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Practical

A simple laboratory class using a *Pseudomonas aeruginosa* auxotroph to illustrate UV-mutagenic killing, DNA photorepair and mutagenic DNA repair

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A simple and cheap laboratory class is proposed to illustrate the lethal effect of UV radiation on bacteria and the operation of different DNA repair mechanisms. The class is divided into two sessions, an initial 3-hour experimental session and a second 2-hour analytical session. The experimental session involves two separate experiments: one dedicated to illustrating the lethal effect of UV radiation and the protective effect of DNA photorepair; the second to explore the operation of DNA repair mechanisms that prioritise survival but introduce mutations. The procedure makes use of a *Pseudomonas aeruginosa* double auxotroph, which serves to detect UV-induced back-mutations to prototrophy. The proposed scheme is carried out by undergraduate students of the Bacterial Physiology and Genetics course, as part of our Biotechnology curriculum. We think that it will be a valuable tool for microbiology students to increase their understanding of basic genetic concepts.

Keywords: UV killing; UV mutagenesis; photoreactivation; mutagenic DNA repair; auxotroph

Introduction

All living forms on the Earth's surface are exposed to UV radiation emitted from the sun, with varying intensity and quality depending on the altitude, the filtering effectiveness of the ozone layer and the degree of organism exposition. The mutagenic potential of UV light derived from the sun is mainly associated with the UV-A (320–400 nm) and UV-B (290–320 nm) regions of the spectrum, because the UV-C fraction (100–290 nm) is completely absorbed by atmospheric ozone (Sinha and Hader 2002). UV-B primarily induces dimerisation of neighbour pyrimidines in one strand of the DNA and blockage of the DNA replication machinery (Deisenhofer 2000). In addition to pyrimidine dimerisation, UV-A promotes formation of reactive oxygen species, which results in chemical modification of the purine and pyrimidine bases and, consequently, altered base-pairing properties (Sinha and Hader 2002). Thus, both types of UV radiation are responsible for mutations in UV-exposed organisms.

It is not surprising to find that sunlight-exposed bacteria have then acquired and evolved a series of mechanisms to cope with the damage induced by UV radiation (Goosen and Moolenaar 2008). Specific UV-induced repair systems include (i) photoreactivation; (ii) removal of the damaged base by specific glycosidases; and (iii) endonucleolytic cleavage by UV-damaged endonucleases. Photoreactivation depends on the activity of the enzyme photolyase, which can resolve pyrimidine dimers

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upon absorption of visible (blue) light by chromophores (Deisenhofer 2000). Additionally, bacteria can recur to general mechanisms to repair UV-induced lesions: (i) nucleotide excision repair (NER) by UvrABC-like systems; (ii) RecA-mediated recombinational repair; and (iii) translesion synthesis by error-prone inducible DNA polymerases. All these mechanisms except photoreactivation are operative in the dark (Goosen and Moolenaar 2008). Moreover, in some bacterial species, high levels of UV-related damage trigger the SOS response, to coordinate induction of several DNA repair mechanisms (Sutton et al. 2000).

Pseudomonas aeruginosa is a widespread Gram-negative bacillum, commonly found in environmental samples (soil, water), as well as colonising the skin and mucosal tissues of mammals and other eukaryotes. If these hosts are immunocompromised, *P. aeruginosa* can behave as an opportunistic pathogen (Brenner et al. 2005). Several *P. aeruginosa* strains have been sequenced, prompted by the interest to find medical solutions for its eradication from the lungs of cystic fibrosis patients, skin infections following burning damage and some intrahospitalary infections (Kung et al. 2010). As *P. aeruginosa* is easily grown in the lab and many genetic tools are available for its study (Choi et al. 2008), it is utilised in undergraduate microbiology courses to illustrate different microbiological phenomena and practices (Harley and Prescott 2001).

The laboratory class described here is carried out by undergraduate students of the Bacterial Physiology & Genetics course, as part of our Biotechnology curriculum. An auxotrophic mutant derived from the sequenced strain *P. aeruginosa* PAO1 is utilised as the test strain (Haas et al. 1977). The aims of this class are to illustrate: (1) the lethal effect of UV radiation on bacteria; (2) the operation of photoreactivation; and (3) the operation of error-prone DNA repair mechanisms.

Methodology

Class outline

The experimental procedure described below can be carried out in a single lab session of about 3 hours, with the simultaneous participation of no more than twenty-five students divided into groups of two or three. A second session of about 2 hours is required for colony count and analysis of the results. Students communicate their findings in a written report.

In our course, the whole experimental setup required the use of only two microcentrifuges, four vortex agitators and two laminar flow chambers as overall equipment, apart from a rotary bath shaker for overnight culture growth and an incubator for colony development on plates. The laminar flow chambers used in our class are equipped with Philips TUV 30W/G30T8 germicidal tubes and Osram Daylight L 36W/765 fluorescent tubes, which are located at a distance of 80 cm from the bench.

Bacterial strain

Pseudomonas aeruginosa was chosen because handling and maintenance are inexpensive and require little training including appropriate safety issues (see below). In addition, it grows rapidly, with doubling times of approximately 45 minutes in rich media, allowing completion of experiments in a few days. The *P. aeruginosa* strain PAO372 (Haas et al. 1977) is a double auxotroph, requiring lysine and arginine for its growth in minimal medium (Figure 1). Strain PAO372 (*lys-58 argH32*) was generated by ethylmethansulfonate mutagenesis of strain PAO362 (*argH32*) (Haas et al. 1977). In turn, PAO362 was derived from wild-type strain PAO1 (ATCC 15692) using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (Isaac and Holloway 1972). Neither mutation has been sequenced, but the results of our UV reversion experiments let us safely assume that both mutations are due to single nucleotide substitutions within *lysA* (PA5277) and *argH* (PA5263) genes. Strain PAO372 has been deposited in the Culture Collection of Switzerland (CCOS, Waedenswil, Switzerland) under the entry code CCOS 692, from where it is freely available upon request. The strain can be stored for up to 2 weeks in the refrigerator (4°C) on nutrient agar plates (NA; 40 g l^{-1} blood agar base; 5 g l^{-1} yeast extract). For long-term storage, saturated liquid cultures can be stored at ultra-low temperatures (–80 to –130°C) as 20% glycerol suspensions.

Growth media and conditions

P. aeruginosa was grown at 37°C with shaking at 200 rpm in nutrient yeast broth (NYB; nutrient broth 25 g l^{-1}, yeast extract 5 g l^{-1}) (Stanisich and Holloway 1972). Cells grown in NYB were spread onto NA plates for UV irradiation in Experiment 1, or onto minimal medium with succinate MMS (Meyer and Abdallah 1978) for UV irradiation in Experiment 2. The MMS medium contained (g 1^{-1} in distilled water): KH_2PO_4, 3.0; Na_2HPO_4, 6.0; NH_4Cl 1.0; NaCl, 0.5; $MgSO_4.7H_2O$, 0.25; $CaCl_2$, 0.02; succinic acid, 3.2. The pH was adjusted to 7.0 by addition of NaOH prior to sterilisation. When required, the autoclaved MMS medium was supplemented with 0.5 mM lysine and/or arginine.

Materials for each group of students

For completion of the two proposed experiments, each group requires: (i) an overnight culture of strain PAO372 (typically a 15-ml Falcon tube containing 5 ml of NYB); (ii) NA plates and MMS plates supplemented with lysine; (iii) saline solution (NaCl 9 g l^{-1}); (iv) cardboard pieces of 5 cm × 10 cm; (v)

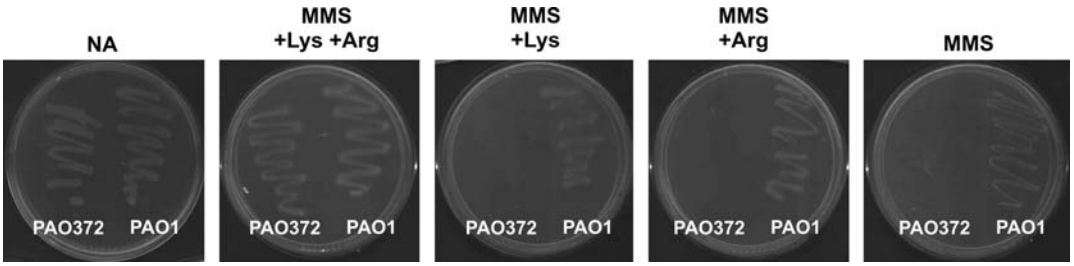

Figure 1. *Pseudomonas aeruginosa* strain PAO372 is a double Lys⁻ Arg⁻ auxotroph.
Notes: NA, nutrient agar (rich medium containing amino acids); MMS, amino acid-free minimal medium with succinate and ammonium; +Lys, medium contains 0.5 mM lysine; +Arg, medium contains 0.5 mM arginine; PAO1, wild-type prototrophic *P. aeruginosa* strain; PAO372, Lys⁻ Arg⁻ double auxotrophic *P. aeruginosa* strain derived from PAO1

a spatula and alcohol for plating; (vi) micropipettes, tips and 1.5-ml microtubes; (vii) a chronometer; and (viii) latex examination gloves, to avoid skin exposure to UV radiation.

Instructions for Experiment 1: UV mutagenic killing and DNA photorepair

1. Plate 100 µl of PAO372 culture onto NA plates, according to the experimental design indicated by the instructor (Table 1).
2. Place all NA plates upward on the laminar flow chamber. Remove the lid of each plate and put it aside; then, cover the left half of each plate with one piece of cardboard.
3. Leave one NA plate covered with its lid to serve as control.
4. Turn on the UV light and start the chronometer.
5. At every exposure time, quickly remove the piece of cardboard and cover the corresponding plate with its lid. Up to this step, we have adapted the procedure delineated by Dr Michelle Furlong's laboratory handout 'UV light lab' (http://a-s.clayton.edu/furlong/BIOL3250/lab/Admin/Updated%20SchedulesFA11.htm) (College of Art & Sciences, Department of Natural Sciences, Clayton State University, USA).
6. Upon completion of the exposure, transfer the group of plates designated as 'UV' to the incubator. The plates corresponding to the group 'UV+white light' must be exposed to the fluorescent white light of the laminar flow chamber for at least 4 hours before being transferred to the incubator.
7. After 48 h of incubation at 37°C, plates can be examined and colonies counted, or stored in the fridge to synchronise plate analysis with those of Experiment 2.

Instruction for Experiment 2: mutagenic DNA repair

1. Centrifuge 1 ml of PAO372 culture for 1 min at 14,000 rpm, discard the supernatant and resuspend cells into 1 ml of saline solution with the help of a vortex agitator.
2. Plate 100 µl of washed cells onto MMS-Lys plates, according to the experimental design indicated by the instructor (Table 1).
3. Place all MMS-Lys plates upward on the laminar flow chamber. Remove the lid of each plate and put it aside. Leave one plate covered with its lid to serve as control.
4. Turn on the UV light and start the chronometer.

Table 1. Experimental design to explore UV-mutagenic killing, DNA photorepair and mutagenic DNA repair in *Pseudomonas aeruginosa*

Experiment 1: UV mutagenic killing and DNA photorepair							
UV exposure time	5 seconds	10 seconds	30 seconds	1 minute	5 minutes	10 minutes	15 minutes
'UV' treatment	✓	✓	✓	✓	✓	✓	✓
'UV + white light' treatment			✓	✓	✓	✓	✓
Control (plate with lid)							✓
Experiment 2: Mutagenic DNA repair							
UV exposure time	5 seconds	10 seconds	15 seconds	20 seconds	30 seconds	45 seconds	60 seconds
'UV' treatment	✓	✓	✓	✓	✓	✓	✓
'UV + white light' treatment			✓	✓	✓	✓	✓
Control (plate with lid)							✓

5. At every exposure time, quickly cover the corresponding plate with its lid.
6. Upon completion of the exposure, transfer the group of plates designated as 'UV' to the incubator. The plates corresponding to the group 'UV+white light' must be exposed to the fluorescent white light of the laminar flow chamber for at least 4 hours before being transferred to the incubator.
7. After 4–5 days of incubation at 37°C, plates can be examined and colonies counted.

Safety considerations

The microbiology laboratory is a place where potentially infectious microorganisms are handled and examined. It should be kept in mind that, at all times, experimental procedures like the one described above carry some measure of associated risk. *P. aeruginosa* is an opportunistic pathogen (BSL-2). Thus, it may be pathogenic in a seriously immunocompromised person. Primary hazards to personnel working with this agent relate to accidental percutaneous or mucous membrane exposures, or ingestion. The students must be instructed to be aware and to comply with the standards set by the CDC Principles of Biosafety for BSL-2 microorganisms (http://www.cdc.gov/biosafety/publications/bmbl5/). Disposable plates and liquid cultures containing *P. aeruginosa* are to be decontaminated, together with handling material (pipette tips, microtubes), by chemical treatment (chlorine) or autoclaved.

Results and discussion

Experiment 1: UV-mutagenic killing and DNA photorepair

Students are instructed to inspect plates and to register the number of colony-forming units (CFU) developed after 48 hours of incubation. If the number of colonies developed in the UV-exposed side of the plate is higher than 300, we just keep the record '>300'. This normally happens for exposure times <5 min. The average number of CFU is then plotted as a function of the UV exposure time for both treatments.

The effect of UV light on survival of *P. aeruginosa* PAO372 is shown in Figure 2. As expected, the PAO372 CFU counts in the irradiated side of the plates declined with the exposure time (Figure 2A). Usually, after 15 minutes of exposure, no colonies are detected. As every NA plate was inoculated with about 10^8 cells, the UV treatment killed 99.9995% of the inoculated cells in only 5 minutes (Figure 2B). A simple CFU count of the PAO372 preculture could be added to the experience (Herigstad et al. 2001), if a more precise estimation of the germicidal rate is desired. The results of PAO372 exposure to UV light are useful to discuss the effectiveness of UV irradiation for sterilisation of surfaces.

The left side of each plate, which was covered with cardboard to avoid UV irradiation, showed a lawn of PAO372 cells (Figure 2A). This serves as a control for a correct plate inoculation. Another inoculated NA plate was exposed to UV light without removing its lid (Table 1); in this plate, a bacterial lawn is always observed after incubation. Inspection of this plate allows students to realise the UV-filtering properties of the plastic material. Comparatively, the group of plates that were exposed to white light after UV treatment developed more colonies at every time point after 1 minute of UV exposure (Figure 2). Indeed, this effect is attributable to the irradiation with white light before incubation. A proportion of cells that otherwise would have accumulated a lethal number of UV-induced damage, succeeded to survive and to give rise to colonies upon exposure to white light.

The observations serve to elaborate on the hypothesis that *P. aeruginosa* does express a DNA photorepair system. In fact, students can query the *P. aeruginosa* PAO1 genome (www.pseudomonas.com) for the presence of annotated DNA photolyase encoding genes. The contribution to the UV-B survival of the *P. aeruginosa phr* gene PA4660, encoding a class 2 deoxyribodipyrimidine photolyase, has been reported (Kim and Sundin 2001) and this paper may be utilised as a source of further discussion.

Experiment 2: mutagenic DNA repair

For this second part of the practical class, students are instructed to examine the plates and to register the number of CFU developed after not less than 4 days of incubation. The longer incubation time is due to the utilisation of a minimal growth medium and to the fact that colonies arise from UV mutagenised cells. The latter is also reflected in the pleomorphic aspect of the colonies.

It is first important to discuss with students that the time frame for UV exposure in this experience is significantly shorter than that of the first experiment, because the aim of the second experiment is to evidence mutagenic DNA repair. Thus, the extent of UV-mutagenic damage must be reduced in order to allow recovery of enough cells to develop countable colonies. Under our experimental conditions, the most appropriate UV-exposure period was 1 minute. This has to be set up for different microorganisms, UV sources and growth conditions.

As described previously, strain PAO372 is a double auxotroph unable to synthesise lysine and arginine (Figure 1). Both mutations are due to single nucleotide replacements. Based on this, the rationale

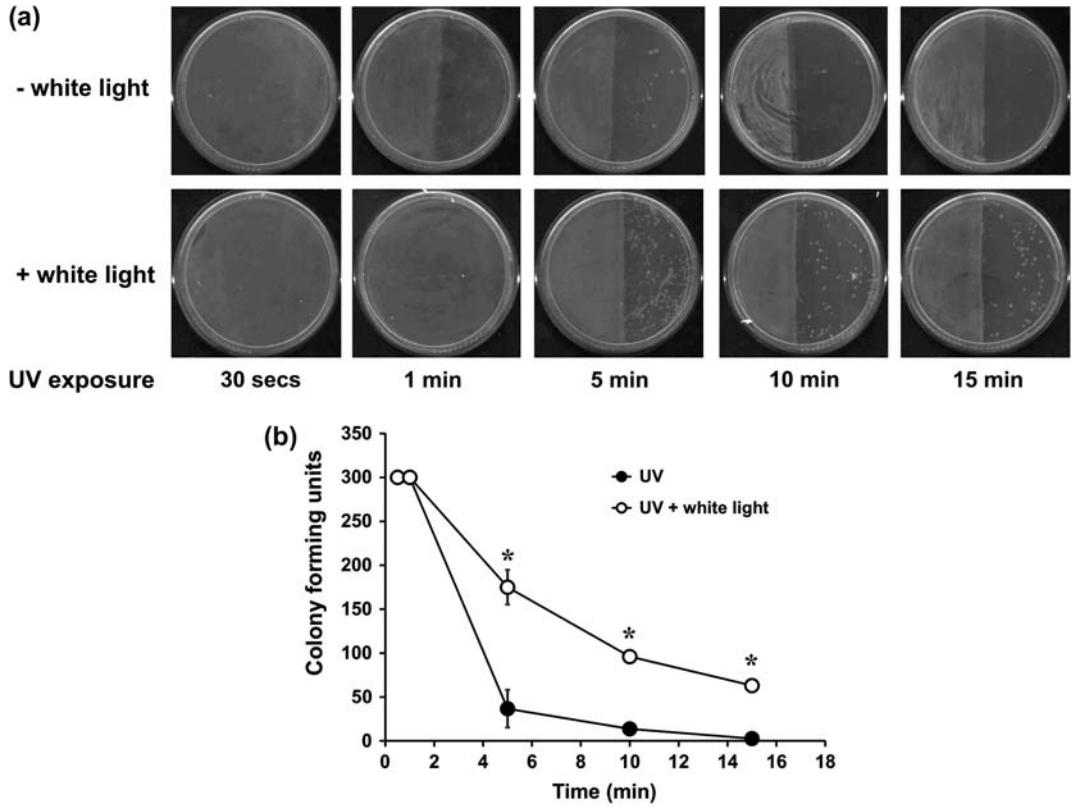

Figure 2. UV-mutagenic killing and DNA photorepair in *Pseudomonas aeruginosa* PAO372. (a) Survival of *P. aeruginosa* upon irradiation with UV light as a function of exposure time and of exposure to white light before growth (photoreactivation conditions). All plates were inoculated with about 10^8 cells and exposed to a germicide lamp for different times as indicated. The left half of each plate was covered with cardboard to shield it from UV irradiation and to serve as a plating control; the right half remained uncovered during the experience. At the end of the corresponding irradiation period, the plates in the top lane (–white light) were immediately transferred to the incubator for colony development without exposition to white light. The plates in the bottom lane (+white light) were exposed to white light for 4 hours and then transferred to the incubator for colony development. (b) Graphical representation of the survival of *P. aeruginosa* upon irradiation with UV light as a function of exposure time and of exposure to white light before growth. Data represent the average number of colonies per plate ± standard deviation (n = 3). Asterisks indicate that the average number of CFU significantly differ among treatments at $P < 0.005$ (t-test)

of this experiment is that in the absence of photoreactivation (no white light exposure after UV irradiation), PAO372 cells will try to repair the DNA damage introduced by UV radiation. If a mutagenic DNA repair mechanism exists in *P. aeruginosa*, it should be possible to recover prototroph revertants from UV-irradiated PAO372 cells (Lehrbach et al. 1979). Thus, a positive selection of back-mutations induced in auxotrophs is an effective way to reveal the operation of an error prone DNA repair process. In one of the two experimental treatments, PAO372 cells are plated on minimal MMS medium supplemented with either lysine or arginine, irradiated with UV, and then transferred to the incubator without exposure to white light. An important control is the plate with the lid (Table 1), because it is already known from the results of Experiment 1 that the plastic lid filters out UV radiation, so this plate serves a control for spontaneous reversion of the auxotrophy. Usually, no or a few colonies are developed in this plate because the back-mutation frequency to arginine and lysine prototrophy is $<10^{-8}$. For this reason, the colonies developed in the treatment plates most probably correspond to revertants originated by mutagenic DNA repair.

Typical results for this experience are shown in Figure 3. For UV-exposure times between 5 and 30 seconds, the number of revertant CFUs increased with the irradiation time. For longer exposure, the number of revertants declined. This is consistent with the fact that this experiment is revealing two concurring phenomena; first, the mutagenic DNA repair that increases with the accumulated DNA damage consistent with the SOS response (Cirz et al. 2006), and second, the extension of DNA damage that cannot be further resolved above certain levels that compromise viability. The simultaneous operation of both processes results in a bell-shaped curve for the number of revertants according to the UV-exposure time (Figure 3).

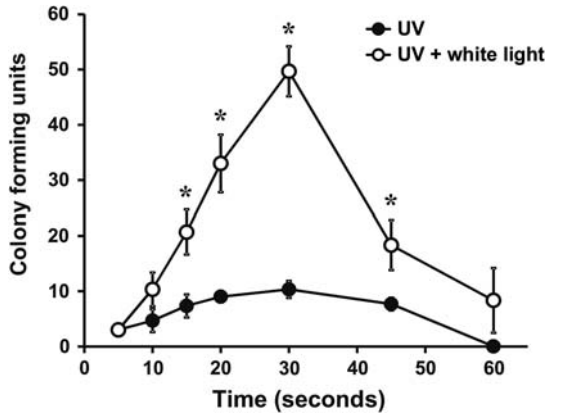

Figure 3. Mutagenic DNA repair in *Pseudomonas aeruginosa* PAO372: effect of UV irradiation on the appearance of Arg^+ prototrophs by back-mutation of auxotrophic strain PAO372. Data represent the average number of colonies per plate ± standard deviation (n = 3), that developed on MMS medium supplemented with 0.5 mM lysine. All plates were inoculated with about 10^8 cells and exposed to a germicide lamp for different times as indicated. At the end of the corresponding irradiation period, one set of plates (•) was immediately transferred to the incubator for colony development without exposition to white light. A second set of plates (○) was exposed to white light during 4 h and then transferred to the incubator for colony development. Asterisks indicate that the average number of CFU significantly differ among treatments at $P < 0.05$ (t-test)

As for Experiment 1, a second group of plates is exposed to white light for several hours before incubation for colony development (Table 1). The effect of DNA photoreactivation is clearly detected as a significant increment in the number of revertant CFUs compared to the same plates that were not exposed to white light after UV irradiation (Figure 3) As observed for Experiment 1, the photoreactivation of UV-damaged bases allows a greater proportion of cells to avoid accumulation of lethal mutations, thus resulting in a higher number of revertants that would otherwise be compromised in their viability (Figure 3).

Again, inspection of the *P. aeruginosa* PAO1 genome sequence may serve as a source of further inquiry-based discussion. For instance, the sequence of the *lysA* and *argH* genes may be explored to look for consecutive thymines, the target of UV-B radiation, which dimers may be subject to mutagenic repair in the absence of white light. In the same direction, the genome database may be queried for the presence of annotations encoding error-prone DNA polymerases, like *dinB* (PA0923; Sanders et al. 2006).

Conclusions

We here propose a simple experimental setup to explore the effects of UV radiation on the viability of *Pseudomonas aeruginosa* cells, as well as the operation of the DNA repair mechanisms referred to as photoreactivation and mutagenic DNA repair. Previously published laboratory exercises have made use of mutant strains deficient in repair systems to evaluate the effect of UV on survival (Bauer 1998; Healy and Livingstone 2010), or simply evaluated the effect of photoreactivation on survival of wild type strains (Delpech 2001; Zion et al. 2006). To our knowledge, this is the first practical exercise that: (1) exploits positive selection of auxotroph back-mutations to prototrophy; and (2) provides a simple experimental test to reveal the operation of mutagenic dark repair mechanisms. The simple procedure described here can be adapted to any microorganism for which single-nucleotide mutants conferring auxotrophy were available.

The design of the practical lab session follows that of scientific experiments, in which students collect, analyse and interpret their own data, and utilise them to support or not the hypotheses discussed during the theoretical part of the course. The scheme requires one experimental class of about 3 hours, and a second class of about 2 hours for plate counts and analysis of results. The procedure is straightforward, cheap, does not require expensive or heavy equipment, and it is reliable and reproducible. If restrictions exist at an academic department to utilise BSL-2 microorganisms, the entire experimental scheme is adaptable to available auxotrophs derived from BSL-1 bacteria. Furthermore, the experimental design is still open to variations, such as testing mutants affected in different DNA repair systems, comparing the back-mutation frequency of the other auxotrophic marker, or testing which region of the white light spectrum is required for photoreactivation by using appropriate filters. Moreover, the results obtained with this laboratory exercise lead to inquiry-based discussions that can be approached through genome sequence analysis and training in hypothesis-driven experimental design. For all the reasons stated above, we believe that this is a valuable tool for microbiology students to increase their understanding of basic genetic concepts.

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