Application of $XeCl_{308nm}$ excimer laser radiation to mutate industrial microorganisms

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Abstract

In this study, we have investigated the effects of an $XeCl_{308\ nm}$ excimer laser radiation on bacterial mutagenesis. Our experiments have revealed that the mutagenesis inducted by the $XeCl_{308\ nm}$ excimer laser radiation is independent from RecA protein, the regulator of the SOS response, unlike $UV_{254\ nm}$ radiation that is not mutagenic for *Escherichia coli* mutants lacking the RecA protein. This found suggests that the $UV_{308\ nm}$ laser radiation might be mutagenic also in microorganisms naturally lacking the SOS response. To test this hypothesis, we applied our innovative mutagenesis approach on *Nonomuraea* ATCC 39727, an industrial strain producing an antibiotic, which is relatively refractory to $UV_{254\ nm}$ radiation-induced mutagenesis. Our results demonstrated the efficiency of $XeCl_{308\ nm}$ excimer laser radiation to induce mutagenesis in *Nonomuraea* ATCC 39727.

INTRODUCTION

Bacteria are excellent producers of several potent bioactive compounds. Many of the products currently used for human or animal therapy, in pharmaceutical and food industry and in agriculture are produced by microbial secondary metabolism or by chemical modification of a microbial products. However, it is rare for these microorganisms to produce biological molecules at concentration so high as to initiate the production on an industrial scale. Therefore, an important challenge in industrial microbiology is to improve the secondary metabolites production by microorganisms.

Mutation-selection procedures are widely used in biotechnology to improve the performance of producer microorganisms. The mutagenic effects of ultraviolet (UV) light of microorganisms are fairly well known from studies with UV lamps. The interaction of UV light with cellular systems is responsible for photochemical, photothermal, photodecomposition and photodinamic processes. When short-wavelength radiation strikes biological material, DNA is damaged. It can be repaired or can cause cell killing, mutagenesis or, in mammalian cells, carcinogenesis [1-2].

Mutagenic and lethal mechanisms of $UV_{254\ nm}$ radiation have been elucidated in several microorganisms. RecA, a multi-functional protein, plays a fundamental role in bacterial response to $UV_{254\ nm}$ radiation [3-4]. *Escherichia coli* strains harboring loss-of-function mutations in *recA* gene are extremely sensitive to far-UV by germicidal lamps [5]. DNA photoproducts are maximally represented by pyrimidine dimers, and it is believed they are responsible of the far-UV lethal effects [1]. The extreme sensitivity of *recA*-deficient strains to far-UV radiation is dependent on the inability to both repair DNA by means of the post-replicative recombination pathway, and to activate additional error-free and error-prone (mutagenic) DNA repair pathways (the so-called SOS response) [6-7]. Owing to the failure to activate the SOS response, *recA*-deficient strains are also inefficiency to $UV_{254\ nm}$-induced mutagenesis. This is a frequent condition that limits diffusion of the $UV_{254\ nm}$ mutagenesis for research or industrial purposes because many strains are naturally SOS response-defective [8].

Since the eighties, due to the development of excimer lasers, new frontiers in the study of UV applications have been opened. In this context we, analyze the effects of the $UV_{308\ nm}$ laser radiation generated by an homemade $XeCl_{308\ nm}$ excimer laser [9], using *E. coli*, as a model organism, and *Nonomuraea* ATCC 39727, as a microorganism of industrial interest.

Our experimental approach is also of wide interest because, in contrast to far-$UV_{254\ nm}$ radiation, the environmental radiation band around 308 nm is only partially attenuated by the atmosphere and has a potential impact on biological systems. Moreover, studies of the biological effects of coherent (laser) radiation are rare in the literature, and, in particular, the response of *recA*-defective strains to

$UV_{308\,nm}$ XeCl excimer laser radiation has not been investigated systematically before.

EXPERIMENTAL SETUP

Strains and Growth Conditions

The *E. coli* strains were grown in Luria-Bertani (LB) broth or LB agar. *Nonomuraea* sp. ATCC 39727 was obtained from the ATCC. The composition (per liter) of the complex media used in this study for *Nonomuraea* growth is listed below.

– Medium YS: 2 g yeast extract, 10 g soluble starch (pH 7.3)

– Medium 707: 5 g peptone, 3 g yeast extract, 1 g MgSO4 • 7 H2O (pH 7.0).

When requested, agar was added at a concentration of 1.8%.

Irradiation of *E. coli* Strains by $UV_{308\,nm}$ XeCl ecimer laser or $UV_{254\,nm}$ germicidal lamp and UV-radiation survival tests

The *E. coli* strains were grown to late logarithmic phase (optical density = 1.0 at a wavelength of 550 nm) in LB broth at 37°C with vigorous shaking. Diluted and non diluted cells were plated on LB agar mini-plates (2 cm in diameter) and exposed to UV radiation using one of two sources: a $UV_{308\,nm}$ XeCl excimer laser or a $UV_{254\,nm}$ germicidal lamp. The laser fluences were fixed at 25 or 50 mJ cm^{-2} per laser shot, and the laser beam was focused on the surface of the mini-plates using a convergent lens. We administered multiple laser shots of the same fluence. Surviving cells were quantified after overnight incubation at 37°C by counting the number of colony-forming units at the different dilutions. In our experiments, we verified preliminarily that the laser treatment did not significantly increase the temperature of the biological samples excluding thermal effects. Indeed, using a platinum probe (Pt-RTD l00Ω) inside the agar plates next to the irradiated surface, we observed a maximum increment of 1.85°C after 500 laser shots (at 1 Hz repetition rate). In parallel experiments, *E. coli* strains were irradiated with the $UV_{254\,nm}$ germicidal lamp at a radiance of 0.3 mJ $cm^{-2}s^{-1}$. Irradiation was performed by opening the LB agar mini-plates under a SpectrolineR model ENF-260C/F

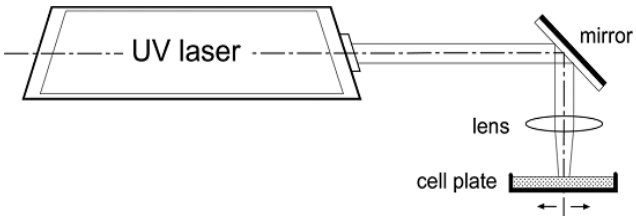

Fig. 1. Experimental apparatus utilised for cells irradiation.

$UV_{(254)}$ germicidal lamp (Spectronics Corporation, Westbury, NY) at a distance of 15 cm for different times in the absence of daylight illumination. As a control, dilutions from the same cultures were spotted onto non irradiated plates. UV-light fluences were measured by a light-sensitive photodiode (S1336-I8BQ). Exposure to the germicidal lamp during our experiments never resulted in an increase in the temperature of the LB agar medium by more than 0.1°C, as measured by a platinum probe (Pt-RTD 100 Ω) that was placed at the surface of the medium.

Determination of Spontaneous and UV-Radiation-lnduced Mutation Frequencies in *E. coli*

E. coli cells grown to an optical density of 1.0 at a wavelength of 550 nm were collected by centrifugation and gently resuspended in LB broth to concentrate them. To determine $UV_{308\,nm}$-radiation-induced mutation frequencies, 0.45-µm pore-sized White HAWP Millipore membranes were placed on the surface of LB agar mini-plates (2 cm in diameter). Bacteria (about 2 x 10^{10} cells) were plated onto membranes and exposed to the UV-radiation. Exposure was carried out at room temperature in the absence of daylight illumination. After irradiation, bacteria were incubated at room temperature for 30 min to allow the cells to recover and repair UV radiation damage. Then the Millipore membranes were removed and transferred onto LB agar mini-plates supplemented with rifampicin (36 µg ml^{-1}) and incubated at 37°C for 24h. Serial dilutions were treated in parallel and plated on LB agar mini-plates to determine the viable colony-forming units. Mutation frequencies were determined by dividing the number of resistant colony forming units ml^{-1} by the total number of viable colony-forming units ml^{-1}. For statistical analysis, the data were obtained from three independent experiments. In each experiment, 10 independent cultures of each strain were tested, and median values of mutation frequencies to rifampicin resistance were determined. For each strain subjected to a given $UV_{308\,nm}$- or $UV_{254\,nm}$- radiation treatment, the median values did not differ more than 20%.

Irradiation of *Nonomuraea* spores by UV308nm XeCl excimer laser and survival tests

Nonomuraea sp.ATCC 39727 spores (about $5 \times 10^{8} ml^{-1}$) were collected by centrifugation and re-suspended in medium 707 (for re-hydration). Non-diluted or diluted spores (10µl) were then plated on YS agar mini-plates (2 cm diameter) exposed to $UV_{308\,nm}$ radiation. The laser fluences were fixed at 25 or 50 mJ/cm^2 per laser shot, and the laser beam struck the surface of the mini-plates thank

by a convergent lens. Figure 1 shows the experimental apparatus. We applied laser shots of the same fluence for different times. Survived cells were quantified following over-night incubation at 37 °C, by counting the number of colonies forming units (cfu) at the different dilutions. Because of the high fluences used in our experiments, we preliminarily verified that the laser treatment did not increase significantly the temperature of the biological samples excluding thermal effects. Using a platinum probe (Pt-RTD 100Ω) inside the agar plates next to the irradiation surface, we observed a maximum increment of 1.85 °C after 500 laser shots (at 1Hz repetition rate). After irradiation, samples were incubated at 28 °C for two weeks to determine the viable cfu.

Determination of spontaneous and UV_{308nm}-induced mutation frequencies in *Nonomuraea* spores

Nonomuraea spores were irradiated on solid surface. This procedure was necessary to obtain effective mutagenesis by $UV_{308\ nm}$. In order to perform the irradiation, 0.45 μm-pore sized White HAWP Millipore membranes were placed on the surface of YS agar mini-plates (2 cm diameter). Non-diluted (about 5 × 108 ml^{-1}) or diluted spores (10μl) were plated onto membranes and exposed to XeCl excimer laser. Exposure was carried out at a room temperature, in the absence of daylight illumination. After irradiation, spores were incubated at room temperature for 30 min to allow the cells to recover and repair UV damage. Subsequently, the Millipore membranes were removed and transferred onto YS agar mini-plates (2 cm diameter) supplemented with streptomycin (50 μg ml^{-1}) or in control YS agar mini-plates without antibiotic, and incubated at 28°C for two weeks. Mutation frequencies were determined by dividing resistant cfu ml^{-1} by the total viable cfu ml^{-1}. For statistical analysis, data were obtained by three independent experiments. In each experiment, 10 independent cultures of each strain were tested and median values of mutation frequencies to rifampicin resistance were determined.

RESULT AND CONCLUSION

We started our study by determining the effect of $UV_{254\ nm}$ radiation of *E. coli* strains. The strain JC1553 harbours the recA mutation and it is congenic to the recA-proficient strain JC411.
The results confirmed previous data on the extreme far-UV-radiation sensitivity of strains carrying the recA mutation as a result of the inability to repair UV-radiation-induced damage to DNA [10-11]. At $UV_{254\ nm}$-radiation doses ranging between 0 and 9 mJ cm^{-2} and in the absence of photoreactivation, the survival curves had complex shapes that were distinct in recA-proficient and in recA-defective strains. In the recA-proficient strain, the decrease in survival as a function of $UV_{254\ nm}$-radiation dose was smaller at lower doses (<3 mJ cm^{-2}) than at higher doses (>3 mJ cm^{-2}). An opposite behavior was observed in the recA-defective strains, which showed less effect at higher doses (> 1 mJ cm-^{2}) than at lower doses (< 1 mJ cm^{-2}) (Figure 2 A).

In parallel experiments *E. coli* strains were subjected to $UV_{308\ nm}$ coherent radiation. In the survival plot, a net change in the slope was apparent at doses higher than 5000 mJ cm^{-2}; cell killing increased only slightly with further increasing in $UV_{308\ nm}$-radiation dose. The same effect was observed with the recA-defective strain at about the same doses (Figure 2 B).

The different behaviors of the recA-defective strain after exposure to the germicidal lamp or to the excimer laser suggested that there were different lethal and genotoxic mechanisms for the two UV-radiation sources. To better elucidate this difference, we analyzed the mutability of recA-

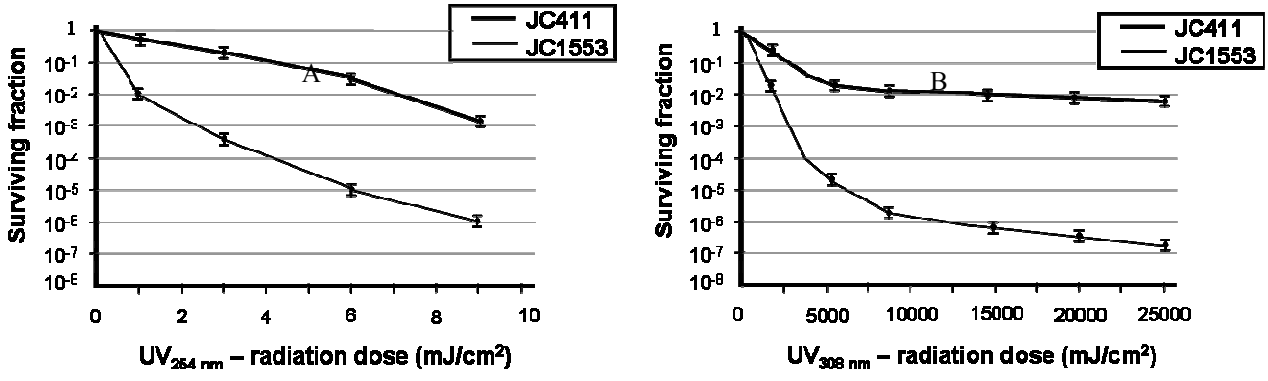

Fig. 2. Survival of recA-proficient and recA-defective *E. coli* strains exposed to lamp-generated $UV_{254\ nm}$ radiation (A) and to excimer laser-generated $UV_{308\ nm}$ radiation (B). Surviving fraction are plotted as a function of UV radiation dose.

proficient and recA-defective strains upon exposure to the $UV_{308\,nm}$ excimer laser radiation. Frequency of mutation to rifampicin resistance was used as a parameter of the mutability.

Under our experimental conditions, the recA-defective strain JC1553 exhibited spontaneous mutation levels that were a little higher (about two- to three-fold) than the congenic recA-proficient strain JC411 (Table 1). As expected, the laser treatment resulted in more than a fivefold increase in mutation frequencies to rifampicin resistance in JC411 (recA-proficient) at doses higher than 2500 mJ cm^{-2}. In particular, the maximal increment, 6.2-fold, was observed at 2500 mJ cm^{-2} with a surviving fraction of $7xl0^{-2}$. Unexpectedly, the $UV_{308\,nm}$ laser treatment was much more mutagenic for JC1553 (recA) than for JC411, resulting in an impressive increase in mutation frequencies as a function of dose. At 2400, 3600 and 6000 mJ cm^{-2} the increment was 720-, 1200- and 1900-fold, respectively (Table 2).

The greater mutagenic effect of the laser compared to the lamp in the recA genetic background may be due to the different DNA photochemistry of the two radiation sources. It is generally assumed that, in contrast to far-UV radiation, the effects of near-UV radiation on DNA are mostly indirect and are mediated by photosensitizers [1]. Cellular DNA exposed to near-UV radiation plus sensitizers is damaged in a variety of ways,

Table 1
Mutation Frequencies and Surviving Fractions of *E. coli* recA -Proficient and recA -Defective Strains Exposed to Germicidal Lamp-Generated $UV_{308\,nm}$ Radiation

Strain	$UV_{308\,nm}$ radiation dose (mJ/cm^2)	Surviving fraction	Mutation frequency per 10^9 cells, median no.
JC411	0	1.0	9.0
	2500	7.0×10^{-2}	55.8
	5000	5.0×10^{-2}	45.0
	10000	3.0×10^{-2}	42.5
JC1553	0	1.0	29.0
	1800	3.2×10^{-2}	3625
	2400	3.0×10^{-4}	21000
	3600	1.8×10^{-4}	35000
	6000	0.6×10^{-4}	56000

Table 2
Mutation Frequencies and Surviving Fractions of *E. coli* recA -Proficient and recA -Defective Strains Exposed to Germicidal Lamp-Generated $UV_{254\,nm}$ Radiation

Strain	$UV_{254\,nm}$ radiation dose (mJ/cm^2)	Surviving fraction	Mutation frequency per 10^9 cells, median no.
JC411	0	1.0	9.5
	3	2.1×10^{-1}	22.7
	4	1.1×10^{-1}	40.8
	6	2.8×10^{-2}	146.3
JC1553	0	1.0	18.6
	0.4	1.2×10^{-2}	42.8
	0.6	8.4×10^{-2}	53.9
	0.8	2.7×10^{-2}	67.9

with DNA strand breaks and interstrand crosslinks being the most common effects.

Photoproducts of the aromatic amino acids phenylalanine, tyrosine and tryptophan act as major sensitizers for the action of near-UV radiation [14]. Near-UV-radiation photolysis of tryptophan releases superoxide ions and hydrogen peroxide and the near-UV-radiation photoproducts of tryptophan are especially toxic for recombination-deficient mutants [15].

This found suggests that the $UV_{308\ nm}$ radiation might also be mutagenic in microorganisms naturally lacking the SOS response, a condition that frequent limits the diffusion of the $UV_{254\ nm}$-radiation-induced mutagenesis for research or industrial purposes [8], and it encourages the setting of new mutagenesis protocols based on XeCl excimer laser radiation.

To value this hypothesis, in this study, we have determined the effect of $UV_{308\ nm}$ radiation on survival of Nonomuraea ATCC 39727 spores. Results demonstrated a moderate sensitivity of the strain to the radiation (Figure 3). Survival was about 2.7 x 10^{-3} at values of about 5000 mJ cm^{-2}. After determining the survival curve, we analyzed the mutability of Nonomuraea ATCC 39727 upon exposure to the $UV_{308\ nm}$ excimer laser radiation. Mutation frequency to streptomycin resistance was used as a parameter of the mutability. Doses ranging between 0 and 2500 mJ cm^{-2} were used, being the survival fraction at values >2500 mJ/cm^2 too small (<10%) to obtain a statistically significant number of viable mutants. Results demonstrated the efficiency of the $UV_{308\ nm}$ laser mutagenesis with a microorganism that, as well as other actinomycetes [8], is relatively refractory to mutagenesis by UV_{254nm} radiation generated by germicidal lamps.

Mutation frequencies increased as a function of the dose. At about 2500 mJ cm^{-2}, the mutation frequency was about 15-fold higher than in the control (un-irradiated) sample, and the survival fraction approximated 10% (Figure 4). In conclusion, using recA-proficient and recA-defective *E. coli* strains, we have demonstrated that the $UV_{308\ nm}$ laser radiation is mutagenic also in microorganisms lacking the SOS response. Indeed, the application of UV_{308nm} laser mutagenesis protocol to select *Nonomuraea* ATCC 39727 streptomycin-resistant mutants has been very effective at variance with $UV_{254\ nm}$ radiation which is not mutagenic for this industrial strain. Further studies will be necessary to elucidate the molecular mechanism by which $UV_{308\ nm}$ radiation induces mutagenesis in microorganisms naturally SOS-defective. Nevertheless, our results have relevant applicative potentialities to improve mutagenesis-selection procedures for research and industrial purposes.

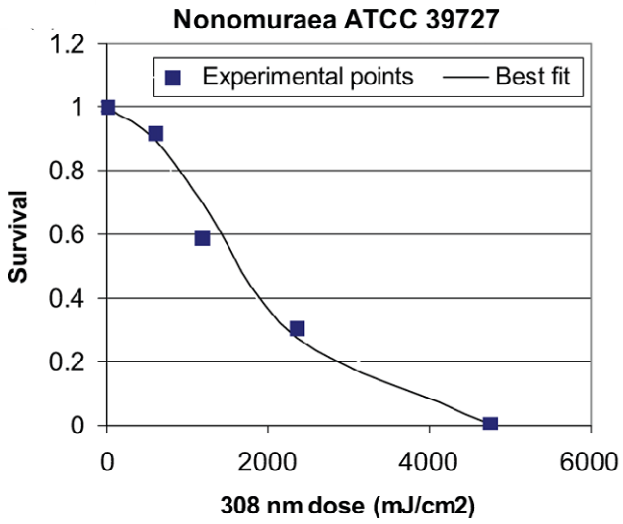

Fig. 3. Survival of *Nonomuraea* ATCC 39727 spores exposed to excimer laser-generated $UV_{308\ nm}$ radiation. Survival fractions and standard deviations are plotted as a function of $UV_{308\ nm}$ dose.

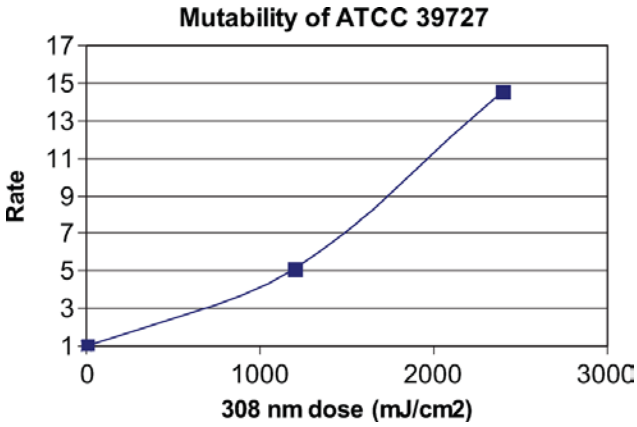

Fig. 4. Mutation frequency to streptomycin-resistance of *Nonomuraea* ATCC 39727 exposed to excimer laser-generated $UV_{308\ nm}$ radiation. Values represent means and standard deviations of the median values obtained in the three independent experiments.

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