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GENETIC MODIFICATION AND SELECTION OF MICROORGANISMS FOR GROWTH ON MARS

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Genetic engineering has often been suggested as a mechanism for improving the survival prospects of terrestrial microorganisms when seeded on Mars. The survival characteristics that these pioneer microorganisms could be endowed with and a variety of mechanisms by which this can be achieved are discussed, together with an overview of some of the potential hurdles that must be overcome. Also, a number of biologically useful properties for these microorganisms are presented that could facilitate the initial human colonisation and ultimately the planetary engineering of Mars.

1. INTRODUCTION

The planetary engineering of Mars suitable for habitation by humans is likely to be in the very minimum a two stage process [1,2]. The first stage defined by Haynes [3] is called ecopoiesis and the end point of this can be viewed as the creation of an ecosystem suitable for and filled by anaerobic and possibly micro-aerobic organisms. The second stage of planetary engineering is called terraforming and this involves the development of an oxygen/nitrogen atmosphere and a climate suitable for human habitation [1,2,4-6]. Most proposals for planetary engineering, at least during ecopoiesis, invoke the use of genetically modified or specially selected organisms [3,5,7-10] in the establishment of a Martian biosphere. However, none of the previous papers have addressed the issues of how such organisms may be constructed and what properties they require. This paper therefore introduces the concept of DNA technology (molecular biology) and focusing on microorganisms presents a preliminary description of the selection, design and engineering of such organisms for use on Mars. (Note, we do not invoke the potential but as yet unproved technology of nanotechnology [11]).

2. SELECTION OF BACTERIA FOR MARS - THE SEARCH FOR A MARSBUG

The microbial world, in contrast to that of higher organisms, is characterized by metabolic versatility that allows the colonization of an incredible range of exotic and hostile habitats. This versatility is on the one hand, a consequence of the wealth of metabolic and physiological functions found in microorganisms and on the other, a consequence of their genetic promiscuity. Thus microbes are ideal candidates as pioneer organisms for Mars. (For a generalized presentation of a bacterium see fig. 1).

A number of wild type pioneer photosynthetic microorganisms (without the need for genetic modification) have been proposed for use on Mars. Friedmann [9] proposed the cyanobacterium *Chroococcidiopsis* as a pioneer microorganism because it inhabits an unusually large range of diverse niches, including severe environments, and is thus an ideal candidate for introduction on Mars. Other microorganisms that

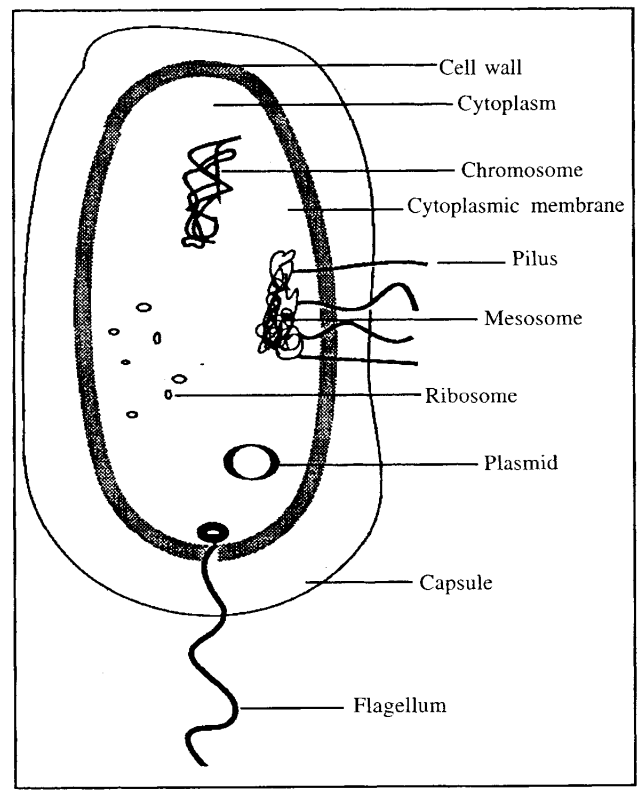

Fig. 1 A generalized representation of a bacterium. The capsule is a gel like layer that surrounds some bacteria, internal to this is a uniform dense layer called the cell wall. Between the cell wall and the cytoplasm is the cell membrane through which molecules are transported and pass. The chromosome is the main unit of genetic storage in bacteria and some species contain an additional smaller information system called a plasmid. Both the chromosome and plasmid are circular molecules and are made of DNA. Bacteria contain ribsomes which are used to synthesize proteins and some bacteria contain a flagellum which are used for active motility. Mesosomes are intracellular membranes that contain specialised functions such as nitrogen fixation.

could be utilised as candidate pioneer microorganisms are bacteria that inhabit extremes of environments, such as salt lakes and hot springs. These organisms, called extremophiles, include psychrophiles, thermophiles, halophiles and acidophiles. However, in general, extremophiles combine some metabolic adaptations to adversity that permit tolerance, with strategies that allow avoidance [12]. For example, although some extremophiles could be very resistant to drying and large temperature variations (metabolic adaptation) they might have to live under translucent stones (avoidance) to escape UV radiation.

Therefore, in choosing a microorganism for genetic adaptation one could select microorganisms from extreme environments on Earth that resemble some of those conditions expected on Mars. However, most metabolic processes within organisms fall within set physiological criteria and as discussed above, microorganisms adapted to harsh conditions are more likely to habit protected niches, rather than have evolved specialized metabolic functions. Thus, it would be a great advantage to combine the traits necessary to allow survival and growth in an unprotected environment into a pioneer microorganism [13]. Genetic modification and selection allows this possibility.

The pioneer microorganism will need to express a number of survival characteristics (phenotypes). The two most important properties (phenotypes) for a pioneer microorganism are first, they must be photoautotrophs [14] and second, anaerobic (i.e. respire without oxygen). Survival characteristics can be divided into two possible types. The first concern those functions necessary for day to day survival on a partially altered Mars. These would include (but not necessarily be limited to;

(1) osmotic (salt and heavy metal) tolerance;

(2) resistance to UV radiation;

(3) cold tolerance;

(4) tolerance to limited nutrients;

(5) tolerance to limited water and

(6) resistance to oxides and

(7) adaptation to decreased intracellular pH due to the CO_2 atmosphere.

The second type concerns periods when conditions become intolerable for survival and the genetic heritage of the microorganism must be preserved. One such mechanism to prevent this is the formation of resistant bodies known as endospores.

One candidate microorganism for the basis of genetic modification could be *Bacillus polymyxa* [15] because it has a number of properties suitable for a pioneer microorganism. First, it is a facultative anaerobe, which is able to grow with or without molecular oxygen and therefore is ideal to grow in an oxygen free environment (as in the atmosphere proposed for and during ecopoiesis). Second, it will produce endospores, which enable it to survive long periods of nutrient or moisture depravation. Third, it can fix nitrogen both aerobically and anaerobically and fourthly it exhibits heavy metal tolerance.

3. GENETIC ENGINEERING - A SIMPLE MATTER OF CUT AND PASTE

All organisms so far discovered on the Earth (apart from some viruses) use a molecule called deoxyribonucleic (DNA) to store the genetic information (genotype) that determines their phenotype (e.g. how they are shaped, control their metabolism etc.). The information is stored in the form of chemical bases, of which there are four; adenine (A), guanine (G), cytosine (C) and thymine (T). These bases are organized in groups of three, called codons. The central dogma of molecular biology is that DNA is copied to RNA (chemically similar to DNA), by cellular enzymes in a process called transcription, and the RNA is then processed by specialized cellular machinery called ribosomes to produce a protein (fig. 2). This step is called translation. The codons are arranged within a unit called a gene that determines what protein is to made. The genes are arranged in physical structures called chromosomes (all bacteria have one chromosome). Protein is composed of units called amino acids and each codon corresponds to one amino acid. Thus the genetic informa-

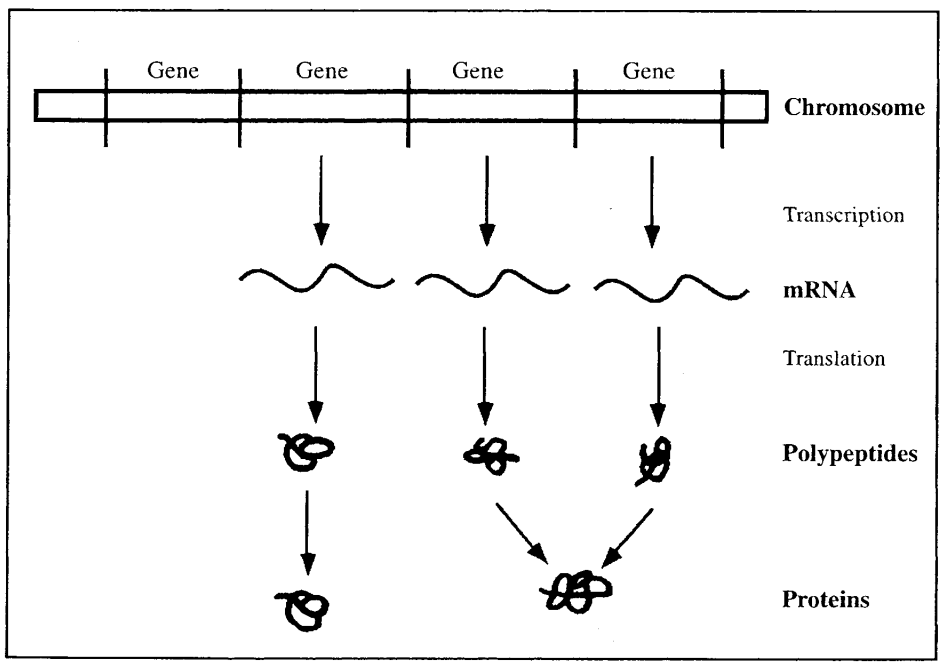

Fig. 2 The genes are arranged along a chromosome and are transcribed into messenger RNAs (mRNAs) and are then translated into polypeptides by ribosomes. A protein can be composed of one or more polypeptides, from the same or different genes.

tion is analogous to a computer programme, genes are read by ribosomes which produce proteins as output. The genetic code also contains the information that ultimately determines when a protein is made, in what quantity and for what duration etc (elements that control these processes are called promoters and enhancers). In addition special proteins called repressor proteins can interact with a promoter to reduce the level of transcription (and hence ultimately the amount of protein made).

DNA can be manipulated using special proteins. For example restriction enzymes (first isolated from bacteria) cut DNA at specific points along a DNA molecule and are therefore viewed as molecular scissors. The DNA molecule can then be joined (pasted) to other DNA molecules using an enzyme called DNA ligase. Thus, the technology is available and has been used extensively for a number of years to characterize and transfer genetic material from one organism to another. These organisms are then termed recombinant. (An excellent introduction to DNA technology and molecular biology can be found in Alberts *et al.* [16].

4. GENETIC MODIFICATION AND SELECTION

Once one or more microorganisms have been selected, one can then begin genetic modification and produce Genetically Engineered Mars Organisms (GEMOs) [13]. Bacteria have evolved powerful mechanisms both for the lateral spread of genetic information (e.g. via promiscuous plasmids and transposons) and for the inhibition of gene acquisition (e.g. by means of restriction enzymes and modification systems). Such gene transfer mechanisms enable members of microbial communities to acquire new phenotypes that provide selective advantage when environmental conditions change and hence are considered to constitute a fundamentally important mechanism for the adaptation to change. However, barriers to gene transfer are also thought to be principal mechanisms providing genetic stability and species identity.

In designing a GEMO, it would be useful to be able to raise and lower barriers to gene transfer. Engineering barriers to lateral gene spread [17] would also be of great interest in terms of increasing the ecological predictability of GEMOs intended for release on Mars. Genetic circuits to suppress the lateral spread of cloned genes from microorganism to microorganism in the environment have been developed for terrestrial bacteria [18], and this technology could be adapted for GEMOs. It may be undesirable for genes to be transferred between different GEMOs. For example this could lead to uncontrolled growth, or unforeseen genetic consequences such as the death of certain GEMOs. In experiments using barrier circuits, the transfer of a cloned gene linked to it decreased by four to five orders of magnitude. In order to prevent the undesired spread or growth of microorganisms, one could also include suicide or killing genes [19] that could remain latent and, for example, be triggered by environmental factors such as an increase in ppO_2 or infection by a bacteriophage (a virus that infects bacteria). Indeed, because bacteriophages integrate into bacterial DNA (lysogeny), they could be adapted to genetically modify bacteria in the environment on Mars.

A number of properties outlined above and described below could be incorporated into the genetic material of microorganisms to be used as GEMOs using genetic manipulation. However, one could not be certain that such new traits would not be either detrimental or of any use. Therefore, it will be necessary to test all such microorganisms under a variety of simulated Martian conditions (i.e. in Marsjars) prior to their spread into the Martian environment. Also, the employment of Marsjars would enable the organisms to become (at least partially) adapted to the Martian environment and this would be a great advantage. For example, repeated desiccation of the terrestrial cyanobacteria *Nostoc commune* resulted in an enhanced desiccation independence for photosynthesis and nitrogen fixation [20]. Thus similar experiments can be performed here and now on the Earth to investigate selection. Indeed Marsjars and growth experiments have been used to study the survival of terrestrial organisms under certain simulated Martian conditions [21-23].

Normally genetic information can be introduced in two places within a bacterium, the chromosome and/or a plasmid. (Plasmids are present in certain bacteria and represent extra chromosomal DNA; they normally code for one to a few survival characteristics, such as antibiotic resistance or heavy metal tolerance). One could design a plasmid with the entire new genetic information for the Marsbug, or integrate some of the information into the chromosome and some into the plasmid. Although plasmids are easier to genetically manipulate and the technology has been in use for many years, plasmids have a number of disadvantages, both in the laboratory and in the field. For example, they can become lost if the bacterium no longer needs them. These problems have resulted in the widespread use of an alternative genetic transfer system for engineering organisms for unconfined (i.e. non-laboratory) applications, called minitransposon vectors. Minitransposons enable very stable recombinant phenotypes to be engineered with minimal number of manipulations.

In addition to inserting heterologous genes into the chromosome of various species of bacteria, transposon vectors enable indigenous promoters to be used to express recombinant genes, even in the absence of any information on promoter structure and its regulatory activity in the native host. Of great importance for the GEMO is the establishment of regulatory pathways, which would control gene expression. For example, one can programme gene expression to be temperature sensitive, or react to light. It would be advantageous to control expression of a gene via a regulatory pathway, rather than have a gene produce a product all of the time. This is wasteful in terms of metabolites and cellular energy and could also be detrimental (e.g. certain metabolites become toxic at high concentrations). Promoters responsive to carbon, nitrogen, iron and phosphate starvation have been characterized in many bacteria and in principle could be used to activate endospore formation to protect the GEMO when such nutrients become limiting.

5. GENE EXPRESSION ON MARS

Engineering gene expression in GEMOs for Mars is not so much the problem of making well characterized promoters work away from the laboratory. Rather, it is a problem of determining which promoters are functioning in the environment in question, (e.g. low atmospheric pressure, limiting nutrients etc) and then asking whether or not they can be used to express the gene(s) of choice [13].

Another potential problem in GEMOs could be gene rearrangements, a natural property that occurs in many bacteria (for a review see [24]). Such rearrangements could cause inactivation of either the recombinant and/or wild type gene(s) in the GEMO. If the gene(s) inactivated coded for protein(s) that was essential for the GEMOs survival, then the GEMO would die and the altered genotype would not persist in the population. However, if the inactivated gene(s) were non-essential for survival then the altered genotype may persist and could come to dominate a particular population, especially if a growth advantage was conferred by the rearrangement. Whatever the

outcome, the danger would be that the GEMO might not express the phenotype that is was selected and/or modified to display.

There are a number of ways around this problem. The first would be to ensure that all the recombinant genes in the GEMO were in some way advantageous for the GEMO to have, i.e. for survival and/or growth. Thus, even if gene rearrangements were to occur, the majority of the population would express the engineered phenotype. An alternative solution would be to reduce the frequency of such rearrangements. However, gene rearrangements are one of the main mechanisms by which bacteria respond immediately to a new environment and thus it may be undesirable to inhibit or retard this process.

GEMOs released on Mars cannot be forced to do something that is incompatible with their ecological fitness and/or energetic balance. Engineering organisms for release will necessitate a considerable understanding of the way promoters involved in regulatory pathways have been assembled during evolution, thus enabling them to respond to a changing environment (as will occur during planetary engineering).

In order to monitor and trace the spread of different populations of GEMOs, for example to investigate population structure, one could mark the organisms in a number of ways (e.g. see [25] and references therein). All marker systems provide the ability to determine viable and total cell concentrations. The most important advance provided by marker systems is the ability to measure marked cell activity without the requirement for extraction of cells from the environment.

5.1 Survival properties

The endowment of GEMOs with a number of survival traits using genetic modification and selection will greatly enhance their ability to grow, reproduce and retain their genetic heritage on Mars during climatic modification. However, the climate of a partially altered Mars is still likely to be deleterious [10]. For example, desiccation, high incidence of UV radiation etc (Table 1; adapted from [2, 26-28]) and therefore the environmental effects on growth in such conditions need to be considered.

5.1.1 Tolerance to peroxides

The superoxide radical, H_2O_2 and the hydroxyl radical are highly reactive with DNA, lipids and proteins which are all components of microorganisms. Cells protect themselves against oxidant toxicity either by scavenging the oxidants with enzymes such as catalase, superoxide dismutase and reductases or, in the case of DNA damage, by repairing the damage after it has taken place. Some microorganisms are physiological able to survive some peroxide stress [29] and others have been rendered tolerant to high levels of oxidant [30]. Pretreating the bacteria with a low concentration of oxidant can protect against the effects of high concentrations of oxidant and is thought to result in the increase capacity for DNA repair. A number of proteins are involved in the response to peroxides and are controlled by a protein called OxyR, which is directly regulated by oxidation. Thus pre-treating GEMOs may render them more tolerant to oxides, which have been hypothesized to occur in the Martian soil.

5.1.2 Osmotic adaptation

Salinity and osmotic stresses have been reported to effect stress induced gene expression in different ways [31]. Salinity and osmotic stresses induce the expression of many common proteins but also proteins unique to either salt or osmotic stress. It is important that the GEMO(s) are tolerant to such stresses as these can effect cellular productivity. For example by affecting dinitrogenase, the enzyme involved in nitrogen fixation [31].

Osmotic adaptation of halophilic (salt loving) and halotolerant (salt tolerant) microorganisms requires osmotic equilibrium across the bacterial membrane and as water is freely permeable, a cytoplasm of similar osmotic strength as the surrounding medium. Two strategies of adaptation have evolved among halophilic and halotolerant microorganisms: The first is the potassium chloride (KCl) type, and second is the organic osmolyte type. Halobacteria tolerate high cytoplasmic concentrations of KCl due to specially adapted enzymes and cell structures [32,33]. Bacteria using the second strategy use a more variable adaptation, the consequence of which enables them to maintain normal enzymatic machinery.

Aerobic microorganisms have been shown to use either one of the above strategies, whereas the few anaerobic microorganisms that have been investigated (Haloanaerobium, Halobacteroides, Sporohalobacter, Acetohalobium species) all employ KCl type osmo-adaptation [34 and references therein]. Thus it may be possible for GEMOs to possess both types of systems, although only one may be necessary. As the first GEMOs will be anaerobic the KCl type will be more appropriate. Such systems can be experimentally evaluated in Marsjars prior to the introduction of GEMOs on Mars.

5.1.3 UV Resistance

UV radiation causes DNA damage and DNA is one of the main targets of UVB (280-320 nm) and UVC (less than 280 nm) radiation [35]. The incidence of UV radiation on a partially modified Mars is still likely to be high due to the lack of O_2/O_3. Although, the atmospheric pressure will be increased somewhat, it is likely that the amount of UV radiation falling on the surface will still be higher than that found on Earth (see Table 1). However, during chemical and biological evolution, microorganisms directly exposed to higher than average levels of UV

TABLE 1: *Conditions on present day Earth and Mars, and Mars after ecopoiesis and terraforming*

Parameter	Earth	Mars	Ecopoiesis	Terraforming
Insolation (Wm^{-2})	1371	593	Increase up to 1370	
Mean surface temperature (K)	288	215	280	
$ppCO_2$ (mb)	30	7	>10	
ppO_2 (mb)	210	0.006	10	>130
ppN_2 (mb)	790	.12	1-10	>300
UVflux (Wm^{-2})	None	6	Reduction	Zero

radiation have evolved three main responses to UV radiation damage; DNA repair, motility and the production of protective pigments.

DNA repair involves a number of enzymes, including the activation of photolyase, which catalyzes the repair of DNA damaged by far UV (UV-C) radiation and DNA editing enzymes. A large number of bacterial plasmids have been determined to affect cell survival and mutagenic responses to UV irradiation and have been found to contain some genes that encode UV resistance [36]. Such genes can be isolated, characterised and incorporated into the GEMO. Some of the DNA repair processes lead to high levels of mutation among surviving bacteria and this may be beneficial to GEMOs to enable them to more fully adapt to adverse or new conditions on a changing Mars. However, using genetic means, it will be necessary to ensure that the properties with which one programmes the GEMOs, for example nitrogen fixation, are not affected by this adaptation mechanism.

DNA repair has also been characterized in the extremely radio resistant bacterium *Deinococcus radiodurans* and the process of repair is thought to be extremely efficient [37; 38 and references there in]. Bacteria of these species are also extremely resistant to desiccation, and together with the fact that they are amenable to genetic manipulation would also make them ideal microorganisms to be used in the construction of a GEMO.

Microbial activities and growth habits also could have contributed to protection from high UV irradiance. Negative phototaxis (moving away from light) and phototrophic reactions (growth reactions caused by light) can cause a microorganism to move away from a damaging intense light source and thus achieve greater shielding from damaging UV irradiance. The mating habitat (layers of microorganisms) [39 and references therein] is another potential protective mechanism [7]. Cells in the interior of mat communities are protected from UV exposure by the cells in the surface layers. Following death by exposure, the surface layer of cells continues to provide UV protection to the living cells, below the mat. If the growth and replacement rates of the protected cell layers below the upper layers are fast enough to exceed the death and lysis rate of the surface most cells, the population will persist. However, the limited availability of surface water on Mars may preclude the widespread use of this growth habitat.

A number of proteins thought to be involved in shielding an organism from UV radiation have been isolated from bacteria and algae that are exposed to high fluxes of UV-A (320 to 400 nm) and UV-B radiation. Biochemical defense against this include blocking potentially harmful wavelengths with UV-absorbing compounds, which have been isolated from a variety of microorganisms including marine [40], Antarctic and desert cyanobacteria [41]. Indeed, in desert microorganisms, the UV-A/B protecting pigment has been found to be roughly 10% of the dry mass of the bacterium [41]. The genes that code for such pigments could be engineered into and expressed by pioneer microorganisms to protect them from the high incidence of UV radiation.

5.1.4 Tolerance to high intracellular acid concentrations

Because of the relatively high atmospheric partial pressure of CO_2 the interior of a GEMO is likely to be more acidic than the interior of the wild type organisms from which the GEMO was derived. Such changes in pH could affect enzyme structure and metabolic processes. For example, changes in pH of the growth media affected nitrogen fixation in the thermophilic green sulphur bacterium *Chlorobium tepidum* [42]. The GEMOs could be rendered acid tolerant using genetic manipulation. For example, an acid tolerant strain of *Rhizobium leguminosaram Biovar Trifolii* has been genetically manipulated to grow at pH 4.4 [43, 44].

5.1.5 Endospore formation

When bacteria of the species *Bacillus* encounter nutrient depravation or harsh climatic conditions they undergo morphological change (shape change) to form endospores and this process is under genetic control [45-48]. Endospores have been shown to be extremely resistance to the effects of UV radiation (although some genetic damage does occur), cosmic radiation, vacuum etc [49, 50]. When conditions become more favorable, germination occurs and the bacteria continue growing and reproducing. The genetics of endospore formation are well characterized, and if a bacteria not belonging to the *Bacillus* species is used as a GEMO, then it may be possible to manipulate the GEMO to form endospores in a manner similar to *Bacillus*. However, such manipulation has not yet been attempted on the Earth and it is likely to prove a challenge (although not impossible) given the number of control and regulation pathways involved.

Note that amongst desert and Antarctic microorganisms there are no spore formers [51]. This is because spore formation and germination are energy intensive processes and such communities have a limited availability of nutrients. When water becomes limiting, desert microorganisms tolerate desiccation and restart metabolic activity when water becomes available [51]. Such alternative survival mechanisms should also be taken into account for the early pioneer GEMOs. A dormant pioneer would have no active metabolic processes and would therefore not contribute to the planetary engineering effort [51].

However, one concern might be that the combined affects of low pressure, UV radiation and a low water potential (which would all be greater on a partially altered Mars compared to the Earth) might render the GEMO more susceptible to DNA damage. Although, such damage could be offset by an increased DNA repair capability, there would be biochemical and genetic limits to this capability. Therefore spore formation as a mechanism of avoiding such an unfavorable combination of climatic parameters may offer a protective mechanism for early GEMOs. Once the climate becomes more clement (i.e. an increase in atmospheric pressure with a concomitant decrease in UV radiation), then desiccation tolerance would be an excellent mechanism in GEMOs for avoiding a lower water potential, without the necessity of expending metabolites on an energy intensive mechanism such as spore formation. Laboratory experiments will be needed to investigate such physiological and metabolic parameters to decide in the selection of the most appropriate GEMO(s).

5.2 General properties

Although the GEMOs are likely to require the above survival traits, they can also possess a number of other useful properties. For example, photosynthesis, nitrogen fixation and denitrification etc. Three such properties that could be conferred or enhanced in GEMOs are outlined below.

5.2.1 Photosynthesis

Plants and photosynthetic bacteria gather and utilize sunlight by employing specialized cellular components and machinery. At present on Earth, photosynthesis is an integral part of the ecosystem. Because of the photosynthetic process, atmospheric carbon dioxide is recycled every 300 years and oxygen every

2000 years [71]. A greater understanding of photosynthesis will be required for Mars [10], where the amount of insolation (sun light) is less than half that of Earth.

Many diverse pigments are used by photosynthetic organisms to efficiently harvest the spectrum of energy derived from the sun that drives photosynthesis. In the prokaryotic cyanobacteria and the eukaryotic red algae, light harvesting is carried out by a group of pigmented proteins, called phycobiliproteins, that become constituents of a macromolecular complex called the phycobilisome (PBS). The primary constituents of the PBS may be produced in massive amounts in a number of photosynthetic microorganisms and may constitute 50% of the soluble protein of the cell. Photosynthesis has been extensively studied in photosynthetic bacteria and a number of genetic mechanisms are available for genome manipulation [for a review see 52].

Light harvesting systems are also able to respond to unpredictable changes in illumination. A 100-fold fluctuation in solar radiation can occur within seconds, often out of phase with the biochemical requirements of the organism. Excess light can lead to photo-inactivation and destruction. Mechanisms therefore exist to regulate light absorption and to repair damaged reaction centers. Such mechanisms would prove beneficial if the insolation on Mars is increased using solettas [72] or undergoes large fluctuations, perhaps during unexpected climatic alterations.

Also the PBS can be utilized as a store of essential nutrients during starvation. General responses to nutrient limitation include changes in both cellular morphology and physiology. In response to the depletion of nutrients the PBS can be destroyed, which would not be ultimately beneficial to the GEMO (although this is more preferable than cell death). However a protein called zeaxanthin may offer limited protection in nutrient limited cells and production of this molecule could be triggered when the GEMO encounters harsh conditions.

5.2.2 *Nitrogen fixation*

The utilization of nitrogen gas as a source of nitrogen is called nitrogen fixation [for a review see 53] and is a property of only certain free living aerobic and anaerobic bacteria. In addition, symbiotic bacteria fix nitrogen only when present in nodules or on roots of specific host plants. As far as is currently known, no eukaryotic organism can fix nitrogen in nature.

The biological process of nitrogen fixation is catalyzed by an enzyme called dinitrogenase (or just nitrogenase) and as the name suggests consists of two protein components [54, 55]. This enzyme has been purified from a large number of nitrogen fixing organisms. The entire nitrogen fixing (*nif*) region (comprised of at least 15 genes) has been assembled in foreign hosts (who do not constitutivly express dinitrogenase) and some of these hosts have been shown to exhibit nitrogen fixation. Therefore, as well as bacteria one might be able to modify a lower eukaryote such as yeast for use as a GEMO to carryout nitrogen fixation.

Nitrogen fixation is under environmental and ultimately genetic control and the coordinate expression of the *nif* genes is controlled at two levels (in some bacteria). The machinery of the first level, the nitrogen regulation gene (*ntr*) system senses and responds to the intracellular concentration of fixed nitrogen [56]. The second level of control is encoded by one of the ntr regulated gene units. This unit activates transcription [57]. In contrast to this, cyanobacteria do not have similar control genes and instead employ a different set of nitrogen control (*ntc*) genes [58].

A useful lesson to be learned at this stage is that other proteins and gene systems may be required for the effective expression of the cloned genes. For example, proteins called chaperonins may be required for functional/efficient nitrogen fixation [59]. The chaperonin proteins GroEL and GroES are thought to play a role in the solublization (dissolving in water) and folding of the *nif* transciptional activator. Without this activator nitrogen fixation would not occur.

The amount of nitrogen present in the Martian atmosphere under any planetary engineering scenario so far discussed will need to be increased, so nitrogen fixation will probably only be required in the long term to set up a biological nitrogen cycle [10].

5.2.3 *Denitrification*

One of the main problems in the creation of a Martian biosphere suitable for human habitation will be the presence of a buffer gas such as nitrogen [4]. The partial pressure of nitrogen on Mars is very low (Table 1) and several mechanisms have been proposed to increase the partial pressure of this gas [1, 2, 5]. The conversion of an anaerobic biosphere (i.e the end point of ecopoiesis) into an aerobic biosphere is likely to be a long process compared to the establishment of an anaerobic biosphere. Therefore, one mechanism to facilitate an increase in ppN_2 over time would be to use microorganisms that can convert nitrate deposits into N_2 [10]. A similar mechanism, again using microorganisms, has been proposed by Friedmann *et al.* [60] to release CO_2 from carbonate deposits. Using genetic modification and selection, these properties could be made more efficient and additional alterations could increase survival of such GEMOs early in ecopoiesis [13].

The ability to grow anaerobically by reducing ionic nitrogenous oxides to gaseous products is distributed among a diverse number of eubacteria [61] and in the extremely halophilic branch of the Archaebacteria [62]. This respiratory process, in which nitrogen oxides serve as electron acceptors results in the concomitant generation of ATP (an energy containing molecule) and is designated denitrification or dissimilatory nitrate reduction [63]. (As opposed to assimilatory N-reduction in which nitrates are converted to ammonium and then to amino acids).

The enzymes associated with denitrification are synthesized under anaerobic conditions or when conditions become anaerobic, although denitrification can occur in the presence of oxygen [64]. In some cases, enzyme induction may even require low concentrations of oxygen [65]. The nitrate reductases associated with denitrification and respiration (i.e. dissimilation) are, with one exception, membrane bound enzymes that catalyze the reduction of nitrate to nitrite and couple this reduction to the translocation of protons. The enzymes require many compounds, for example molybdenum. Such components, called co-factors, are usualy required for enzyme specificity or activation.

Certain purple nonsulphur photosynthetic bacteria, which ordinarily carry out the assimilatory reduction of nitrate, also reduce nitrate to nitrite. These include *Rhodopseudomonas sphaeroides* f. sp. *denitrificans*, which also reduces nitrite to dinitrogen [66].

6. USES OF GEMOS AND SOME SPECULATIONS

Initially GEMOs could be employed by the first Martian colonists either in greenhouses [67] and/or to aid with self contained biological life support systems. For the planetary engineering of Mars the modified microorganisms could be employed to digest mineral deposits, for example carbonates

[60], hypothesized to occur in the Valles Marineris [68] and release the CO_2. Such sites could possibly be determined from orbit and would thus aid in the distribution of GEMOs [13]. For example, detection of carbonate deposits from orbit using thermal neutrons [69]. GEMOs could also be used in the establishment of biological cycles, for example biological carbon, sulphur, phosphorus and nitrogen cycles [10], perhaps also providing the organic matter that other organisms could then utilize. Genetic modification could also be used to improve the growth rate of endolithic microorganisms that have been described in the dry valleys of Antarctica [70] and proposed by Friedmann [9] as pioneer microorganisms. Such adapted organisms would have extremely favorable prospects of survival and more importantly growth, under potentially deleterious climatic conditions expected on a partially altered Mars.

7. CONCLUSIONS

The introduction of microorganisms on Mars will greatly facilitate colonization, both during initial attempts and in establishment of a stable ecosystem, either in enclosed habitats or at the end of ecopoiesis or terraformation. During the initial stages of ecopoiesis climatic conditions on Mars will be limiting for most terrestrial microorganisms. By using genetic modification and directed selection under simulated Martian conditions, it may be possible to greatly enhance the survival capability of microorganisms during the alteration of the Martian climate to more clement conditions. Such microorganisms could be used to facilitate any planetary engineering effort. For example, they could be used to release CO_2 and N_2 from putative carbonate and nitrate deposits.

The genetic alteration of microorganisms will not be so much of a problem of introducing foreign genes into the organism but more a matter of understanding and controlling the regulatory pathways for the expression of such genes. However, such understandings will provide valuable insights into genetics, not only for increasing the productivity of microorganisms on Mars but possibly also for Earth.

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REFERENCES

1. C.P. McKay, O.B. Toon and J.F. Kasting, "Making Mars habitable," *Nature*, **352**, 489-496 (1991).
2. M.J. Fogg, "A synergic approach to terraforming Mars," *JBIS*, **45**, 315-329 (1992).
3. R.H. Haynes, "Ethics and planetary engineering. 1. Ecce ecopoiesis: Playing God on Mars," in D. Macniven (Ed), *Moral Expertise*, 161-183. Routledge, London and New York (1990).
4. M.J. Fogg, "The creation of an artificial dense Martian atmosphere: A major obstacle to the terraforming of Mars," *JBIS*, **42**, 577-582 (1989).
5. J.B. Pollack and C. Sagan, "Planetary engineering," in J.Lewis and M. Matthews, (Eds), *Resources of Near Earth Space*, Univ. Arizona Press (1991).
6. R.M. Zubrin and C.P. McKay, "Technological requirements for terraforming Mars", *AIAA* **93-2005**, 1-13 (1993).
7. W.R. Kuhn, S.R. Rogers and R.D. MacElroy, "The response of terrestrial organisms to the Martian environment: A modeling study," *Icarus*, **37**, 336-346 (1979).
8. C.P. McKay and R.H. Haynes, "Should we implant life on Mars?," *Scientific American*, **263**, 144 (1990).
9. E.I. Friedmann and R. Ocampo-Friedmann, "A primitive cyanobacterium as pioneer microorganism for terraforming Mars," *Adv.Space Res.*, **15**, 243-246 (1994).
10. D.J. Thomas, "Biological aspects of the ecopoiesis and terraformation of Mars: Current perspectives and research," *JBIS*, this issue (1995).
11. M.D. Nussinov, S.V. Lysenko and V.V. Patrikeev, "Terraforming of Mars through terrestrial microorganisms and nanotechnological devices," *JBIS*, **47**, 319-320.
12. M.J. Fogg, personal communication (1995).
13. J.A. Hiscox, "Modification of microorganisms for Mars," *The Terraforming Report*, in press (1995).
14. R.H. Haynes and C.P. McKay, "The implantation of life on Mars: Feasibility and motivation," *Adv.Space Res.*, **12**, 133-140 (1992).
15. P.A. Rusin, J.E. Sharp, K.L. Oden, R.G. Arnold and N.A. Sinclair, "Isolation and physiology of a manganese-reducing *Bacillus polymyxa* from an Oligocene silver bearing ore and sediment with reference to Precambian biogeochemistry," *Precambrian Research*, **61**, 231-240 (1993).
16. B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts and J. Watson, *Molecular Biology of the Cell*, 2nd Edition. Garland Publishing. New York (1989).
17. S.E. Lindow, N.J. Panopoulus and B.L. McFarland, "Genetic engineering of bacteria from managed and natural habitats," *Science*, **244**, 1300-1307 (1989).
18. M. Herrero, V. de Lorenzo and K.N. Timmis, "Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria," *J. Bact.*, **172**, 6557-6567 (1990).
19. S. Molin, L. Bue, L.B. Jensen, C.S. Kristensen and M. Giuskov, "Suicidal genetic elements and their use in biological containment of bacteria," *Ann.Rev. Micro.*, **47**, 139-166 (1993).
20. S. Scherer and Z.-P. Zhong, "Desiccation independence of terrestrial Nostoc commune ecotypes (Cyanobacteria)," *Microbial Ecology*, **22**, 271-283 (1991).
21. S.M. Siegel, L.A. Rosen and C. Giumorro, "Effects of reduced oxygen tension on vascular plants. IV. Winter rye germination under near Martian conditions and in other non terrestrial environments," *PNAS*, **48**, 725-728 (1962).
22. A.A. Imshenetsky, N.F. Pisarenko, L.A. Kuziurina and V.M. Yakshina, "Physiology of xerophytic micro-organisms growing under Martian conditions," *COSPAR*, **XV**, 47-52 (1976).
23. D.M. Moll and J.R. Vestal, "Survival of microorganisms in Smectite clays: Implications for Martian exobiology," *Icarus*, **98**, 233-239 (1992).
24. R. Haselkorn "Developmentally regulated gene rearrangements in prokaryotes," *Ann.Rev.Gen.*, **26**, 113-130 (1992).
25. J.I. Prosser, "Molecular marker systems for detection of genetically engineered microorganisms in the environment," *Microbiology*, **140**, 5-17 (1994).
26. T.R. Meyer and C.P. McKay, "The resources of Mars for human settlement," *JBIS*, **42**, 147-160 (1989).
27. P. Catermole, Mars, *The Story of the Red Planet*, Chapman and Hall, London (1992).
28. M.J. Fogg, "Terraforming: A review for environmentalists," *The Environmentalist*, **13**, 7-17 (1993).
29. A.A. Arrage, T.J. Phelps, R.E. Benoit and D.C. White, "Survival of subsurface microorganisms exposed to UV radiation and hydrogen peroxide," *Appli. Environ. Micro.*, **59**, 3545-3550 (1993).
30. O.M. Hartford, and B.C.A. Dowds, "Isolation and characterization of a hydrogen peroxide resistant mutant of *Bacillus subtilis*," *Microbiology*, **140**, 297-304 (1994).
31. T.A. Fernandes, V. Iyer and S.K. Apte, "Differential responses of nitrogen fixing cyanobacteria to salinity and osmotic stresses," *Appli. Environ. Micro.*, **59**, 899-904 (1993).
32. H. Eisenberg and E.J. Wachtel, "Structural studies of halophilic proteins, ribosomes, and organelles of bacteria adapted to extreme salt concentrations," *Ann. Rev. Biophys. Biophys. Chem.*, **16**, 69-92 (1987).
33. H. Stan-Lotter, M. Sulzner, E. Egelseer, C.F. Norton and L.I. Hichstein, "Comparison of membrane boundATPases from extreme halophiles isolated from ancient salt deposits," *Orig. Life and Evol. Biosphere*, **23**, 53-64 (1992).
34. B. Ollivier, P. Caumette, J.-L. Garcia and R.A. Mah, "Anaerobic bacteria from hypersaline environments," *Microbiol. Rev.*, **58**, 27-38 (1994).
35. J.M. Paramio, C. Bauluz and R. De Vidania, "Comparative effects of near-

UV light (360 nm) and 8-methoxypsoralen plus near-UV on plasmid DNA," *Cell Mol. Bio.*, **37**, 125-137 (1991).
36. P. Strike and D. Lodwick, "Plasmid genes affecting DNA repair and mutation," *J.Cell Sci. Supplement.* **6**, 303-321 (1987).
37. M.J. Daly, L. Ouyang, P. Fuchs and K.W. Minton, "In vivo damage and recA-dependent repair of plasmid and chromosomal DNA in the radiation-resistant bacterium *Deinococcus radiodurans*." *J. Bact .*, **176**, 3508-3517 (1994).
38. K.W. Minton, "DNA repair in the extremely radio resistant bacterium Deinococcus radiodurans," *Mol. Micro.*, **13**, 9-15 (1994).
39. B.K. Pierson, V.M. Sands and J.L. Frederick, "Spectral irradiance and distribution of pigments in a highly layered marine microbial mat," *Appli. Environ. Micro.*, **56**, 2327-2340 (1990).
40. J.M. Shick,W.C. Dunlap, B.E. Chalker, A.T. Banaszak andT.K. Rosenzweig, "Survey of ultraviolet radiation-absorbing mycosporine-like amino acids in organs of coral reef holothuroids,"*Marine Ecol. Progress Series* **90**, 139-148 (1992).
41. S. Scherer, T.W. Chen and P. Büger, "A new UV-A/B protecting pigment in the terrestrial cyanobacterium*Nostoc commune*," *Plant Physiol.*, **88**, 1055-1057 (1988).
42. T.M. Wahlund and M. T. Madigan. "Nitrogen fixation by the thermophilic green sulfur bacterium *Chlorobium tepidum*," *J. Bact.*, **175**, 474-478 (1993).
43. H. Chen, A.E. Richardson, E.Gartner, M.A. Djordjevic, R.J. Roughley and B.G. Rolfe, "Construction of an acid tolerant *Rhizobium leguminosaram Biovar Trifolii* strain with enhanced capacity for nitrogen fixation" *Appli.Environ.Micro.*, **57**, 2005-2011 (1991).
44. H. Chen, A.E. Richardson and B.G. Rolfe, "Studies of the genetic basis of acid tolerance in*Rhizobium leguminosaram Biovar Trifolii*,"*Appli.Environ. Micro.*, **57**, 2005-2011 (1993).
45. J.A. Hoch, "Regulation of the phosphorelay and the initiation of sporulation in *Bacillus subtilis*," *Ann.Rev.Micro.*,**47**, 441-465 (1993).
46. J.D. Chung, G. Stephanopoulos, K. Ireton and A.D. Grossman, "Gene expression in single cells of *Bacillus subtilis*: Evidence that a threshold mechanism controls the initiation of sporulation,"*J. Bact.* **176**, 1977-1984 (1994).
47. M. Pedraza-Reyes, F. Gutiërrez-Corona and W.L. Nicholson, "Temporal regulation and forespore-specific expression of the spore photoproduct lyase gene by sigma-G RNA polymerase during Bacillus subtilis sporulation," *J. Bact.*, **176**, 3983-3991 (1994).
48. J. Zhang, H. Ichikawa, R. Halberg, L. Kroos andA.I.Aronson, "Regulation of the transcription of a cluster of *Bacillus subtilis* spore coat genes," *J. Mol. Biol.*, **240**, 405-415 (1994).
49. C. Lindberg and G. Horneck, "Action spectra for survival and spore photoproduct formation of*Bacillus subtilis* irradiated with short-wavelength (200-300 nm) UV at atmospheric pressure and in vacuo," *J. Photochem. Photobiol.*, **11**, 69-80 (1991).
50. J. Koike, T. Oshima, K.A. Koike, H.Taguchi, R. Tanaka, K. Nishimura and M. Miyaji, "Survival rates of some terrestrial microorganisms under simulated space conditions," *Adv. Spa. Res.*,**12**, 271-274 (1992).
51. E.I. Friedmann, personal communication (1995).
52. P.A. Scolnik and B.L. Marrs, "Genetic research with photosynthetic bacteria". *Ann. Rev. Micro.*, **41**, 703-726 (1987).
53. E.W. Triplett, G.P. Roberts, P.W. Ludden and J. Handelsman, "What's new in nitrogen fixation," *ASM News*, **55**, 15-21 (1989).
54. D.R. Dean, J.T. Bolin and L. Zheng, "Nitrogenase metalloclusters: Structures, organisation and synthesis," *J. Bact.*, **175**, 6737-6744 (1993).
55. J. Kim and D.C. Rees, "Nitrogenase and biological nitrogen fixation," *Biochemistry*, **33**, 387-397 (1994).
56. B. Magasanik, "Genetic control of nitrogen assimilation in bacteria,"*Ann. Rev. Gen.*, **16**, 135-168 (1982).
57. D.W. Ow and F. M. Ausubel, "Regulation of nitrogen metabolism genes by *nif*A gene product in *Klebsiella pneumoniae*," *Nature*, **301**, 307-313 (1983).
58. I. Luque, E. Flores andA. Herrero, "Molecular mechanism for the operation of nitrogen control in cyanobacteria," *The EMBO Journal*, **13**, 2862-2869 (1994).
59. D. Govezensky, E.S. Bochkareva, A. Zamir and A.S. Girshovich, "Chaperonins as potential gene regulatory factors" *J. Biol. Chem.*, **269**, 14003-14006 (1994).
60. E.I. Friedmann, M. Hua and R. Ocampo-Friedmann, "Terraforming Mars: Dissolution of carbonate rocks by cyanobacterium," *JBIS*, **46**, 291-292 (1993).
61. R.M. Jeter and J.L. Ingraham, "The denitrifying prokaryotes," In*A Handbook on Habitats, Isolation, and Identification of Bacteria*, Ed. M. P. Starr, H. Stolp, H. Trnper, A. Balows, H. G. Schlegel, 913-925. New York: Springer-Verlag (1981).
62. L.I. Hochstein and G.A.Tomlinson, "Denitrification by extremely halophilic bacteria," *FEMS Micro.Lett.*, **27**, 329-31 (1985).
63. W.J. Payne, "Reduction of nitrogenous oxides by microorganisms," *Bact. Rev.*, 37, 409-452 (1973).
64. L.D. Boody and K.J.P. Davis, "Persistence of bacterial denitrification capacity under aerobic conditions-the rule rather the exception," *FEMS Micro. Ecol.*, **45**, 185-190 (1987).
65. W.J. Payne, P.S. Ripley and C.D. Cox, "Separate nitrite, nitric oxide, and nitrous oxide reducing fractions from *Pseudomonas perfectomarinus*," *J.Bact.* **106**, 356-361 (1971).
66. B. Kundu and D.J.D. Nicholas, "Denitrification in *Rhodopseudomonas sphaeroides* f. sp. *denitrificans*," *Arch. Micro.*, **141**, 57-62 (1985).
67. C. Lindberg and G. Horneck, "Planetary protection considerations for Marsnet and Mars sample return missions," *Adv.Spa. Res.*, **15**, 277-280 (1994).
68. C.P. McKay and S.S. Nedell, "Are there carbonate deposits in the Valles Marineris, Mars?," *Icarus*, **73**, 142-148 (1988).
69. W.C. Feldman and B.M. Jackosky, "Detectability of Martian carbonates from orbit using thermal neutrons," *J. Geophys. Res.*, **96**, 15589-15598 (1991).
70. E.I. Friedmann, "Endolithic microorganisms in the Antarctic cold desert," *Science*, **215**, 1045-1053 (1982).
71. J. Barber and B. Anderson, "Revealing the blueprint of photosynthesis," *Nature*, **370**, 31-34 (1994).
72. P. Birch, "Terraforming Mars quickly," *JBIS*, **45**, 331-340 (1992).

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