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Irradiation of *Escherichia coli* in the Visible Spectrum with a Tunable Organic-Dye Laser Energy Source

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Pulsed laser energy was shown to be effective in inhibiting the growth of *Escherichia coli*. The irradiation source was derived from a tunable organic-dye laser utilizing rhodamine 6G (590 ± 5 nm) solutions as lasing media. The organisms, suspended in nutrient broth, were irradiated both with and without an exogenous photosensitizer. One photosensitizer (toluidine blue) did not appreciably alter the inhibitory effect observed. In the presence of acridine orange, however, some additional growth occurred.

Radiation derived from a laser output provides a bactericidal effect on microorganisms that is far more intense than radiation of similar frequency obtainable by conventional methods. Because organic matter most easily absorbs radiation from that portion of the spectrum providing the highest photon energies (i.e., ultraviolet [UV] light, X rays, etc.), this application has been slow in development, patiently awaiting the technological advances that provide just such a high-energy laser capability. It has been reported, however, that bacteria such as *Escherichia coli* appear to be somewhat transparent to continuous-wave laser radiation in the visible red region and thus are not affected without exogenous photosensitizers (6). A comparable study (4) using cultures of *Serratia, Staphylococcus aureus, Pseudomonas*, and *Pneumococcus*, all essentially equivalent to *E. coli* in susceptibility to visible radiation, also showed that inhibition was largely dependent on the ability of the organism to assimilate an appropriate dye, which in turn absorbed the visible radiation and transferred the energy to the cell. A third investigation (1) reported on the relative inefficiency of ruby laser radiation on various unstained bacteria, *E. coli* included.

As a check on the efficacy of visible laser radiation for sterilization purposes, we report a preliminary study that is a precursor to more definitive investigations concerning the effect of wavelength and intensity of laser energy for bactericidal reactions. There may even be applications to the study of biocatalytic photochemical reactions. Both broad-band and tunable monochromatic outputs, obtained by lasing the dyes rhodamine 6G and 7-diethylamino-4-methylcoumarin, were used to irradiate samples of *E. coli*. Exogenous sensitizers used were toluidine blue and acridine orange. The laser source provided a pulsed output whose instantaneous energy was considerably greater than that reported in earlier continuous-wave studies.

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MATERIALS AND METHODS

Laser energy source. The tunable organic-dye laser system consisted of a 0 to 10-kV power supply, 2.0-microfarad micropulser capable of manual or automatic (0 to 60 pulses/s) operation, an elliptical reflector enclosing a 5-mm-bore linear flashlamp and 3-mm-bore quartz dyecell at each foci, as well as various optics depending on the desired lasing conditions. For maximal broad-band output, a high-reflectance, dielectric-coated mirror was used at the back end and a 50% reflectance, dielectric-coated, partially transmitting mirror at the front end. Tunable monochromatic outputs were achieved with an 1,800-grooves/mm aluminum-coated diffraction grating blazed in the first order for 500 nm in place of the high-reflectance mirror. The laser output at maximal radiant-flux density (power per unit cross-section) had a rise time of 700 nanoseconds as determined by conventional measurements using a photomultiplier tube with an oscilloscope readout. The maximal energy flux was measured with a laser calorimeter in conjunction with a microvoltmeter and was found to be 201 mJ/cm^2 per pulse.

Preparation of the organism. The microorganism used for the study was *E. coli* (0127-08). The bacteria were first grown on a Bio Cert tryptic soy agar slant

and later placed at 4 C. By aseptic techniques, a loopful of the culture was transferred to 20 ml of nutrient broth (Difco) and incubated at 37 C for 24 h. When an exogenous photosensitizer was used, 5 μliters of dye in aqueous solution was added at the time of initial inoculation to yield a resultant dye concentration of 5×10^{-6} M.

Experimental irradiation procedures. Identical 0.2- or 0.5-μliter droplets of the organism in nutrient broth were deposited on twin microscope cover glasses. The smaller sample was used with focused-beam irradiation and the larger was used when the beam was not focused. A 0.2-μliter droplet had a nominal diameter of 1.4 mm, whereas a 0.5-μliter sample had a diameter of 2.0 mm on the cover glass. Since the unfocused laser beam diameter was 3.0 mm, total drop coverage was possible. In focused irradiation work, a 10-inch (about 25 cm) focal-length converging lens was used to intensify the beam. Positioning the droplet 5 inches (about 12.5 cm) from the lens quadrupled the radiant-flux density. The droplets on the cover glasses were placed on separate Lucite mounts in airtight cavities. One sample served as the unirradiated control; the other sample was irradiated through the cover glass. A totally reflecting mirror aligned perpendicular to the laser optical axis was located behind the droplet to maximize efficiency.

After exposure to a predetermined total energy flux that varied from 0.08 to 130 J, the drop cover glass was removed from the mount and dropped into a sterilized test tube containing 10 ml of sterile distilled water. The test tube was shaken by standard techniques. When a 0.2-μliter sample was used, a 0.2-ml pipette was used to pipette 0.2 ml into each replicate petri dishe. It was found that three or four replicates were necessary to obtain a meaningful average.

When a 0.5-μliter drop was used, a 0.1-ml pipette was used for the above procedure. The object of pipetting at these volumes was to insure that the final plate count ranged between 30 and 300 colonies.

Aside from the exposure to laser irradiation, the control sample went through the identical procedure: into 10 ml of distilled water, shaken to disperse the droplet, and pipetted into replicate petri dishes. Both the irradiated and control samples remained in distilled water for 10 min. Approximately 15 ml of MacConkey agar was added to each 0.1- or 0.2-ml sample in the petri dish and swirled to permit the organisms to distribute themselves uniformly in the agar. An incubation period of 20 to 24 h at 37 C was found to be optimal for plate counting.

The laser beam was superimposed on the drop by physically adjusting the drop so that a visual check could ascertain coverage. It was determined that nearly perfect alignment of the laser beam on the droplet was necessary for inhibition. The reason for this requirement was that the energy profile, normally Guassian, fell very sharply away from the center of the intense portion of the beam. Early runs were inconsistent because alignment of the beam on the droplet was attempted by visually locating the drop in the middle of the laser beam. Since the beam spot was extraordinarily bright, it was not always possible to pick out the intense portion of the beam. At this time it was assumed that the beam energy profile was exactly Guassian with the most intense spot at the center. Unfortunately, a later check with laser safety goggles showed that the intense portion was offset. Subsequent alignment accomplished with the use of goggles proved to be adequate.

Mere alignment of the beam on the drop did not always insure perfect irradiation. Thermal gradients within the dyecell had a tendency to cause the laser beam spot to move unpredictably, shifting at times by a length equivalent to the diameter of the droplet itself. Automatic pulsing operations did, however, alleviate the problem to some degree. Sometimes, though, upon automatic pulsing, the mode structure of the beam changed with every pulse. Although Gaussian profiles were obtained with manual pulses, automatic pulsing, which was constrained to a pulsing rate of 2 pulses/s, caused the flowing medium to heat unequally, thereby upsetting the ideal lasing conditions which ultimately resulted in an impure mode performance. As we learned earlier (P. K. Takahashi, Ph.D. thesis, Louisiana State Univ., Baton Rouge, 1971), imperfect mode structures produced beam patterns that did not properly cover the droplet. For example, a Gaussian, or TEM_{00}, mode suitably covers the hemispherical drop, since the intense portion of the beam passes through the thickest portion of the drop. A combination of TEM_{01} and TEM_{10} modes form an annular profile that is almost totally useless for irradiation operations, since the null portion passes through the thickest part of the drop and the intense ring passes through the thinnest.

Several other inconsistencies made reproducibility often difficult to achieve. Each 5-mm-bore-diameter flashlamp was different. At the lowest automatic pulse rate setting on the micropulser, pulsing rates differed between flashlamps from 2 to 4 pulses/s. If the rate was 2 pulses/s, a 10-kV setting resulted in each flash occurring at 10 kV. However, at 4 pulses/s, even though the power supply was set at 10 kV, each flash occurred at 9 kV. Furthermore, the ouptut power of each pulse varied somewhat from pulse to pulse. In addition to the above, the dye solution itself degraded with time. Coumarin solutions, in particular, had to be replaced as frequently as every two daily irradiation runs. Rhodamine 6G solutions, however, remained usable for several weeks.

The possibility of a significant temperature rise due to energy absorption by the droplet during exposure to laser radiation was tested experimentally. The effect was ascertained to be negligible ($\ll$1.0 C temperature rise of a fast-response thermocouple).

In summary, although consistency in laser pulse performance was not always achieved, the use of 60 to 250 irradiation pulses was sufficient to compensate for the variation in energy flux from one experimental run to the next. The system and procedures used to irradiate *E. coli* proved to be quite satisfactory for demonstrating the efficiency of visible laser light.

RESULTS

We determined that from 76 unstained and toluidine blue-sensitized runs at an average total energy flux of approximately 25 J/cm^2 the

fraction survival was 0.52. The unstained or nutrient broth samples did not significantly differ in survival fraction from the toluidine blue-sensitized samples. We suspect that the nutrient broth for the irradiation conditions utilized had an equivalent photodynamic effect upon all samples. However, 12 acridine orange-sensitized, unfocused-beam irradiation runs all showed increases in growth after irradiation. Of these, nine broad-band runs irradiated at 590 ± 5 nm and at an average energy level of 16.5 J/cm² experienced a fraction of survival of 1.49. Similarly, three broad-band runs irradiated at 450 ± 5 nm and at an average energy level of 5.0 J/cm² showed a fraction survival of 1.35. It should be noted that each run involved from six to ten samples because of replication and the need for controls.

Table 1 summarizes the relative effect of a variety of laser irradiation conditions on the fraction survival of *E. coli*. It is unfortunate that higher inhibition efficiencies were not achieved. The relatively low laser output energy, as can be verified from the Luckiesh (5) tables, constrained survival fractions to a range between 2.09 and 0.17; the results above 1.0 suggest growth in excess of the control. In addition, replicate samples derived from the same droplet

TABLE 1. *Summary of the laser irradiation effects on the survival of E. coli*

Energy flux (J/cm²)	Lasing dye[b]	Photosensitizer[c]	Mean survival fraction	No. of runs[a]	Plate counts[a]			
					Sample		Control	
					Min.	Max.	Min.	Max.
(A) Laser beam unfocused								
0.41	R6G	None	0.68	2	40	167	155	280
0.89	C	None	0.79	2	74	107	115	245
2.0	R6G	None	0.72	6	114	168	191	239
2.3	R6G	None	0.55	4	74	133	214	288
3.0	R6G	None	0.29	5	45	93	154	230
4.1	R6G	None	0.35	2	70	210	190	284
5.0	C	None	0.60	3	168	211	178	277
5.0	C	A.O.	1.35	3	125	244	36	115
6.4	R6G	T.B.	0.51	3	80	130	226	274
8.1	R6G	None	0.51	2	39	132	122	255
8.1	R6G	T.B.	0.47	2	55	148	214	294
11.4	C	None	0.81	2	109	153	151	204
15.1	R6G	None	0.40	2	102	134	231	287
15.1	R6G	T.B.	0.50	2	89	237	218	284
15.1	R6G	A.O.	1.51	2	210	302	57	251
16.4	R6G	A.O.	2.09	2	238	298	95	255
18.0	R6G	None	0.40	2	50	170	126	290
20.1	R6G	T.B.	0.39	2	37	63	175	286
20.1	R6G	A.O.	1.14	3	209	300	71	219
22.5	R6G	T.B.	0.27	2	34	167	208	298
22.5	R6G	A.O.	1.59	2	145	280	98	220
(B) Laser beam focused								
0.08	R6G	None	0.86	2	154	229	220	279
0.08	R6G	T.B.	1.06	2	69	154	63	185
0.08	R6G	A.O.	1.92	2	136	211	35	165
13.0	C	None	0.55	2	62	123	186	290
13.0	C	A.O.	0.20	2	30	110	134	258
24.4	R6G	None	0.51	2	23	213	200	249
24.4	R6G	T.B.	0.54	2	48	127	181	250
32.5	R6G	None	0.49	2	93	220	205	290
32.5	R6G	T.B.	0.67	1	37	184	160	210
45.7	R6G	None	0.50	4	47	159	126	237
49.0	R6G	T.B.	0.49	2	42	117	128	291
49.0	R6G	A.O.	0.51	2	69	116	113	159
65.0	R6G	None	0.43	4	30	94	164	261
65.0	R6G	T.B.	0.38	5	35	146	155	258
65.0	R6G	A.O.	0.67	2	65	184	191	281
98.0	R6G	T.B.	0.05	2	31	49	218	288
130.4	R6G	None	0.17	2	45	87	130	222
130.4	R6G	T.B.	0.38	1	63	176	190	272

[a] Each run was replicated three or more times (using at least three plates per run). Abbreviations: Min., Minimum; Max., maximum.

[b] Lasing dyes: R6G, Rhodamine 6G; C, 7-diethyl-4-methylcoumarin.

[c] Exogenous photosensitizers: T.B., Toluidine blue; A.O., acridine orange.

varied by roughly ±20% because of inherent errors in the relatively small sample size and associated plating effects. However, it is important to emphasize that the irradiated samples, save for the results that suggest enhancement of growth as observed with acridine orange, all straddled survival fractions below 1.0. Thus, there appears to be little doubt from our statistical study of these reported results that growth inhibition was achieved (with an extremely high level of confidence exceeding 0.99). Based on several irradiation experiments with thermocouple probes, temperature rise in the droplet was considered insignificant as contributing to this effect.

In this work, rhodamine 6G had a broad-band output in the region 590 ± 5 nm, and coumarin lased at 450 ± 5 nm. It was found that both broad-band and tunable monochromatic outputs of equivalent intensities showed comparable inhibitory effects. For the tunable irradiation runs, the rhodamine 6G wavelength was varied from 556 to 610 nm and coumarin from 441 to 475 nm; the band width in each case was less than 0.1 nm.

The energy flux-survival relationship for this irradiation system was developed and related to the published data for *E. coli* survival under conventional light irradiation (3). This nonlinear relationship, whose derivation is documented elsewhere (Takahashi, Ph.D. thesis), follows: $X/X_0 = \exp[-0.398E_cW(\lambda)] = \exp(-kE_L/\lambda)$, where X = viable cell concentration after irradiation, X_0 = viable cell concentration before irradiation, E_c = energy flux imparted by conventional light source (in joules per square centimeter), $W(\lambda)$ = relative effect of wavelength (5), k = laser radiation kill coefficient, E_L/λ = energy flux imparted by organic-dye laser at λ (in joules per square centimeter), and λ = wavelength of radiation. Our experimental results were evaluated by relating the microbiological plate count ratio of irradiated and control samples to X/X_0 and directly measured E_L, the energy flux output of the organic-dye laser. For a direct comparison of the relative inhibitive and growth effects for various lasing conditions, the laser radiation kill factor, k, was determined by routine computerized least-squares statistical study of the data. When k is equal to zero there is no inhibition. A positive k value indicates inhibition, and a negative k value suggests growth that exceeds the control. The statistical analysis also provided the correlation coefficient, R, for each set of parametric runs. Except for the rhodamine 6G/unfocused/acridine orange and the 7-diethyl-4-methylcoumarin/unfocused/none sets, which resulted in borderline values of the correlation coefficient, the remaining R values were well within statistical confidence. Thus, the correlation coefficients derived from these data strongly suggest that laser irradiation was the primary factor for inhibition and that other factors did not appear to be significant. A more quantitative interpretation of these initial experiments (Table 2) does not appear warranted at this time.

DISCUSSION

We draw from these experiments observations that appear significant in several respects. (i) Pulsed laser light appeared to be more efficient than either conventional or continuous-wave laser light in inhibiting the growth of *E. coli;* (ii) with the exception of the acridine orange-sensitized samples, unfocused irradiation was more efficient than focused irradiation in inhibiting the growth of the organism; (iii) an apparent increase in growth was observed in samples sensitized with acridine orange and irradiated with unfocused laser light.

Except for the acridine orange-sensitized samples, all pulsed laser irradiation systems were more effective than conventional light in inhibition of the organism; that is, under identi-

TABLE 2. *Comparison of a variety of laser irradiation conditions on the fraction survival of E. coli*

Lasing dye	Beam condition	Exogenous photosensitizer	Total no. of runs	Avg energy flux[a]	Mean fraction survival	Relative kill factor	Correlation coefficient
Rhodamine 6G	Unfocused	None	25	7.8	0.49	1.73	0.73
Rhodamine 6G	Focused	None	16	55.7	0.47	0.60	0.90
Rhodamine 6G	Unfocused	T.B.	11	13.7	0.44	1.87	0.97
Rhodamine 6G	Focused	T.B.	15	55.5	0.48	0.47	0.99
Rhodamine 6G	Unfocused	A.O.	9	18.7	1.49	−3.92	0.46
Rhodamine	Focused	A.O.	6	58.3	0.60	0.28	0.93
Coumarin	Unfocused	None	7	5.1	0.67	0.68	0.53
Coumarin	Unfocused	A.O.	3	5.0	1.35	−1.19	1.00

[a] Obtained by adding the individual run energy fluxes and dividing by the number of runs.

cal irradiation conditions, the higher the value of k the more effective was the kill effect (Table 2). This result supports the Harm study (2), where a single-exposure high dose was shown to be more effective than fractionated or continuous dosages of the same total energy for inactivating *E. coli*. The organic-dye laser used in this investigation imparted an extremely high radiant-flux density of 2×10^5 W/cm^2 in a time interval approaching 1 microsecond. The rapid pulse indicates, however, that the total energy accumulation of contact was low.

It can be surmised, therefore, that unfocused-beam irradiation is more efficient than the focused output simply because our optical system provided the most effective use of the laser beam for this situation by repetitive reflection through the biological sample. This was not feasible for the focused-beam experiments. It was also noted by qualitative measurement that droplet absorption of the laser beam was very low on a per-pass basis; however, with the totally reflecting mirror aligned behind the sample, multiple passes enchanced the irradiative effect of the unfocused beam.

It has not been ascertained, however, whether the growth increase, that is, the negative k value, was a true catalytic effect or a simple physical cleavage, i.e., splitting of microbial chains, pairs, or colonies. Additional study will be necessary to resolve the mechanics of this so called "reverse" effect. We also recognize that there was some absorption across the visible portion of the spectrum by the nutrient broth in which the organism was suspended. This absorption was greatest at the wavelengths of irradiation. As a result, this suggests that the actual energy absorbed by the organism was less than reported, thus enhancing the irradiation procedure and tending to make the effect even more pronounced than our study indicates. It does, however, somewhat qualify the measurement of energy absorbed by the organism reported here. Future work should utilize nonabsorbing suspending media such as buffers or saline solution so that meaningful quantitative energy measurements can be obtained.

Since UV radiation at 265 nm is approximately 30,000 times more effective than the visible at 600 nm (2), a concerted effort is being made to achieve a tunable UV laser output at reasonable energy levels. We anticipate that our future tunable UV laser studies will eventually be even more stimulating and rewarding than the present visible laser light probe. Unfortunately, the existing frequency-doubled tunable UV laser systems are only about 10^{-4} times as powerful as those instruments operating in the visible spectrum. Technology is just now beginning to produce tunable UV laser irradiators powerful enough for microbiological research.

Certainly, though, the effects of visible laser light should be examined in greater detail to determine the reasoning behind the intriguing results that we have experienced. To obtain microbial survival fractions approaching 10^{-5} to 10^{-2}, however, visible laser radiant-flux densities at least 1,000 times more powerful are required, and these powerful tunable lasers are only now becoming available.

A comprehensive study analyzing the effects of tunable laser radiation from 250 to 700 nm on several types of bacteria, under unsensitized and photodynamic as well as focused and unfocused conditions using a sufficiently powerful laser, is the logical next step toward the application of the laser to processes of sterilization, selective sterilization, and catalysis.

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