Reports

Bacterial Resistance to Ultraviolet Irradiation Under Anaerobiosis: Implications for Pre-Phanerozoic Evolution

Abstract. *The concept that low concentrations of atmospheric oxygen and consequent unattenuated ultraviolet irradiation limited the emergence of Phanerozoic life, the Berkner-Marshall hypothesis, is no longer tenable. Anaerobic bacteria, which probably evolved far earlier than Metazoa, were irradiated in a special chamber under strictly anaerobic conditions. Both intrinsic resistance and photoreactivation by visible light were discovered in obligately and facultatively anaerobic microbes. Atmospheric scientists have shown that small amounts of oxygen would have limited pre-Phanerozoic surface ultraviolet irradiation to fluxes well below those used in the anaerobic experiments described. Since adequate ultraviolet protection mechanisms evolved early, the late Proterozoic appearance of Metazoa probably was not related to high fluxes of solar ultraviolet radiation.*

It is widely believed that unattenuated ultraviolet radiation during the pre-Phanerozoic aeons (from 3.5×10^9 until about 0.6×10^9 years ago) limited life to marine environments, precluding colonizations of the land. This concept, known as the Berkner-Marshall hypothesis, was developed by Berkner and Marshall (*1*, *2*) and has been widely perpetuated; for example, "typical contemporary microorganisms would be killed in a matter of seconds if exposed to the full intensity of solar radiation in this wavelength region (250 nm)" (*3*). We now present new data that challenges the Berkner-Marshall hypothesis. We question that ultraviolet light precluded occupation of terrestrial habitats at any time in the past since the early Archean (*4*).

There is no consensus concerning the quantity of atmospheric oxygen before and during the Archean aeon from about 3.8×10^9 until 2.5×10^9 years ago. Some argue that the Archean atmosphere contained reducing gases and was anoxic (*5*). Walker (*3*) suggests that the atmosphere contained oxygen, but far less than it does at present; in contrast, Dimroth and Kimberley (*6*) believe that the atmosphere contained significant quantities of oxygen, comparable to those at present. Some attenuation of potentially lethal ultraviolet irradiation would have occurred even if oxygen were at 0.01 or 0.10 its present atmospheric level, according to recent calculations (*7*) that supersede those of Berkner and Marshall (*1*). In any case, in our research we assumed the worst possible case: a complete lack of oxygen and no consequent development of a protective ozone shield.

The pre-Phanerozoic fossil record, although certainly incomplete, provides evidence for microbial life in the form of several abundant microbiotas (*8*) and stromatolites (*9*). Stromatolites, generally laminated carbonate or siliceous rocks, have been interpreted as bioorganic sedimentary structures formed by the metabolism and growth of communities, primarily filamentous cyanobacteria. The microbial growth pattern of trapping and binding of sediment to form mats is called the "matting habit." In addition, large quantities of reduced carbon in early Archean rocks probably come from microbial photosynthesis (*10*). A land microbiota comparable to today's "desert crust" communities may have existed during the pre-Phanerozoic (*11*). The abundance of pre-Phanerozoic life implies either that ozone shielded microbes or that the microorganisms had already evolved sufficient mechanisms of protection from potentially lethal ultraviolet light. Our results suggest at least the latter.

Four points argue against the Berkner-Marshall hypothesis; the first two are based on our work:

1) The existence of photoreactivation in obligate anaerobic bacteria; that is, the effects of irradiation with lethal doses of ultraviolet are reversed by visible light. This photoreactivation, as well as enzymatic ultraviolet repair processes that do not require light (dark repair), are well known for aerobic bacteria (*12*), including cyanobacteria (*13*).

2) High intrinsic ultraviolet resistance of obligate anaerobes relative to facultative anaerobes and aerobes.

The third and fourth are based on our previous experimental results and those of others (*14*, *15*).

3) Protection of microbial communities afforded by the matting habit in which organisms in the mat are protected by those at the very surface (*14*).

4) Protection of microbes by inorganic ions such as nitrate and nitrite (*14*). Because a variety of organic compounds absorb ultraviolet light at 260 to 280 nm it is likely that in solution they too afford natural protection although this point lacks experimental documentation.

Because of methodological difficulties there are no previous reports of ultraviolet sensitivity, photoreactivation, or protection in obligate anaerobic microorganisms. These were overcome by the construction of a special anaerobic glove box equipped with an ultraviolet light source in which routine bacteriological techniques could be used (*4*).

Clostridium sporogenes (*16*) was grown under strict anaerobic conditions; the gas mixture for both irradiation and incubation was 87 percent nitrogen, 10 percent carbon dioxide, and 3 percent hydrogen. In all cases, dissolved oxygen was less than 0.01 part per million (the sensitivity of the electrode) in the chamber (an Orion oxygen electrode was used as monitor). Per liter, the liquid medium contained sodium chloride, 5 g; glucose, 10 g; sodium thioglycolate, 2 g; casaminoacids, 20 g; sodium formaldehyde sulfoxylate, 1 g; and resazurin, 0.001 g, an oxygen indicator. For solid media, 20 g of agar was added and the plates were incubated in Brewer jars. The facultative anaerobes *Escherichia coli*, *Bacillus subtilis*, and *Sarcina lutea* were cultured in medium consisting of beef extract, 30 g; peptone, 5.0 g; and 15.0 g of agar added to 1.0 liter of tap water. The only obligate aerobe used for comparison, irradiated aerobically, and plated aerobically was *Pseudomonas aeruginosa*. The medium consisted of (per liter) tryptone, 10 g; peptone, 10 g; K_2HPO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 1.5 g; and agar, 15 g. All bacteria were grown at 37°C except *P. aeruginosa* which was grown at 25°C.

All the facultative anaerobes were irradiated in phosphate buffer (*p*H 7) under strict anaerobic conditions at 254 nm [General Electric (G25T8) yielding a continual fluence of 3.21 erg mm^{-2} sec^{-1}, as measured by a Kettering radiant meter]. Cell suspensions were adjusted to an optical density of 0.2 by a Klett-Somerson colorimeter in Bellco side-arm flasks; this corresponded to 6×10^7 to 7×10^7 cell/ml. The adjusted suspensions were placed in a 100-mm petri dish corresponding to a fluid depth of 1 cm. During irradiation, the cell suspension was ro-

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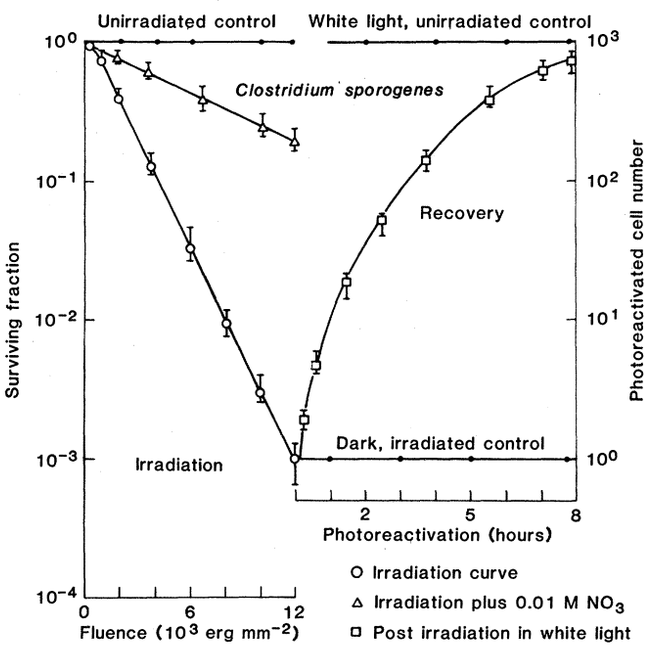
tated by an anaerobic gas jet at a rate of approximately 30 rev/min. At intervals during irradiation, samples were removed and plated on solid growth media. All results show minimum survival since liquid-holding recovery (LHR) effects were not permitted. Photoreactivating illumination after irradiation was provided by a tungsten light source at 2000 lux as measured by a Luna-Pro light meter. Colonies were counted after 4 days. The irradiations and plate counts were repeated at least three times for each species.

The amount of ultraviolet irradiation required to reduce survival of *C. sporogenes* by 99.9 percent was 1.2×10^4 erg/mm^2 (Fig. 1); *S. lutea* (Fig. 2) and *E. coli* (Fig. 3) required 3.0×10^3 erg/mm^2 and *B. subtilis* (Fig. 4) required 5.7×10^3 erg/mm^2 to reduce survival by 99.9 percent; *P. aeruginosa* required 2.08×10^3 erg/mm^2 for a similar reduction of survival (99.9 percent).

The visible light control checking for photoreactivation under anaerobic conditions are shown on the right of Figs. 1 to 4. *Clostridium sporogenes* was more than 80 percent photoreactivable, whereas all of the facultative anaerobes were



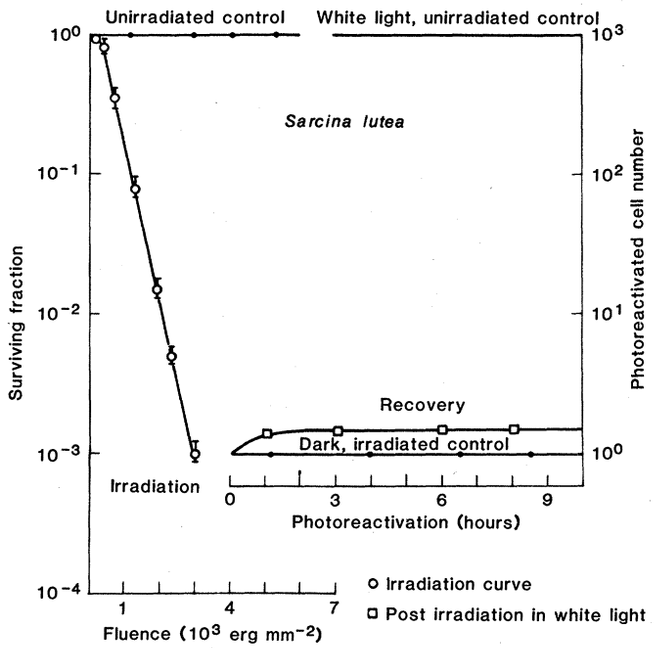
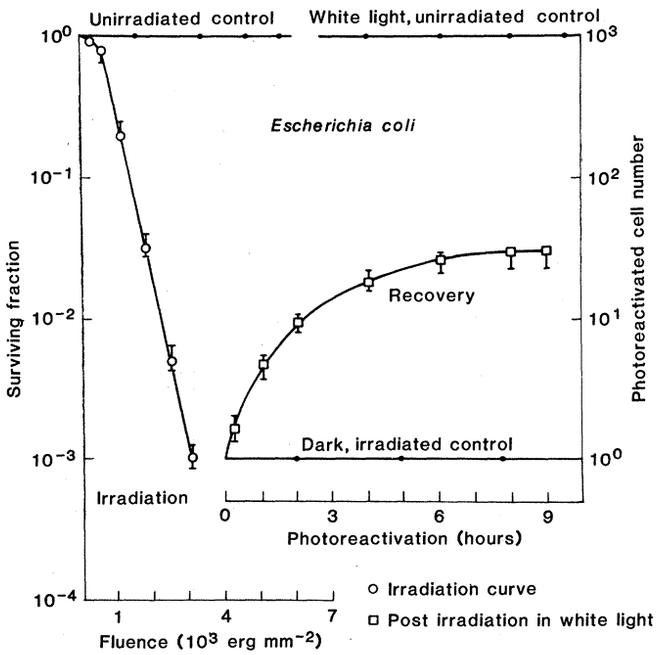


Fig. 1 (left) Inactivation of vegetative cells of *Clostridium sporogenes* that had been anaerobically irradiated with ultraviolet as a function of ultraviolet dosage; (open circles, left) in phosphate buffer; (triangles, left) with added 0.01*M* sodium nitrate; (squares) the surviving fraction of irradiated *Clostridium* after exposure to white light at 2000 lux are shown in the photoreactivation curves at the right. Fig. 2 (right). Inactivation of *Sarcina lutea* anaerobically irradiated with ultraviolet (left) and exposure to white light (2000 lux) after irradiation (right); phosphate buffer, no nitrate.



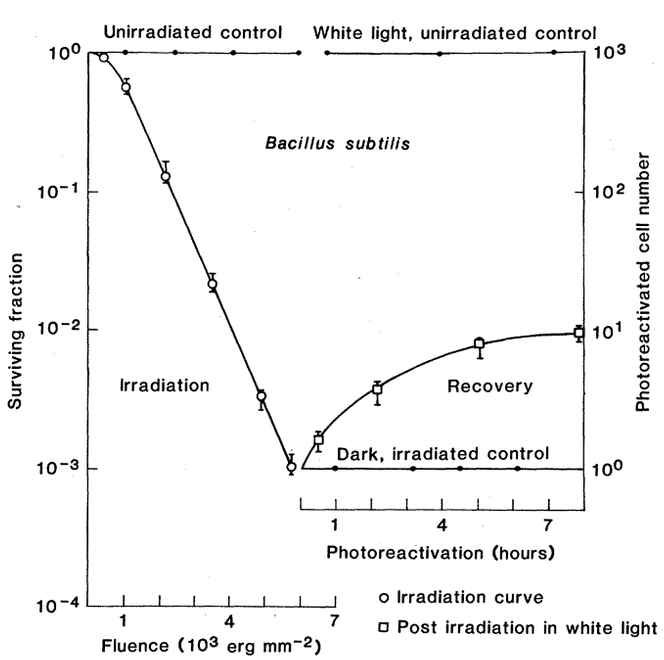


Fig. 3 (left). Inactivation of *Escherichia coli* anaerobically irradiated with ultraviolet (left) and exposure to white light (2000 lux) after irradiation (right); phosphate buffer, no nitrate. Fig. 4 (right). Inactivation of vegetative cells of *Bacillus subtilis* anaerobically irradiated with ultraviolet (left) and exposure to white light (2000 lux) after irradiation (right); phosphate buffer, no nitrate.

less than 10 percent. The reduced ability of facultative anaerobes under anaerobic conditions to photoreactivate may indicate an oxygen enhancement of the photoreactivating system. Some 80 to 90 percent photoreactivating efficiency in bacteria under aerobic conditions has been reported (*12*). *Bacillus subtilis* and the ciliate *Blepharisma*, however, show photoreactivation under anaerobic but not aerobic conditions (*15*). Photoreactivable lethal effects of far ultraviolet were the same when *E. coli* ultraviolet-sensitive mutants were irradiated under anaerobic or aerobic conditions (*12, 17*). In any case, our results show conclusively that at least one obligate anaerobe, *C. sporogenes*, is resistant to ultraviolet and can be efficiently photoreactivated by visible light.

We propose that these ultraviolet responses are legacies of pre-Phanerozoic conditions. The lack of ultraviolet attenuation during the early pre-Phanerozoic would have imposed intense selection pressure for protection and efficient repair of photodamage to macromolecules. Protection mechanisms acting together were probably available, including enzyme-mediated photorepair and photosensitive motility. In a series of experiments in which matted clumps of *Lyngbya* were irradiated with ultraviolet light, it was concluded that this growth habit afforded significant protection to cyanobacterial communities (*14*). The matting capability depends on the presence of mucilaginous sheaths of such filamentous photosynthetic organisms. These sheaths, which are poorly characterized chemically, and morphologically tend to be species-specific, seem to be necessary prerequisites for stromatolite production (*18*). Other means of protection against potentially lethal ultraviolet irradiation is fortuitous chemical absorption at about 250 nm by organic compounds, such as purines and pyrimidines (*2*), and by spore formation. Spores may be three to four orders of magnitude more resistant to ultraviolet irradiation than vegetative cells of the same species (*18*). The "oceanic skin," a surface microlayer composed of complex organics and enriched in metallic elements, may be ultraviolet protective (*19*). Nitrate salts also strongly absorb in this spectral region: in $10^{-2}M$ solutions all microorganisms tested withstood an increase of ultraviolet dosage of one to two orders of magnitude (Fig. 1, left). The irradiation dosages were chosen to be at least equivalent to those estimated from present solar intensity in the unattenuated (ultraviolet) as calculated

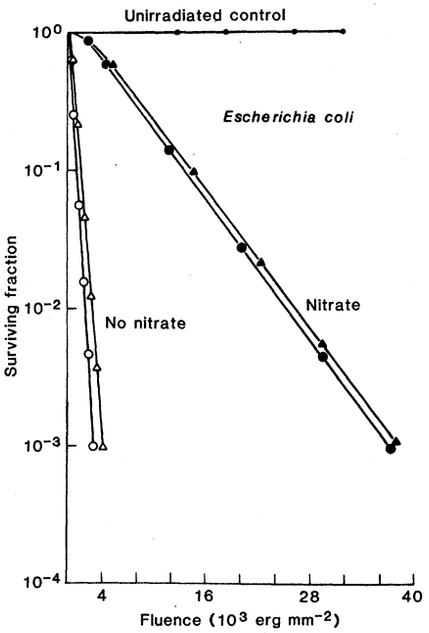


Fig. 5. Protection by sodium nitrite of *Escherichia coli* during ultraviolet irradiation. (Open circles) Inactivation of anaerobically irradiated cells; (triangles) inactivation of aerobically irradiated cells.

(*7*): a solar flux of 2.2 erg mm^{-2} sec^{-1} at a solar declination of 0° at 30° north of the equator. The flux in our experiments, 3.2 erg mm^{-2} sec^{-1}, was even higher. Furthermore most estimates of solar luminosity suggest that it was lower during the Archean than it is at present (*20*).

If all the protective mechanisms are simultaneously imposed in experiments attempting to simulate the Archean environment, both anaerobes and aerobes including oxygenic photosynthesizers survive continuous irradiation of at least 24 hours, whereas unprotected organisms die within a half hour or so. By astronomical inference, day length during the early Archean is thought to have been far shorter, 5 to 6 hours (*21*). If this is true, there would have been frequent opportunities for dark ultraviolet repair processes to operate. Furthermore there most likely has been a relaxation of selection pressure on ultraviolet resistance since the appearance of atmospheric oxygen. Thus we conclude that ultraviolet radiation did not pose a serious threat to Archean microorganisms. As today, the threat of desiccation must have been far more serious. The development of a low-lying soil terrestrial microbiota which probably existed early in the Proterozoic if not before depended on growth in moist habitats and the evolution of mechanisms of desiccation resistance such as those employed by desert crust organisms (*11*). Berkner and Marshall (*1*) devised their hypothesis at least in part to explain the late appearance of animal fossils at the Phanerozoic boundary. However, there is now a more adequate though multifactored understanding of the "late appearance" question (*22, 23*). Thus in great admiration for its boldness and integrative power we point out that on atmospheric, biological, and geological grounds the Berkner-Marshall hypothesis is no longer tenable.

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