, not just for disinfection but also for detection. As a model for detection based on unmodified TiO_2 photocatalysis, we suggest the photocatalytic scenario of titania-affected prophage (DNA of *Lactococcus phage*, *Siphoviridae* P335, x411-f and x415-f) induction to lytic cycle accompanied with their release from lysogenic Gram-positive bacteria (*L. lactis*).

Experimental

The strains of *Lactococcus lactis* bacteria were obtained from a collection of the Department of Biotechnology and Bioecology of the Belarusian State Technological University (Table 1). All cultures were allocated from a surface of green parts of plants and identified by phenotype with the use of molecular-genetic tests. Bacteria were cultivated by standard methods at 30 °C in liquid or on the condensed peptide, M17 media containing 0.5 wt%

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Table 1 Biological objects used for photocatalytic inactivation studies

Subjects	Species	Strains (feature)
Lactic acid bacteria (*L. lactis*)	*Lactococcus lactis*, ssp. *lactis*	411 415 509 404/2
	Lactococcus lactis, ssp. *cremoris*	502
	Lactococcus lactis, ssp. *diacetilactis*	503
Lactococcus phages (family *Siphoviridae*)	936	BIM BV-30 (virulent phage)
	P335	x411-f (temperate phage) x415-f (temperate phage)

of glucose.[16–18] The nanodispersed TiO_2 Hombikat UV 100 from Sachtleben Chemie was used as the photocatalyst.

A two-layer method was used for revealing negative colonies of phages.[19] The bottom layer consists of M17 agar (1.0 wt%) media containing glucose (0.5 wt%), $CaCl_2$ (5 mM l^{-1}) and glycin (0.75 wt%). The top layer differed from the bottom layer only by the agar concentration (0.4 wt%). For evaluating the sensitivity of the phages to photocatalytic action of TiO_2, 1 ml prophage lysates with concentration *ca.* 1.2×10^9 plaque-forming units (PFU) ml^{-1} and 5 mg of TiO_2 powder or SiO_2 powder (used in the control experiments) were placed into a Petri dish with a diameter of 25 mm. For photocatalytic experiments, a 365 nm line of a 120 W high-pressure Hg lamp was used; the light intensity was 15 mW cm^{-2}. The exposed suspensions were diluted by 10^3 and cast over agar layers with model culture *L. lactis* 411 onto M17 medium. The incubation period was 24 h. After incubation the PFU ml^{-1} was determined, taking into account the dilution factor.

To induce the lytic cycle in prophages, lysogenic bacteria cultures in a stationary growth phase were irradiated with UV light. Lysogenic bacteria (*ca.* 10^9 colony-forming units (CFU) ml^{-1}) were diluted 100-fold with 0.15 M NaCl solution. 3 ml of bacteria suspension and 20 mg of powder were placed into a sterile Petri dish with diameter of 60 mm and stirred continuously during irradiation. Immediately after exposure, suspensions were cultivated using the two-layer method (the M17 media) described above.

PCR analysis was performed according to Ref. 20.

For the preparation of samples for TEM investigations, the phagolysate (~10^9 PFU ml^{-1}) was centrifuged at 6000 rpm for 10 min (+ 4 °C) and the supernatant was centrifuged at 20 000 rpm for 10 min (+ 4 °C). The precipitate was then diluted by adding 1 ml 0.1 M ammonium acetate solution, centrifuged at 20 000 rpm for 10 min (+ 4 °C) and diluted by adding 15 ml of 0.1 M ammonium acetate solution. Then 10 µl of suspension, together with 10 µl of 2% tungsten phosphoric acid solution were placed onto a copper grid. The liquid was removed after 1 min by touching the edge of the grid with blotting paper. The samples were dried at room temperature.

Results and discussion

Bacteriophage inactivation

Phage BIM BV-30 with intermediate sensitivity to chemical inactivating factors, as well as Gram-positive bacteria *L. lactis* have been chosen as model systems for kinetic study of TiO_2-assisted photoinactivation (Table 1).

We performed the experiments of bacteriophage titration on the tested bacteria after UV irradiation (λ = 365 nm) in presence of titanium dioxide and silicon dioxide. Inactivation of bacteriophages under UV light irradiation was drastically enhanced in the presence of TiO_2 photocatalysts: 20 min irradiation decreased the phages reproduction activity by a factor of 33. The pathophysiological activity of the TiO_2 photocatalyst against bacteriophages can be attributed to high oxidation power inherent in reactive oxygen species produced at the surface of titania particles under supra-bandgap illumination. TEM investigations show that the oxidation by reactive oxygen species causes photostimulated denaturation of the capsid and tail (Fig. 1). Finally, it affects specific adsorption of bacteriophage and/or damages molecules of nucleic acids

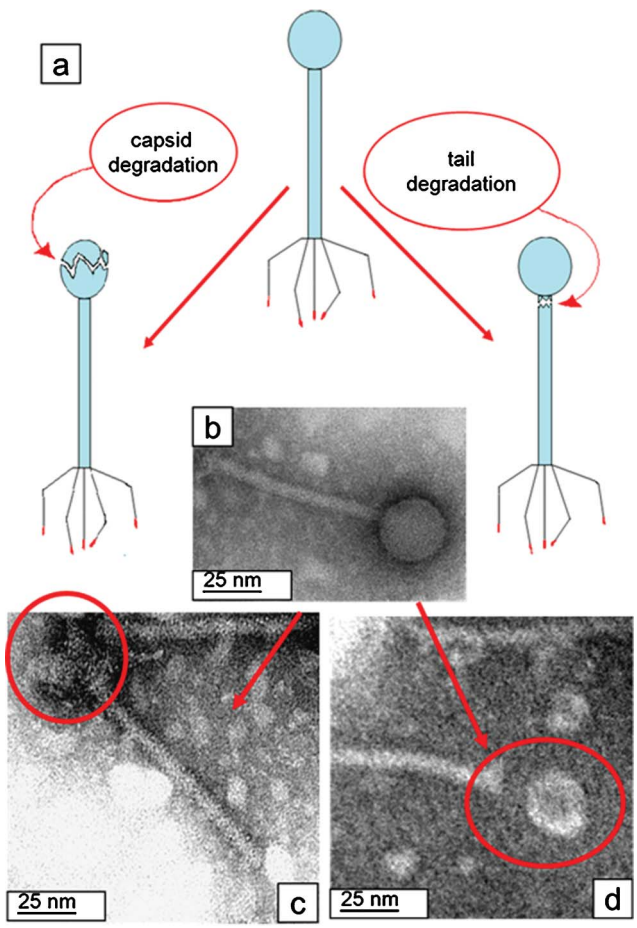

Fig. 1 (a) Schematic representation of phage deactivation. (b) Transmission electron microscopic (TEM) images of initial phages. (c, d) TEM images of phages after UV irradiation in the presence of titania.

Table 2 Bacteria survival and induction of prophages to the lytic cycle under UV irradiation in the presence of a photocatalyst

Strains	C_o,[a] CFU ml^{-1}	C_o,[b] CFU ml^{-1}	S,[c] %	Concentration of phages (PFU ml^{-1}) after UV irradiation[d] with	
				TiO_2	SiO_2
L. lactis 411	1.2×10^9	4.3×10^8	35.8	2.3×10^2	0
L. lactis 415	1.4×10^9	6.7×10^8	47.9	1.1×10^2	0

[a] C_o-initial bacteria concentration. [b] C-bacteria concentration after UV irradiation with TiO_2. [c] Bacteria survival $S = C/C_0 \times 100\%$. [d] Irradiation time was 2 min.

The photogenerated reactive oxygen species cause a DNA chain breakage, followed by the fragmentation of the DNA molecules. We observed the significant reduction of luminescence intensity of phage DNA strips in agarose gel after separation by electrophoresis. Furthermore, we detected an absence of changes in DNA electrophoretic mobility and intensity of luminescence of the strips formed by assays of DNA exposed to the UV irradiation in solution without reagents or with photocatalytically-inert SiO_2 (Fig. 2). Similar results were obtained for the DNA assay kept for 60 min in darkness in the presence of TiO_2. An assay of DNA exposed in the presence of TiO_2 (60 min, λ = 365 nm) showed appreciable reduction of luminescence intensity in gel-electrophoresis and, therefore, a decrease of initial molecule concentration. Obviously, during UV-irradiation the reactive oxygen species generated on a surface of TiO_2 cause the destruction of DNA accompanied by formation of large number of fragments.

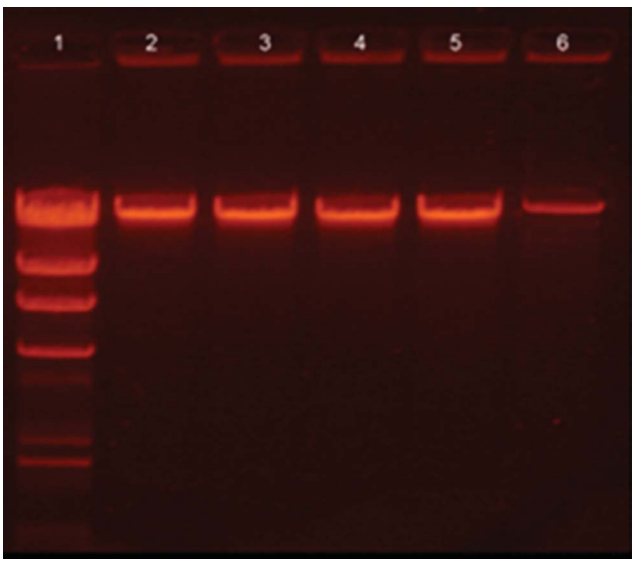

Fig. 2 Bacteriophage BIM BV-30 DNA gel-electrophoresis. (1) DNA-ladder λ/HindIII, (2) native phage DNA, (3) DNA after UV irradiation in the absence of photocatalyst, (4) DNA after UV irradiation in the presence of SiO_2, (5) blank experiment (DNA was kept in darkness in the presence of TiO_2), (6) DNA after UV irradiation in the presence of TiO_2.

Photocatalytic induction of prophages to lytic cycle

Lysogenic (*i.e.*, containing DNA of prophage) and non-lysogenic *L. lactis* strains have been chosen as model systems for demonstrating the possibility of photocatalytic detection of lysogeny, employing TiO_2. Prominent features of lysogenic bacteria populations is the inheritance of prophages. However, the lysogenic state is extremely unstable due to the antagonism of repressor proteins of the lytic cycle, and antirepressors.

The results of the photocatalytic experiments, collated in Table 2, provide evidence for the ability of the titania photocatalyst to induce the lytic cycle in the population of lysogenic cells. Even short-term (2 min) irradiation of lysogenic strains in the presence of titania photocatalyst leads to induction of bacteriophages to lytic cycle. On the contrary, UV irradiation in the presence of photocatalytically-inactive SiO_2 does not lead to negative colonies of free phages at after 2 min, nor after 2 h of irradiation.

The induction of bacteriophages to the lytic cycle due to photocatalytic treatment of bacteria *L. lactis* 411 was monitored by TEM. Fig. 3 demonstrates that bacteriophage are leaving *L. lactis* 411 bacterium under the titania-assisted photocatalytic stimulus.

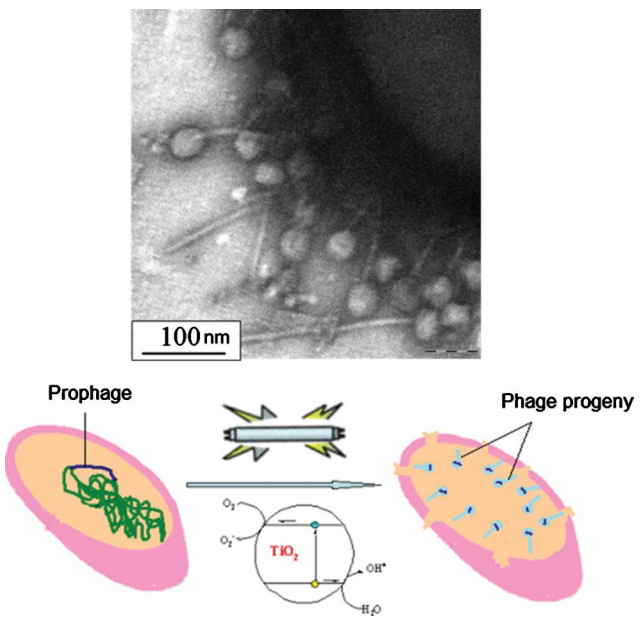

Fig. 3 TEM image showing mature phages leaving bacterium *L. lactis* 411 exposed to UV light in the presence of TiO_2; below the schematic representation of photo induced release of bacteriophage from lactic bacteria is given.

The virulent phages (Table 1) may originate from damage of temperate phages. Thus, any factors that damage the structure of DNA are potentially able to provide transformation of the temperate phages into the virulent form, and induce the lytic cycle in a population of lysogenic cells.

Table 3 The effect of UV irradiation in the presence of catalyst on the concentration of bacteria *L. lactis* and their prophages

Bacteria	Initial concentration of cells, CFU ml^{-1}	Concentration of cells (CFU ml^{-1}) after UV irradiation	Concentration of phages in the irradiated suspensions (PFU ml^{-1}) in the presence of	
			TiO_2	SiO_2
L. lactis 411	1.2×10^7	4.3×10^6	2.3×10^2	0
L. lactis 415	1.4×10^7	6.7×10^6	1.1×10^2	0
L. lactis 404/2	2.0×10^7	5.9×10^6	0.8×10^1	0

For the detection of lysogenic bacteria, two approaches are traditionally used (i) PCR, (ii) photochemical method[21] consisting of a long-term strong UV irradiation (LTSUVI) followed by registering of suspension turbidity (Fig. 4). The proposed third approach, (iii) titania-assisted pathway of photocatalytic DNA biodetection is much more efficient, saving both time and energy.

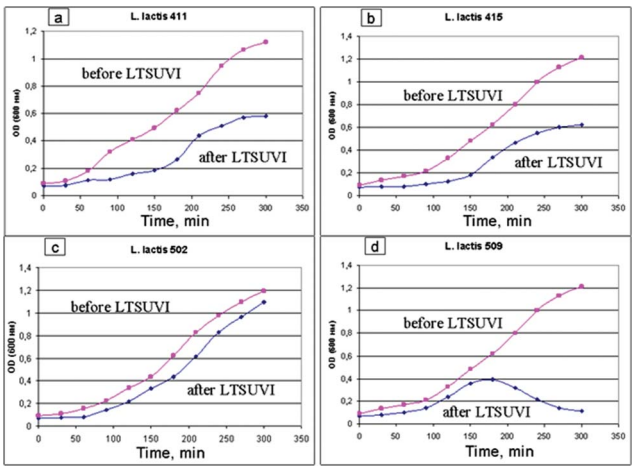

Fig. 4 Time dependencies of optical density for bacterial cultures before and after long-term strong UV irradiation (LTSUVI): (a) *L.lactis* 411, (b) *L.lactis* 415, (c) *L. lactis* 502, (d) *L. lactis* 509.

The visual changes in the optical density of suspensions with time after LTSUVI (λ = 240 nm) were determined for the 20 cultures of *L. lactis* isolated from the surface of the green parts of plants. However, we did not register the expected stop of pattern growth of the culture. Fig. 4 shows the time dependencies obtained for the chosen strains. Thus there were doubts as to whether, after LTSUVI, the bacteria are lysogenic or not. The cells of *L. lactis* ssp. *lactis* 411, are lysogenic as shown by PCR analysis. The second strain, *L. lactis* subsp. *cremoris* 502, a non-lysogenic bacteria, was susceptible to several virulent phages. The cells of both strains showed slightly discernible growth retardation after UV irradiation. The free phages were not identified in the suspensions of UV-irradiated bacteria by cross test: phage-negative colonies were not registered. The LTSUVI method thus can't provide 100% accuracy in bacteria lysogeny detection. It is necessary to repeat such tests several times, or resort to using the PCR method, which is not everywhere available. We can solve the problem using the photocatalyst; simultaneously we can drastically decrease the time of irradiation and detect bacteria lysogeny in all cases.

We performed the PCR analysis of *L. lactis* DNA using oligonucleotide primers P335A and P335B that are specific to the unique sequences of the genomes of temperate phage species P335. We observed prophage-like structures in cells of three strains. The presence of amplification products of the required size (196 bp) formed in the process of PCR analysis of chromosomal DNA of bacteria *L. lactis* subsp. *lactis* 415, *L. lactis* subsp. *lactis* 411 and *L. lactis* subsp. *lactis* 404/2 indicate the presence of the DNA of the temperate phage P335 species, or prophage-like elements in the genomes of these bacteria. These strains were used in further experiments on photocatalytic induction of prophages in the presence of titanium dioxide photocatalyst to determine photocatalytic method efficiency.

Table 3 presents data on the survival of bacteria and the effect on induction of prophages to the lytic cycle after 2 min UV-irradiation (λ = 365 nm) of cell suspensions in the presence of the semiconductor photocatalyst (TiO_2) and the inert substance (SiO_2). The production of DNA fragments was observed for two of the tested strains to ensure, by contrast, that no DNA fragments were present in the suspensions, observed after UV irradiation in contact with the dispersed SiO_2.

Furthermore, PCR analysis demonstrates the presence of DNA fragments of the temperate phage species P335 in the bacteria *L. lactis* ssp. *lactis* 404/2 in the tested bacteria after UV irradiation in the presence of TiO_2, whereas the free phages were not detected. The latter fact can be attributed to the typical to *Lactococcus* and their phages phenomenon of inheritance of chromosomal DNA prophage-like elements.

Primers 5′ GAAGCTAGGCGAATCAGTAAACTTGCTAG 3′ and 5′ CGGCTATCTCGTCAATTGT TCCGGTTGC 3′ were used to detect temperate phage DNA of group P335 in bacteria *L. lactis* 411 and *L. lactis* 415 (Fig. 5). Bacteria *L. lactis* 411 and *L. lactis* 415 with TiO_2 phages were propagated on non-lysogenic strain *L. lactis* subsp. *cremoris* 502. The lysates (x411-f and x415-f) were analyzed by PCR with the above mentioned primers. Both lysates have provided occurrence characteristic for phages of group P335 (Fig. 5a). Fig. 5b shows the formation of negative colonies

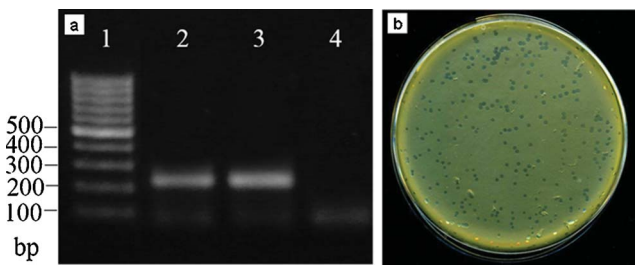

Fig. 5 (a) DNA fragments after amplification of characteristic DNA of moderate phages: (1) DNA-marker, (2) x411-f, (3) x415-f, (4) *L. lactis* 502. (b) Negative colonies formed after analysis of the lysogenic bacteria suspension after UV irradiation with TiO_2.

after analysis of the lysogenic bacteria suspensions after UV irradiation with TiO_2. Thus, the method suggested here is effective for titania-assisted detection of DNA and lysogenic bacteria.

Conclusions

In particular, we studied the kinetic activity and the mechanism of bacteriophage deactivation and compared it with bacterial deactivation. We demonstrated that the novel titania-assisted photocatalytic method of bacteriophage detection has 100% efficiency during bacteriophage release from lysogenic bacteria. The kinetic activity of the deactivation of bacteriophages dramatically exceeds that of bacterial deactivation. We also showed that the time for detection of lysogenic bacteria is less than for bacterial deactivation. Thus, release of bacteriophages from bacteria is not accompanied by the inactivation of bacteriophages. The procedure of photocatalytic lysogenic bacteria detection includes the following steps: (a) obtaining of daily lysogenic bacteria cultures by the standard methods; [16–18] (b) cultivation of lysogenic bacteria in a sterile physiological solution and addition of the nanodispersed titania photocatalyst to the resultant suspension; (c) UV-irradiation of the mixed suspension; (d) seeding of the irradiated culture by a standard two-layer method[19] with the subsequent crops incubated for some days; (e) calculation of number of negative colonies and concentration of phages (PFU ml^{-1}). The method suggested here of lysogenic bacteria detection is significantly cheaper and simpler in comparison to PCR considering that LTSUVI decreased the time of detection from 2 h to 2 min.

Acknowledgements

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