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(NASA-CR-134328) [AEROMONAS PROTEOLYRICA BACTERIA IN AEROSPACE ENVIRONMENTS] Final Progress Report (Texas A&M Univ.) 25 p HC $4.25 CSCL 06M

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Previous work under NASA contract NAS 9-7951 suggested the possibility that spacecraft environments may provide selective pressure for strains of bacteria that are more proteolytic or that produce genetic alterations which result in increased proteolysis. On the basis of these findings it was suggested that the M E E D include a bacterial species whose extracellular enzymes are easily quantitated and which have some overt biological activity. Aeromonas proteolytica was chosen because one of its extracellular enzymes, an endopeptidase, produced in exceptionally high levels, could be measured accurately and conveniently by use of a urea denatured hemoglobin substrate and that the enzyme when produced in these relatively high amounts can induce intracutaneously hemorrhage and necrosis in laboratory animals. In addition, a hemolysin is also found in growth filtrate which is active in lysing human erythrocytes (1).

While this organism is not part of the endogeneous flora of man, it does epitomize a group of organisms which may be present on human tissue. If altered these organisms could pose medical problems of unknown magnitude, i.e. if one or both of these substances, produced in small amounts by many endogeneous microbes, were to be produced in unusually high quantities or altered in such a way as to be more effective in its activity, a serious health problem might exist for space travellers.

Preflight studies necessarily included such problems as: (1) development of a low or non- nutrative holding medium in which the organism would be suspended, (2) the establishment of base line information for the standardization of the assay for endopeptidase levels and hemolytic titers, (3) to determine a method by which intracutaneous hemorrhage could be quantitated in guinea pig tissue, and (4) the response of these organisms to parameters of spaceflight and experimentation.

Organism

The strain of Aeromonas proteolytica used in this study was a subculture of the original isolate of Merkel and Traganza (3). It was obtained from Dr. J. M. Prescott, Department of Biochemistry-Biophysics, Texas A&M University and was maintained on Tryptic Soy Agar (Difco).

Endopeptidase Assay

The endopeptidase activity was measured using a urea denatured hemoglobin substrate. For the assays 1.0 ml of a 1:40 dilution of the culture filtrate containing the enzyme is incubated with 5.0 ml of the hemoglobin substrate for five minutes in a 37°C water bath. At the end of this period 10 ml (w/v) of trichloracetic acid is added to terminate the enzyme action and to precipitate the undigested protein, which is removed by filtrtation through Whatman No.3 paper. The absorbance of the filtrate and control is read at 280 nm on an Hitachi Perkin-Elmer spectrophotometer and the activity expressed in units. One unit of endopeptidase activity is defined as the amount of enzyme producing an absorbance of 1.0 in 5 minutes at 37°C under the assay conditions described.

Hemolysin Assay

The degree of hemolysis produced by the culture filtrate was determined on 2 percent human type O, Rh positive erythrocytes, diluted in physiological saline. Doubling dilutions of the filtrate are made in physiological saline (with a final concentration of 0.2% sodium azide to prevent contaminating growth) through 1:1024. A modified microtiter method was utilized dispensing 5 drops of the culture filtrate and 3 drops of the 2.0% human erythrocytes in wells of a Microtest II Tissue Culture Plate (Falcon Plastics). The hemolysin titer is defined as the reciprocal of the highest dilution which gave complete clearing

in the well, at the end of a 24 hour incubation period, at 37°C.

Hemorrhagic and Necrotic Activity

Crude growth filtrate, when inoculated intracutaneously into guinea pigs produces immediate erythema followed within five minutes by hemorrhage and in some cases necrosis. Unless severe, the lesions will scab within 24 hours. The degree of reaction was evaluated by computing the area of the scabbing (assuming an ellipsoidal shape) from the minimum and maximum perpendicular scab diameters. By diluting the filtrate, areas of reaction can be controlled so that a number of tests may be made on the flank of one animal.

Development of Holding Solution

Several low or non-nutrative holding solutions were studied including distilled water; deionized distilled water, artificial seawater, physiological saline, physiological saline buffered with dibasic sodium phosphate, and 1% asparagine in distilled water. Several studies were made by holding a predetermined concentration of organisms in each test solution for 20 days at 20 degrees. Periodic viability checks were made and the percent survival calculated. In preliminary tests, holding solution aliquots of 5.0 ml were used. After selection of buffered physiological saline (o.5gNa_2HPO_4/1) as the holding solution numerous tests were made in 50 lambda quantities. The percent survival consistently ranged from 22 - 33%.

Pre-flight Ultraviolet Irradiation and Mutation Study

The ultraviolet light source was 140 watt, 115 volt, Hanovia utility quartz lamp which produces a broad spectrum of wavelengths. The two wavelengths selected for the irradiations were 254 nm and 280 nm which were obtained by use of specially built band pass filters designed to pass a single wavelength of UV. The light source was calibrated with these filters to

insure accurate transmittance of the proper energies to the samples, utilizing the potassium ferrioxalate actinometery procedure of Hatchard and Parker

In preparation for the irradiation, an inoculum from a stock culture of A. proteolytica was made into 100 ml of 2% casitone (Difco), made with artificial seawater (27 grams per liter of Seven Seas Marine mix from Utility Chemical Company). Growth was at room temperature for 18 hours on a magnetic stirrer running at low speed. Ten ml of this primary culture were transferred to another flask containing 100 ml of 2% casitone in seawater and grown for 24 hours under the same conditions as before. Cells from this secondary culture were sedimented at 7,710 x g for 15 minutes in a Sorvall refrigerated centrifuge at 4°C. The cells were then washed three times by centrifugation in physiological saline containing 0.05 g/l potassium phosphate (K_2HPO_4), and the suspension adjusted to give a reading of 0.1 optical density (OD) at 660 nm on a Bausch and Lomb Spectronic 20. When diluted 1:50, the suspension yielded a viable cell count of approximately 10^6 organisms per ml and an OD of approximately 0.02.

The dilute suspensions were irradiated in 50 lambda capacity cuvettes. The cuvettes were constructed of black plastic to avoid reflection, and possessed a single 25 mm^2 quartz window, designed to provide unrestricted passage of UV light and a flat field of irradiation.

The light source was fastened in a special stand to maintain a 30 cm distance between the sample and the light source and to assure a constant perpendicular angle of irradiation. By using this stand, any variation in degree of reflection and absorption was eliminated. The cuvettes containing the samples were placed under the light source in triplicate for the predetermined

time calculated to give an exposure of the desired intensity. The energy levels used at both wavelenghts were 1.0×10^2, 5.0×10^2, 1.0×10^3, 5.0×10^3, 1.0×10^4, 5.0×10^4, 1.0×10^5, and 5.0×10^5 ergs per cuvette.

Immediately following each irradiation, 10 - fold dilutions of the samples were made through 10^5 in artificial seawater. Spread plates were then made in duplicate on heart infusion agar from each of the dilutions. Fifty clones of survivors from each energy level of each wavelength were selected and transferred to heart infusion agar slants. As a control, three cuvettes were loaded and unloaded in the same manner as the irradiated cuvettes but without exposure to light. Since the irradiations with the two wavelengths were carried out at separate times, using separate primary cultures from different stock slants, a separate control was set up for each wavelength.

The dose response curves of A. proteolytica to 254 nm and 280 nm irradiation follow the same general trend. However the energy level at which a noticeable effect on survival began was slightly lower for the 280 nm group than for the 254 nm group. Above 1×10^3 ergs per cuvette of the 254 nm irradiation a sharp steady decline of survivors began, terminating with total kill at 5×10^5 ergs per cuvette. The same sharp decline occurred for the 280 nm irradiation group, but it began above 5×10^2 ergs per cuvette with total killing at 1×10^5 ergs per cuvette.

Post Irradiation Evaluation

The culture filtrates of A. proteolytica were prepared for the post irradiation evaluation as follows. Fifty ml of 2% casitone in artificial seawater, contained in a 250 ml flask, was heavily inoculated with growth from a heart infusion agar slant and incubated on a reciprocal shaker for

28 hours at room temperature. Pilot studies demonstrated these to be the optimum conditions for maximum enzyme production. The cells were removed by centrifugation at 7,710 x g for 10 minutes in a Sorvall refrigerated centrifuge at 4°C and the cell-free culture filtrate analyzed for endopeptidase activity, hemolysin titer, and in some cases for guinea pigs necrosis.

Analysis of the endopeptidase production established a distribution of the 99 percent confidence limits for the control group. Experimental clones producing endopeptidase activity outside of the 99 percent confidence range were regrown and reassayed four times to ascertain stability of the deviation. Of 11 clones of the organisms exposed to the 254 nm irradiation, none producing activity lower or higher than the calculated range were found.

There was no great deviation in the hemolysin titers produced by the clones exposed to either of the wavelengths of irradiation. However, there was a slightly different distribution of the percent of clones, producing particular titers.

Apollo 16 M E E D Experiment

Preparation of culture: A starter culture of A. proteolytica was prepared by inoculation from a stock slant (Trypticase soy agar) into 100 ml of 2% casitone (made with seawater). The culture was grown for 18 hours at room temperature on a magnetic stirrer set at low speed. One ml flask containing 2% casitone (made with seawater). The culture was grown 24 hours under the above conditions. Fifteen ml from this culture was sedimented by centrifugation at 7,710 x g for 20 minutes at 4°C. The cells were subsequently washed twice with sterile holding solution by

centrifugation. The cells from the final wash were suspended in 10 ml of holding solution and used to prepare cell suspensions adjusted to an OD at 660 nm of 0.1, 0.15, 0.2, 0.25, 0.3, 0.43, and 0.5 A 1:50 dilution of the cells was prepared using each of the seven different OD solutions. Each was checked on a spectrophotometer to ascertain that they did not drop below the required transmittance (%T) of 90 percent. A portion of each was plated on Heart Infusion Agar pour plates to determine the number of surviving organisms. The suspension yielding the largest number of organisms while not exceeding the optical limit was used to fill the flight cuvettes.

The culture of A. proteolytica used was #3-16-72-2 which has an endopeptidase activity of 16 units as determined by the urea denatured hemoglobin substrate assay for endopeptidase.

Post Flight Survival

The flight cuvettes were opened and the contents plated for surviving organisms. Dilutions of 10^3, 10^4, and 10^5 in duplicate were made from each cuvette. After 24 hours incubation counts were made and averages computed.

Analysis of the survival curves indicate that there is essentially and statistically no difference in the survival patterns with increasing dose energy levels over what one would expect from deaths due to storage. There is also no statistical difference in the death response among the three energy levels and full light. This lack of a death was not unexpected due to the demonstration in pre-flight studies of a repair mechanism following the irradiation of starved cells and storage for several days.

Twenty-four hours after plating the contents of each cuvette, 50 clones were selected at random from each of the energy levels at the four wavelengths.

Each was transferred to Heart Infusion Agar (HIA) slants. Each clone was inoculated into 50 ml of 2% casitone broth and incubated for 28 hours at room temperature under constant aeration by shaking. After this growth period the culture was sedimented by centrifugation for 10 minutes at 7710 x g ($40^{o}C$) and the supernatant frozen until assayed. Clones not cultured were transferred to fresh slants every 14 days.

Guidelines for Data Interpretation

Since the ultimate objective of this study was to ascertain whether the spacecraft environment could be responsible for selecting for or mutating cells with a phenotype (with respect ot extracellular enzymes) signigicantly different from normal cells, an evaluation of the normal cell population was determined. Throughout the pre-flight phases of this study it was observed that the test organism A. proteolytica exhibited a degree of variability in the production of endopeptidase as well as nemolysin. The variability was caused by a number of environmental influences ranging from the age of a particular culture to the medium on which it was grown. Unpublished observation by this laboratory seem to indicate that the culture of A. proteolytica used was a heterogeneous population of cells with variable enzymatic ability. The heterogenety was demonstrated when parent culture was streaked in Heart Infusion Agar to achieve isolated colonies. After suitable incubation one colony or clone was used to inoculate four slants, trypticase soy agar made with artificial sea water (27 g/l). The culture in each of these four slants was then assayed repeatedly for endopeptidase production by the previously described methods. The results indicated

that the culture on each of the four slants gave consistent assays except that each slant gave different enzyme values from any of the other three, i.e. each slant contained a population of cells which was still probably heterogeneous, different from the population on any other slant. The major point is that no reliable statistical evaluation should be performed on any data from a different population of organisms, while assuming the two populations to be the same. No such assumption should be made, for instance, between data collected at different times from different sub-cultures. Consequently in order to minimize the chance that non-treatment population shifts may affect a statistical comparison of data, the two masses of data must be collected as nearly simultaneously as possible and they must reflect the results due to treatment only. Any comparative data such as control data must not only be from the same population of cells, but they must be handled in the same manner, and at the same time, except without treatment.

Because of the various treatment conditions three types of control groups were planned. The ground controls would remanin stationary at the same temperature as the M E E D package. The vibration control would be vibrated in the ground in the same manner as the spacecraft. The flight control would be inside the M E E D package but not exposed to any ultraviolet irradiation. All cuvettes, including these controls were loaded at the same time with the same well-mixed cell suspension to give each cuvette the a same population of cells as the next. At the termination of the flight all cuvettes, including the controls were unloaded and processed identically. Any statistical evaluations relating M E E D data to the base line information refer to the ground, vibration controls.

The reason that the data from the preliminary laboratory UV irridation experiments were not used as base line information is twofold: (1) the population of organisms in this experiment was not the same as the population used in the M E E D experiment and, (2) in the laboratory experiment no storage time allowing for genetic repair was planned as was inherently involved in the M E E D experiment while the spacecraft was returning to Earth. Consequently the data from this preliminary experiment should be considered as a separate experiment from the M E E D experiment and the data should only be used in relative basic trends and not base line information.

Mechanism of Variation Due to Treatment

In the case of the M E E D experiment, there were several facets of the treatment which could conceivably cause changes in the extracellular enzymes of the test organism: ultraviolet irradiation, cosmic irradiation, vibration, and weightlessness being the most obvious factors. The mechanism of mutation caused by the cosmic and UV irradiation have been well documented. A mutation in its simplest term can be conceived as a hit at a site on one of the strands of the double stranded chromosome. If this cell was then allowed to divide once, of the resulting two daughter cells, one would be normal and the other would have the mutation on both strands of the chromosome. This replication after mutagenesis is referred to as phenotypic expression. Without phenotypic expression the "hit" cell when plated on a nutrient medium will form a colony consisting of a heterogeneous population of normal and mutant cells. Unless the "hit" cells in the cuvettes could somehow divide once under the low nutritive conditions before they were plated out on nutrient media, this phenomenon would occur.

The mutational effects of weightlessness and vibration have not been documented. It is conceivable, however, that these environmental factors could exert some sort of selective pressure upon the cell populations in each cuvette. The interaction between these environmental selective pressures and an increased mutation rate caused by irradiation could easily result in population shifts, with respect to the parameters being observed.

As previously mentioned because of the time required for the return trip of Apollo 16 after the M E E D irradiation, a more than adequate time for genetic repair was allowed. However, energy for this repair would be needed and the cells should have been in a starved state by the time of the actual irradiation. The energy probably came from the catabolism of the constituents of lysed cells in the suspension. The nutrient level would probably not be enough to support rapid growth, but would probably supply enough for a minimal level of metabolism.

At any rate, repair could conceivably have occurred post-irradiation and pre-splashdown. Because of this phenomenon, any data relating to the "number of mutants recovered" should reflect the fact that a significant percentage of the induced mutations could have already been repaired by the time the cuvettes were plated out, which would not be expressed by the data collected.

Mutation Rates

As mentioned previously two biological phenomena are being evaluated in this study - mutation and selection. Mutational events would probably best be exhibited by an enumeration of isolates giving enzyme values outside certain confidence limits. This type phenomenon is more likely to

drastically affect individuals rather than the population as a whole. Of course the two phenomena cannot truly be separated because selection ultimately depands upon "favorable" or "non-deleterious" mutation as the mechanism of population shifting. Normally, selective pressure causes very gradual changes because of a low "natural" mutation rate. However, one would expect much more rapid population shifts due to selective pressure if the mutation rate were increased. These are the type of conditions found in the M E E D package: increased mutation rates caused by UV and cosmic irradiation as well as unknown selective pressures which may include weightlessness, vibrations, constantly changing magnetic fields, etc. The phenomenon of selection would probably best be expressed by the simple statistics, such as the mean and standard deviation for the population of cells in each treatment. Individual "drastic" mutations may still influence these values, but the bulk of the data softens the effect considerably.

Analysis of Endopeptidase Data

Of the three parameters monitored for each isolate, endopeptidase, hemolysin and guinea pig reaction, the endopeptidase gave the most reliable and extensive results. For this reason these data will be treated first. The mean ($\bar{x}$) and standard deviation (s) of each energy level with respect to wavelength can be found in Table I.

The data in this table seem to show a definite trend--the means and standard deviations of the groups seems to shift upward as the wavelength is increased. This indicates that the organisms exposed to the higher wavelengths yield more to whatever selective pressures are exerted upon them by the spacecraft environment than do the organisms exposed to the lower wavelenghts or the flight controls. It should also be noted that the organisms in the full spectrum exposure cuvettes gave approximately

the same values as those in the 300 nm group. With increased wavelength came also increased variability, indicating a wider separation of individual valves which reflects a higher variability probably caused by mutation.

In addition when part of this data is rearranged in increasing order of energy levels, irrespective of wavelength, it is seen that there is an apparent increase in the means of all isolates for each energy level with respect to energy level. (See Table II) This apparent increase is borne by the fact that when a linear regression analysis is performed on the endopeptidase mean (ordinate) vs. the $\log_{10}$ of the energy level (absissa) data to determine the best straight line that will fit the points, a positive slope of 0.167 is obtained for this line, thus confirming the apparent trend of the data.

As far as the enumeration of outliers or mutants is concerned, several paths are open, depending upon one's preference of interpretation. Specifically, confidence limits may be established on the basis of the distribution of 1) the ground controls only, 2) the ground, vibration and flight controls collectively, or 3) the overall distribution of all the data. Not included as an option was the distribution of the pre-flight "base line" data for reasons previously mentioned. The best normal distribution from which reliable confidence limits can be established should come from as large a group as possible, which would be option 3. However, all non-control data comes from treated samples and should reflect changes due to treatment -- which is the parameter to be measured. Consequently, even though these data represent an ideally sized group for determining distributions, this group may not be the best option because of distribution shifts caused by treatment effects. The smallest group, the ground controls represent no treatwhatsoever and are therefore suitable for determining a model distribution,

except that the sample size (50) is rather small. A better group would be all the controls, except that the vibration and flight control groups represent varying amounts of treatment, excluding only the UV irradiation. However, based on the means and standard deviations of these control groups, there was deemed an insignigicant amount variation among the groups to warrant the exclusion of any control group from the determination of a model distribution. An analysis of variance study did reveal, however, that the ground control group was significantly different from the toher two control groups. These results could have easily been caused by sampling errors (because of the small sized groups) or by any of the various points of error in the assay itself. At any rate, two model distribution groups are available -- the control groups or all groups, including controls. The following data is presented on the basis of the statistics of these two groups. Using the control groups where n is 150, the mean ($\bar{x}$) is 17.052 and the standard deviation (s) is 1.843. Using all groups, where n is 1243, the mean is 17.627 and the standard deviation (s) is 2.292.

Using control statistics at the 0.0005 uncertainty level, an experimental value above or equal to 23.12 units or below or equal to 10.99 units would be considered an outlier. At the 0.01 uncertainty level, an experimental value equal to or outside of the range values of 21.34 and 12.77 units would be considered an outlier. At the 0.05 uncertainty level, an experimental value equal to or outside of the range values of 20.08 and 14.02 units would be considered an outlier.

Using the statistic of all the data, at the 0.0005 level of uncertainty, an experimental value above or equal to 25.17 units or below or equal to 10.08 units would be considered an outlier. At the 0.01 level of uncertainty,

an experimantal value equal to or outside of the range values of 22.96 and 12.29 units would be considered an outlier. At the 0.05 level of uncertainty, an experimental value equal to or outside of the range values of 21.40 and 13.86 units would be considered an outlier. The actual outlier counts using the above values are found in Table III.

If the total outlier count are converted into mutants per 100 isolates processed or percent mutation an interesting trend is observed (See Table IVA). The percent mutants appear to increase with increasing wavelength, when control statistics are used to indicate the distribution at the 0.10 and 0.02 level of uncertainty (Table IVB). However, when total statistics are used the "mutation rate" appears to be independent of the wavelength at the 0.10 level of uncertainty, but not at the 0.02 level of uncertainty (Table IVC). At the 0.0005 level of uncertainty the number of mutants is not large enough to draw accurate conclusions. Unlike the means of each energy level, the number of outliers, regardless of the level of uncertainty, show no significant relationship to the energy level or irradiation irrespective of wavelength (Table V).

Analysis of Hemolysin Data

Besides the endopeptidase assays run on each of the 1243 isolates, the hemolytic activity of the culture filtrates from each isolate was also determined in quadruplicate. The data from these isolates was analyzed by using the hemolysin titer directly (i.e. 2, 4, 8 , 16, 32, 64, etc.) and by using the $\log_2$ of the hemolysin titer (i.e. 1, 2, 3, 4, 5, 6, etc.) as indicators of the amount of hemolysin present. The $\log_2$ of the hemolysin titer was considered a preferable index of hemolysin concentration because it was less likely to reflect experimental errors, especially toward the higher readings.

Using the direct titer, the following confidence limits were established

using a mean ($\bar{x}$) of 33.108 and a standard deviation of (s) of 16.009 as determined from the 1243 isolates:

2-Tailed t-Test	.10		.02		.001	
1-Tailed t-Test	.05	.05	.01	.01	.005	.005
	0	42.344	0	53.246	0	68.695

Using the $\log_2$ of the hemolysin titer, the following confidence limits were established using the same mean and standard deviation:

2-Tailed t-Test	.10		.02		.001	
1-Tailed t-Test	.05	.05	.01	.01	.005	.005
	2.842	9.436	1.478	10.800	-0.456	12.734

Using the preferable index, the mean of the quadruplicate hemolysin titrations, no isolates were considered significant at any reasonable degree of uncertainty. This indicates that the hemolysin determinations were so variable that any attempt to determine outliers using these statistics was futile. This variability could have been caused by experimental error or it could have been merely a property of the heterogenicity of the isolates assayed. Whichever the case the hemolysin data was too variable to discern any trends.

Even with the high standard deviation of the hemolysin titer, attempts were made to determing the correlation coefficients of the hemolysin and endopeptidase data. The following results were determined:

	Endopeptidase	Hemolysin Mean	Log_2 Hemolysin Mean
Endopeptidase	1.0000	0.0999	0.1048
Hemolysin Mean	0.0999	1.0000	0.9995
Log_2 Hemolysin Mean	0.1048	0.9995	1.0000

As can be seen from the correlation coefficients there is almost no correlation between endopeptidase and either the mean hemolysin titer or the mean $\log_2$ of hemolysin titer, confirming previous work by this laboratory.

Analysis of Guinea Pig Reaction Data

In addition to the endopeptidase and hemolysin activity determinations on all samples, several samples were used to determine their necrotic activity towards dermal guinea pig tissue. Several studies were performed initially to determine the effect of injecting different volumes of culture filtrates. It was found that the larger the volume injected (0.5 ml) the more irregular were the results due to a distension of the skin by the filtrate fluid; but at the smaller volume (0.125 ml) there was a greater chance of error due to significant seepage of the filtrate out of the skin through the needle hole. A filtrate volume of 0.25 ml was deemed optimal. Experiments were undertaken to determine if the amount of endopeptidase in the filtrate had any influence on the area of necrosis. Dilutions of a culture filtrate were prepared containing the following endopeptidase activity per ml: 18.81, 15.05, 12.60, 9.41, 6.27, and 3.14. A volume of 0.25 ml of each dilution was injected in duplicate into each of 3 guinea pigs.

The average areas of necrosis were determined for each dilution of endopeptidase:

Dilution No.	EP Units	Av. Area of Necrosis (mm^2)
1	18.81	159.02
2	15.05	126.90
3	12.60	108.97
4	9.41	111.56
5	6.27	80.77
6	3.14	61.59

As can be seen from these data there is a definite correlation between endopeptidase

activity and necrotic activity. The necrotic activity corresponding to 9.41 endopeptidase units appears to be in error. Consequently, using linear regression analysis to fit a straight line to these data, the following equation results:

Including dilution #4 $y = 5.791\ x + 45.129$

Excluding dilution #4 $y = 5.920\ x + 41.300$

When guinea pig reactions were determined on various filtrates, they were run in duplicate or triplicate on the same guinea pig. It was found that there was so much pig to pig variation, as far as sensitivity to the filtrate was concerned, that no reliable data could be collected concerning necrotic activity. However, the preliminary study did confirm a previously reported trend, providing that several similar reacting pigs and at least 6 replicates were used.

TABLE I

Wavelength	Energy Level (ergs per cuvette)	Mean ($\bar{x}$)	Mean ($\bar{y}_{\bar{x}}$)	Standard Deviation (s)	Mean ($\bar{s}$)
Ground Control		16.334		1.6714	
Vibration Control		17.434	17.067	1.6157	1.7399
Flight Control		17.434		1.9327	
254	4.9×10^2	17.526		2.1736	
254	1.2×10^3	18.161		1.4051	
254	3.5×10^3	17.431		1.9427	
254	1.3×10^4	17.362	17.713	2.1484	1.8221
254	3.5×10^4	18.083		1.4406	
280	3.2×10^2	16.845		2.0648	
280	7.6×10^2	18.602		2.0599	
280	1.6×10^3	15.484		1.3684	
280	8.1×10^3	17.900	17.130	1.7084	1.9265
280	1.6×10^4	16.922		2.5554	
280	6.5×10^4	17.025		1.8018	
300	4.9×10^2	18.723		1.9731	
300	9.6×10^2	17.918		2.4514	
300	4.7×10^3	18.560	18.569	3.1382	2.2821
300	9.5×10^3	19.061		2.0767	
300	3.8×10^4	18.581		1.7712	
Full	4.7×10^2	17.930		1.9960	
Full	2.5×10^3	19.092		2.3873	
Full	1.0×10^4	18.501		2.1402	
Full	2.3×10^4	19.161	18.537	1.6708	2.2425
Full	4.6×10^4	18.210		2.0894	
Full	1.5×10^5	18.327		3.1712	

TABLE II

Energy Level	Log_{10} Energy Level	Wavelength	Mean ($\bar{x}$)
3.2×10^2	2.505	280	16.845
4.7×10^2	2.672	Full	17.930
4.9×10^2	2.690	254	17.526
4.9×10^2	2.690	300	18.723
7.6×10^2	2.881	280	18.602
9.6×10^2	2.982	300	17.918
1.2×10^3	3.079	254	18.161
1.6×10^3	3.204	280	15.484
2.5×10^3	3.398	Full	19.092
3.5×10^3	3.544	254	17.431
4.7×10^3	3.672	300	18.560
8.1×10^3	3.908	280	17.900
9.5×10^3	3.978	300	19.061
1.0×10^4	4.000	Full	18.501
1.3×10^4	4.114	254	17.362
1.6×10^4	4.204	280	16.922
2.3×10^4	4.362	Full	19.161
3.5×10^4	4.544	254	18.083
3.8×10^4	4.580	300	18.581
4.6×10^4	4.663	Full	18.210
6.5×10^4	4.813	280	17.025
1.5×10^5	5.176	Full	18.327

TABLE III

Wavelength	Energy Level (ergs/cuvette)	n	Outlier Count								
			Using Control Statistics						Using Total Statistics					
			High (0.0005)	Low (0.0005)	Total (0.001)	High (0.01)	Low (0.01)	Total (0.02)	High (0.05)	Low (0.05)	Total (0.10)	High (0.0005)	Low (0.0005)	Total (0.001)	High (0.01)	Low (0.01)	Total (0.02)	High (0.05)	Low (0.05)	Total (0.10)
Ground Control		50	0	0	0	0	0	0	2	3	5	0	0	0	0	0	0	0	3	3
Vibration Control		50	0	0	0	0	0	0	3	0	3	0	0	0	0	0	0	0	0	0
Flight Control		50	0	0	0	0	0	0	4	2	6	0	0	0	0	0	0	0	1	1
254	4.9×10^2	50	0	1	1	2	1	3	3	2	5	0	1	1	0	1	1	2	2	4
254	1.2×10^3	50	0	0	0	0	0	0	3	1	4	0	0	0	0	0	0	0	0	0
254	3.5×10^3	50	0	0	0	0	0	0	2	2	4	0	0	0	0	0	0	0	2	2
254	1.3×10^4	50	0	0	0	1	0	1	4	2	6	0	0	0	0	0	0	1	1	2
254	3.5×10^4	50	0	0	0	0	0	0	2	1	3	0	0	0	0	0	0	0	1	1
280	3.2×10^2	50	0	0	0	1	0	1	2	1	3	0	0	0	1	0	1	1	1	2
280	7.6×10^2	50	0	0	0	0	0	0	10	3	13	0	0	0	0	0	0	0	3	3
280	1.6×10^3	50	0	2	2	0	2	2	0	5	5	0	2	2	0	2	2	0	3	3
280	8.1×10^3	48	0	0	0	1	0	1	4	1	5	0	0	0	0	0	0	1	1	2
280	1.6×10^4	50	0	1	1	0	1	1	4	3	7	0	1	1	0	1	1	0	3	3
280	6.5×10^4	50	0	0	0	0	0	0	1	4	5	0	0	0	0	0	0	0	4	4
300	4.9×10^2	50	0	0	0	0	0	0	12	2	14	0	0	0	0	0	0	0	2	2
300	9.6×10^2	50	0	1	1	0	1	1	6	4	10	0	1	1	0	1	1	0	2	2
300	4.7×10^3	50	0	2	2	4	2	6	12	3	15	0	1	1	0	2	2	4	3	7
300	9.5×10^4	49	0	0	0	1	0	1	8	0	8	0	0	0	0	0	0	1	0	1
300	3.8×10^4	46	0	0	0	0	0	0	7	2	9	0	0	0	0	0	0	0	2	2
Full	4.7×10^2	50	0	0	0	0	0	0	5	1	6	0	0	0	0	0	0	0	1	1
Full	2.5×10^3	50	0	2	2	1	2	3	14	2	16	0	1	1	0	2	2	0	2	2
Full	1.0×10^4	50	0	1	1	0	1	1	11	2	13	0	1	1	0	1	1	0	1	1
Full	2.3×10^4	50	0	0	0	1	0	1	10	0	10	0	0	0	0	0	0	0	0	0
Full	4.6×10^5	50	0	0	0	0	1	1	7	2	9	0	0	0	0	1	1	0	2	2
Full	1.5×10^5	50	0	4	4	4	4	8	12	5	17	0	4	4	0	4	4	0	4	4

TABLE IVA

Wavelength	Energy Level (ergs/cuvette)	n	MUTATION RATE (% of Isolates Processed)					
			Using Control Statistics			Using Total Statistics		
			0.001	0.02	0.10	0.001	0.02	0.10
Ground Control		50	0	0	10	0	0	6
Vibration Control		50	0	0	6	0	0	0
Flight Control		50	0	0	12	0	0	2
254	4.9×10^2	50	2	6	10	2	2	8
254	1.2×10^3	50	0	0	8	0	0	0
254	3.5×10^3	50	0	0	8	0	0	4
254	1.3×10^4	50	0	2	12	0	0	4
254	3.5×10^4	50	0	0	6	0	0	2
280	3.2×10^2	50	0	2	6	0	2	4
280	7.6×10^2	50	0	0	26	0	0	6
280	1.6×10^3	50	4	4	10	4	4	6
280	8.1×10^3	48	0	2.1	10.4	0	0	4.2
280	1.6×10^4	50	2	2	14	2	2	6
280	6.5×10^4	50	0	0	10	0	0	8
300	4.9×10^2	50	0	0	28	0	0	4
300	9.6×10^2	50	2	2	20	2	2	4
300	4.7×10^3	50	4	12	30	2	4	14
300	9.5×10^3	49	0	2	16.3	0	0	2
300	3.8×10^3	46	0	0	19.6	0	0	43
Full	4.7×10^2	50	0	0	12	0	0	2
Full	1.0×10^3	50	2	2	26	2	2	2
Full	2.5×10^3	50	5	6	32	2	4	4
Full	2.3×10^4	50	0	2	20	0	0	0
Full	4.6×10^4	50	0	2	18	6	2	4
Full	1.5×10^5	50	4	16	34	8	8	8

TABLE IV B

	Control (0.10)	Total (0.10)
Control	9.33	2.67
254	8.80	3.60
280	12.75	2.35
300	22.86	5.71
Full	23.67	3.33

TABLE IV C

	Control (0.02)	Total (0.02)
Control	0	0
254	1.60	0.40
280	1.68	1.34
300	3.27	1.22
Full	4.67	2.67

TABLE V

Energy Level (ergs/ cuvette)	$\log_{10}$ Energy Level	Wave-length	n	$\bar{x}$	s	Total Outlier Count					
						Control Statistics			All Statistics		
						.001	.02	.10	.001	.02	.10
3.2×10^2	2.505	280	50	16.845	2.0648	0	1	3	0	1	2
4.7×10^2	2.672	Full	50	17.930	1.9960	0	0	6	0	0	1
4.9×10^2	2.690	254	50	17.526	2.1736	1	3	5	1	1	4
4.9×10^2	2.690	300	50	18.723	1.9731	0	0	14	0	0	2
7.6×10^2	2.881	280	50	18.602	2.0599	0	0	13	0	0	3
9.6×10^2	2.982	300	50	17.918	2.4514	1	1	10	1	1	2
1.2×10^3	3.079	254	50	18.161	1.4051	0	0	4	0	0	0
1.6×10^3	3.204	280	50	15.484	1.3684	2	2	5	2	2	3
2.5×10^3	3.398	Full	50	19.092	2.3873	2	3	16	1	2	2
3.5×10^3	3.544	254	50	17.431	1.9427	0	0	4	0	0	2
4.7×10^3	3.672	300	50	18.560	3.1382	2	6	15	1	2	7
8.1×10^3	3.908	280	48	17.900	1.7084	0	1	5	0	0	2
9.5×10^3	3.978	300	49	19.061	2.0767	0	1	8	0	0	1
1.0×10^3	4.000	Full	50	18.501	2.1402	1	1	13	1	1	1
1.3×10^4	4.114	254	50	17.362	2.1484	0	1	6	0	0	2
1.6×10^4	4.204	280	50	16.922	2.5554	1	1	7	1	1	3
2.3×10^4	4.362	Full	50	19.161	1.6708	0	1	10	0	0	0
3.5×10^4	4.544	254	50	18.083	1.4406	0	0	3	0	0	1
3.8×10^4	4.580	300	46	18.581	1.7712	0	0	9	0	1	2
4.6×10^4	4.663	Full	50	18.210	2.0894	0	1	9	0	1	2
6.5×10^4	4.813	280	50	17.025	1.8018	0	0	5	0	0	4
1.5×10^5	5.176	Full	50	18.327	3.1712	4	8	17	4	4	4