A new replication medium enables a rapid identification of *φx-174* virus by synchronous fluorescence of Tryptophan.

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Abstract

Development of rapid methods for identification of bacteriophages based on their intrinsic fluorescence is challenging. Pure bacteriophages may be detected based on the strong fluorescence of the amino acid Tryptophan that exist in their proteins. Nevertheless, Tryptophan is a molecule that also exist in high quantities in the bacterial hosts and their cultivation media. In this work, we show that simple separation of the bacteriophage *φx-174* from its *E.coli* host (grown on standard cultivation medium) by filtration is not sufficient for its identification based on the intrinsic fluorescence of its Tryptophan content. This is mostly because of the tryptophan residues that derive from the cultivation medium. We fabricate a new cultivation medium that does not have any significant fluorescence overlap with Tryptophan. By utilization of this new cultivation medium, we can identify *φx-174* based on the spectral fingerprint of its intrinsic Tryptophan content by synchronous fluorescence measurements.

Introduction

Since the dawn of humanity, pathogens were always considered as a major risk for the human health. Over the years different efficient anti-bacterial agents were successfully developed such as anti-biotics and various peptide based drugs[1]. However, in the case of viral attack many

infective diseases have remained cureless.[2] In the recent year, global pandemic (caused by SARS-CoV-2 virus) has spread all around the world causing millions of deaths and severe illnesses. [3] Rather than finding a cure, one of the main tools in the struggle against viral plagues is the detection of the viral presence in infected people. Such a diagnosis enables to keep them isolated and, in this way, to prevent them from infecting additional people. Therefore, the development of sensitive rapid viral identification method is highly important.

Classical identification methods utilize bacterial or mammalian cells as host for the viruses. The cell lysis that is caused by the viruses forms discrete visible zones (plaques) that can be detected by microscopic observation. [4] Purification of viruses from their host cell debris and other contaminants was previously done by different methods of density gradient centrifugation[5], ultrafiltration[6], and different chromatographic methods.[7, 8] The small sizes of viruses makes them invisible to simple microscopic observation and can be done by transmission electron microscopy (TEM) [9] or by utilization of fluorescent labelling agents under advanced microscopic instrumentation[10]. Determination of viral structures was extensively investigated by X-ray crystallography, NMR and cryo-TEM. [11, 12]. While a high purification degree of viruses is essential for their structural and fundamental studies, it may be time consuming or require the utilization of expensive instruments. Therefore, such methods may not be feasible when a rapid identification is required. Identification of virus can be done without an extensive purification process by utilization of biosensors[13–17]. In most cases such biosensors are based on immunoassays in which unique antibodies can target specific viruses. Identification of the bound antibodies can be done by simple gel-electrophoresis or by immunofluorescence methods. [18].

Another identification method which is very precise is the next generation sequencing (NGS) that can sequence the whole genome of the virus in a relatively short time. [19]A big advantage of sequencing the whole viral genome derives from the ability to identify unexpected mutations and being able to follow evolutionary evolvement. [20] Nevertheless, whole genome sequencing is still relatively expensive for a large-scale usage.

A cheaper method for detection that is extensively used in the recent years is polymerase chain reaction (PCR) [11, 12]. This method can detect viruses by amplification of specific sequences in the viral genome. An enhanced sensitivity was achieved by the developed of loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) [21–25].

Different Spectroscopic methods were reported to identify isolated viruses. Raman spectroscopy was reported to be able to identify viruses [26] and also help determining their characterize their structure [27–29]. The amino acids Tryptophan, Tyrosine and Phenylalanine have an intrinsic fluorescence and can be used for the detection of isolated viruses. [30] Intrisic fluorescence can also be measured from nucleic acids, however is relatively low, and in many cases may not be sufficient to be detected [31]. Nevertheless, the fluorescence intensity of nucleic acids can be detected by utilization of labeling agents such as SYBR dyes, that significantly amplified it. [32]

Several scanning methods are commonly available in commercial fluorimeters. The scanning method which is most extensively used is the emission scan in which the excitation is being done with a single wavelength and a wide range of emission wavelengths is collected. A more advanced scanning method is synchronous fluorescence (SF). [33, 34] In this scanning method, both the excitation and emission wavelengths are scanned simultaneously while keeping a constant wavelength interval ($\Delta\lambda$) between them. When the wavelength interval $\Delta\lambda$ between the excitation and emission wavelength is chosen properly, the resulting spectrum will show one or several features that are much more resolvable than those in the conventional fluorescence emission scan. The maximum fluorescence intensity for a particular component occurs when $\Delta\lambda$ corresponds to the difference between the wavelengths of the excitation and emission maxima for that component [35, 36]. By narrowing the spectral bands, contraction of the spectral range, and simplification of the emission spectra - overlapping of spectra can be minimized [37, 38]. While synchronous fluorescence methods have demonstrated as rapid tool for bacterial classification and identification [39–42], and even distinguishing between live and dead bacteria [43], to our best knowledge, no rapid identification of viruses by intrinsic synchronous fluorescence was reported.

Although fluorescence spectroscopy has a great potential for rapid identification of pure viruses based the intrinsic fluorescence of their amino acids, it is not compatible for matrixes with many biological compounds that are also consist of the same amino acids. In this work, we report a novel composition of a medium for the cultivation of the bacterial host of the virus *φx-174*, whose fluorescence contribution does not have any significant overlap with tryptophan. We show that this medium enables the identification of the spectral fingerprint of *φx-174* by a simple by a simple filtration followed by a synchronous fluorescence measurement.

Results

Optimization of Δλ for synchronous fluorescence measurements of Tryptophan

One of the main native fluorescence contributors in biological components is the amino acid Tryptophan (Trp). We wished to assess the possibility to use the SF spectra of Trp as a fingerprint for identification of *φx-174*. For this purpose, we first investigated optimal the measurement optimal Δλ that will give the maximal intensity for Trp. Pure Trp solution (1 µM) was measured using increasing Δλ values. The highest intensity was obtained at Δλ = 60 nm (Fig. 1).

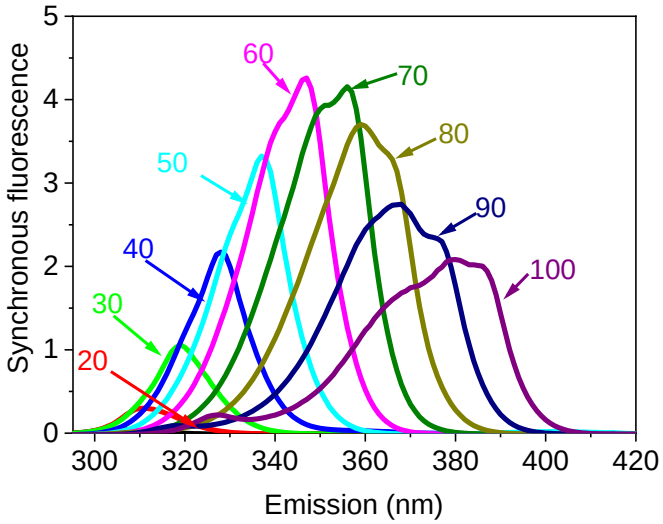

Fig. 1 – SF measurements of pure Trp. SF spectra of pure TRP was measured using increasing Δλ values between 20 – 100 nm. The maximal intensity was obtained at Δλ = 60 nm. Δλ = 20 nm (red), 30 nm (light green), 40 nm (blue), 50 nm (cyan), 60 nm (magenta), 70 nm (dark green), 80 nm (dark yellow), 90 nm (dark blue) and 100 nm (purple).

Tryptophan residues in the bacterial media limits the detection of *φx-174*

A frequently used method for replication of *φx-174* in in petri dishes is called the double agar layer method. [44] In this method the virus is replicated on an *E.coli* layer that is grown on top of a gel bacterial substrate. One of the most frequently used substrates is Nutrient Agar (NA). Whole bacterial cells can be easily separated from *φx-174* by quick simple filtration or centrifugation. However, peptides ore proteins which consist of Trp may also originate form lysed bacterial cells or diffuse from the lower layer of the bacterial substrate. In this case, the separation from the virus is harder to perform. We wished to explore whether lysed cells and diffused molecules from the substrate limit the possibility to identify *φx-174* based on its Trp content. *φx-174* plaques were replicated on an *E.coli* layer on top of NA agar. The *φx-174* were extracted by ammonium acetate buffer for 10 min. The supernatant was removed and filtrated through 0.2 µm filter. The same procedure was repeated for petri dishes without *φx-174.* SF of the filtrates were measured (Δλ = 60 nm, λ_{em} (280 – 450 nm) (Figure. 3). Big peaks with maximal emission of 2.43 and 3.53 were obtained for the filtrates with and without *φx-174* respectively. Fitting analysis of the filtrate's peaks and pure Trp was calculated by

multiple linear regression showing a good fit with RSS values of 0.11 and 0.17 with and without *φx-174* respectively. (Figure. S1). The good fit indicates that the fluorescence in both spectra are the spectral fingerprint of Trp. The obtained results show that the high fluorescence contribution of the NA substrate or lysed bacterial cells that severely overlaps with the net fluorescence that originates from *φx-17*. This overlap disable the ability for identification based on its Trp content.

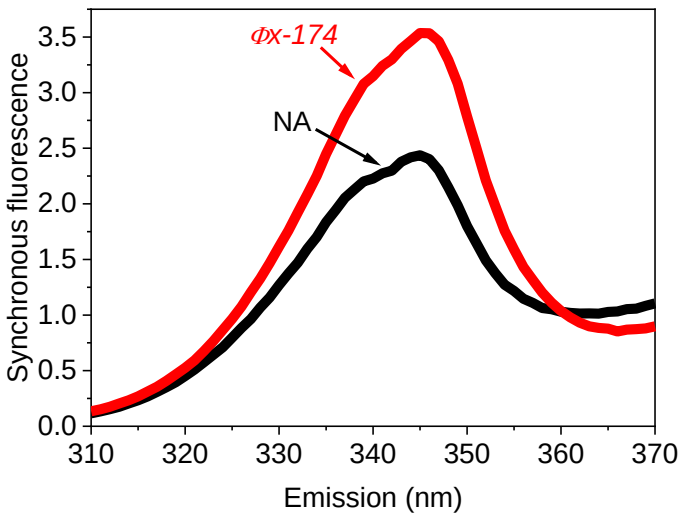

Fig. 2 – Tryptophan residues in the bacterial media limits the detection of *φx-174*. SF spectra of *E.coli* extracts after filtration with and without *φx-174* ($\Delta\lambda$ = 60 nm, λ_{Em} = 310 – 370 nm). Although the intensity of *φx-174* was significantly higher, the spectral fingerprint of Trp was obtained in both spectra. SF of extracts filtrates without *φx-174* (black), and with *φx-174* (red).

Preparation of a low Trp content medium

In order to decrease the Trp fluorescence contribution that derives from the bacterial cultivation medium, we prepared a new medium which consist less Trp than in NA. The main ingredients of the low Trp substrate (LTS) were Agar, glucose, salts mixture and yeast extracts (for more details see experimental section). *E.coli* was successfully cultivated top of the LTS to form a uniformed layer.

Next, we wished to estimate the spectral interference of each individual group of ingredients and the mixture of all ingredients on spectral overlap with Trp. Substrates with Agar, Agar + Glucose, Agar + Salts, Agar + Yeast extracts and Agar + Glucose + Salts + Yeast extracts were prepared. Ammonium acetate buffer was added on top of the substrates and incubated for 10 min. the buffer was removed and filtrated. SF spectra of the filtrates ($\Delta\lambda$ = 60 nm, λ_{Em} = 310 – 370 nm) were measured. (Figure. 3). The substrates of Agar and Agar + Glucose did not have a significant fluorescence contribution. A small fluorescence contribution of 0.1 CPS was obtained from the Agar + Salts substrate, however, the shape of the peak was not similar to the peak of Trp. Interestingly, when all ingredients were added to the substrate, no significant fluorescence was observed. The results show that the presence of sugars and

salts in the substrate prevents its extraction to the ammonium acetate buffer. We postulate that the reason for this could result from interaction between the proteins and the salts or sugars. Another reason may be the increase in the viscosity of the gel which limits the diffusion of the proteins to the upper surface of the substrate.

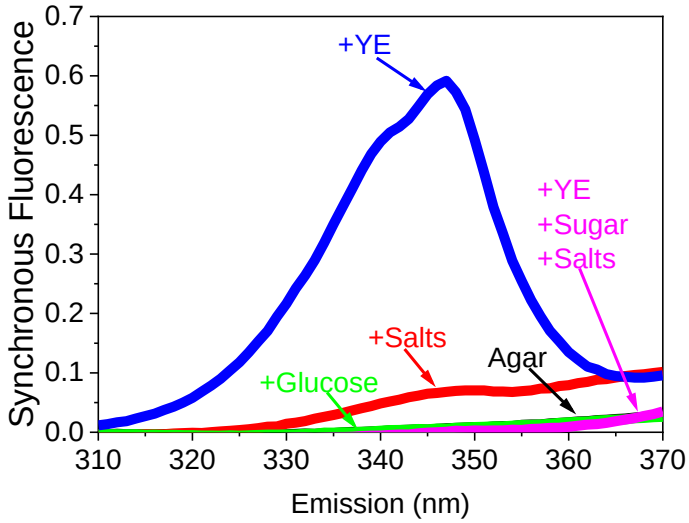

Fig. 3 – SF measurements of the ingredients of the new medium. SF of the ingridients of the new medium were measured to evaluate their fluorescence overlap with Trp. Agar (black), Agar + salts (red), Agar + glucose (green), Agar + yeast extract (blue).

Identification of φx-174 based on its Trp fluorescence fingerprint

Using the LTS has eliminated the extraction of proteins from the substrate to the ammonium acetate buffer. However, proteins other fluorescent materials may be secreted from the *E.coli* cells or be released by dead or lysed cells. we wished to assess whether such materials are extracted into the Ammonium acetate buffer and whether their fluorescence overlaps with the spectral fingerprint of TRP. *E.coli* layer was grown on the LTS with or without addition of φ*x-174*. Amonium acetate buffer was added on the top of the dish for 10 min. The buffer was removed and filtrated through 0.2 µm filter. SF of the filtrates was measured. (Figure. 4). a fluorescence contribution between 330 – 370 nm was obtained from the sample without φx-174. However, it's spectral shape was linear and clearly not originating from TRP. The samples with φx-174 showed the spectral fingerprint of TRP. In order to evaluate the TRP concentration. The spectra of the sample without φx-174 was substructed from the sample with it. a calibration curve of concentration vs. fluorescence intensity at 350nm was prepared by measuring SF spectra of increasing Trp concentrations (0.3, 0.6, 1.2, and 1.8 µM). The Trp concentration of φx-174 was determined to be 0.44 - / + 0.03 µM.

The results shows that when using the LTS substrate (instead of the conventional NA), it is possible to use SF to identify the TRP fluorescence contribution which originates only from φx-174 and not from its environment.

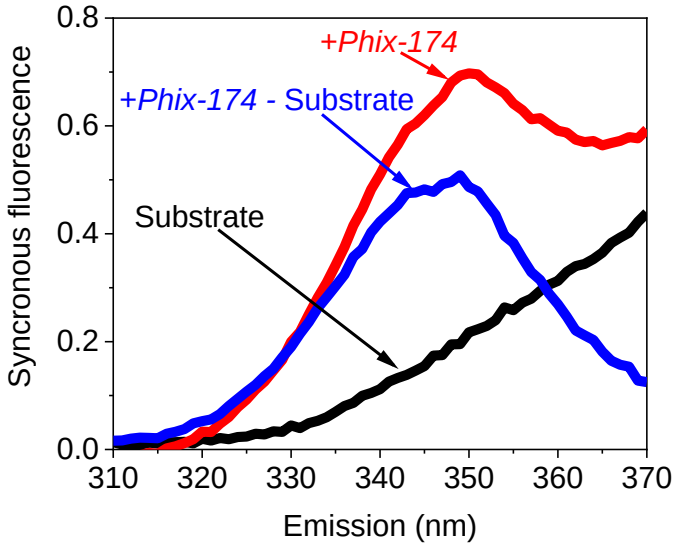

Fig. 4 – Identification of φx-174 based on its Trp fluorescence fingerprint.

Conclusions

In this work, we present a new substrate for bacterial cultivation which consist significantly lower Trp content than NA which is frequently used. We show that by using this substrate as a platform for cultivation of *E.coli* and replication of the virus φx-174, we enable the possibility to identify φx-174 based on its intrinsic fluorescence which originates from its Trp content. This separation and identification method is simple and rapid and may be used in the future for identification of various viral species.

Experimental

Replication of the virus *φx-174*

To replicate the virus *φx-174*, we used the "double-layer" method. Briefly, in this method, a host bacterium (in high concentration) is grown into a solid medium together with infectious phage particles. After the phage is replicate itself in the bacterium, and lyse the bacterium cells, a visible clear zone (plaque) is formed. In our case, to E. *coli CN13* (ATCC 700609, a nalidixic acid resistant strain) a *φx-174* (DSM 4497, Germany) was added and were grown on Nutrient Agar (NA) composed from Nutrient Broth (Difco, USA) with 0.7% Agar (Difco, USA). After over-night incubation (37°C) a viral plaques were formed.

Purification of the virus *φx-174* from whole *E.coli* cells

For separation of viral particles from whole E. *coli* cells, a 5 ml of Ammonium acetate buffer (Ammonium acetate, Riedel-de Haen, Germany, pH=7, 0.1M) were added on the "double-layer" Agar (Petri dish of 100 mm diameter). The buffer was float on the Agar for 10 min and then it pumped out by syringe and filtered by PVDF sterile syringe filter (0.22µ, Millipore, Ireland). As a control, the same procedure was done for "double-layer" Agar without addition of *φx-174* particles prior the incubation (named TEC).

Preparation of the low trp conent medium

The new media consisted from Glucose, 10 gr/L (Merck, Germany); KH_2PO_4, 5 gr/L (Carlo Erba, Italy); $K_2HPO_4 \cdot 3H_2O$, 5 gr/L (Merck, Germany); $(NH_4)_2HPO_4$, 3 gr/L (Merck, Germany); Na_2HPO_4, 4.6 gr/L (Fluka, Switzerland); $MgSO_4 \cdot 7H_2O$, 0.6 gr/L, (Merck, Germany); Yeast extract, 0.5 gr/L (Difco, USA); Agar, 7 gr/L (Difco, USA).

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Conflicts of interest

There are no conflicts to declare.

Author contributions

YF ,YS conceived the idea. YF and YS designed the experiments. YF and YS performed the main experiments. YF, YS and RA wrote the paper. IS and RA supervised the entire research project and provide funding.