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Life and death of dried prokaryotes

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Abstract

The removal of water through air drying damages membranes, proteins and nucleic acids and is lethal to the majority of organisms. Nevertheless, some vegetative cells of bacteria and cyanobacteria survive extreme desiccation. Understanding the mechanisms of their desiccation tolerance is an important issue in cell biology and holds promise for the metabolic engineering of desiccation-sensitive cells.

Keywords: Anhydrobiosis; Desiccation tolerance; Water stress; Anhydrobiotic engineering

1. Introduction

Desiccation tolerance is the ability of cells to undergo nearly absolute dehydration through air drying, without being killed. This is the most severe water deficit stress since the removal of the cell-bound water imposes such structural, physiological and biochemical stresses that cells must adapt or die [6,33]. There may have been strong evolutionary pressure to select mechanisms to withstand such multiple cycles of drying and wetting, and/or prolonged desiccation; conditions that may have constituted a major barrier to the distribution and activity of primitive cells [6]. Ways to withstand extreme desiccation evolved in phylogenetically diverse organisms, including tardigrades, namatodes, cysts of crustaceans, yeast cells, and higher and lower plants [12]. Upon drying, these organisms, called anhydrobiotes, enter metabolic dormancy and resume active metabolism when water becomes available. Spores and akinetes are resistant structures developed by bacteria and cyanobacteria, respectively, and are characterized by reduced water content and undetectable metabolic activities [1,30]. These structures have a greater capacity than their growing counterparts to survive periods of adverse conditions, including extreme desiccation [1,30]. However, to decipher desiccation tolerance of prokaryotes, the topic is not the resistance of dormant forms, but rather the tolerance to extreme desiccation of vegetative cells. Until now, relatively few genera of bacteria were recognized as anhydrobiotic; many of which are in the Cyanobacteria.

To highlight the mechanisms of desiccation tolerance it is necessary to answer the following questions. How dry is an anhydrobiotic cell? How can some bacterial species cope with water deficits while others cannot? How long can cells remain viable in the air-dried state? Understanding the mechanisms of desiccation tolerance holds promise not only because it may solve an important problem in cell biology but also because it may find biotechnological applications in conferring desiccation tolerance on otherwise desiccation-sensitive microorganisms. The achievement of the stabilization of dried cells will allow for the preparation of biosensors and storage of laboratory clones and strains used in the manufacture of food.

2. Desiccation tolerance vs. osmoadaptation

Though air drying (matric stress) and hypertonicity result in the efflux of cellular water, they represent two different stresses. The immediate environment of air-dried cells under matric stress is the atmosphere, while that of cells under osmotic stress is an aqueous solution, albeit one of diminished water potential [33]. Even the water deficit experienced by extreme halophilic bacteria is considerably less than that imposed upon anhydrobiotic cells in which the removal of all but 0.1 g water/g dry weight is easily achieved [6]. Desiccation-sensitive bacteria die when

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the cellular water content is reduced to 0.3 water/g dry weight [34]. The water contents measured for spores and akinetes, 21–58% and 63%, respectively, suggest that these cell types are not anhydrobiotic [33]. In truly desiccated cells the residual water is not even sufficient to maintain a monolayer of water around the macromolecules, without which metabolic enzyme-catalyzed reactions are obviously not possible. Hence, desiccated cells do not grow and the time they remain air-dried may represent the greater part of their life.

Faced with osmotic stress, microorganisms have two options to counterbalance the extracellular osmotic pressure and maintain cellular functions [39]. Halophiles allow the influx of inorganic ions, chiefly K^+ ions, as they develop macromolecules to cope with or even take advantage of the high salt concentration accumulated by halophiles when there exists high extracellular osmotic pressure [39]. The majority of prokaryotes accumulate compatible solutes, such as K^+ ions, glutamate, glutamine, proline, trehalose, glycine betaine, and glucosylglycerol [39]. Compatible solutes favor the maintenance of the native state of proteins by being preferentially excluded from their hydration shell, and determining a thermodynamically unfavorable state which would become even more unfavorable if the protein unfolded to provide more exclusion domains [2,8]. Thus, during extreme desiccation, mechanisms other than preferential exclusion are required to achieve stabilization of macromolecules which have been stripped of solvent monolayer [2]. A common feature of anhydrobiotes is the presence of high concentrations of trehalose or sucrose, two disaccharides which replace cell water (see below). Responses to osmotic stress can, however, be considered as a consequence of the initial stages of slow air drying, when water activity is sufficient to allow a certain degree of growth, and cells can achieve a water balance through osmotic adjustment.

The extreme water deficit experienced by desiccated prokaryotes cannot be reproduced through the addition of variable amounts of salt to the growth medium, as high ionic concentrations are toxic to cells [33]. Cells can be exposed to various degrees of controlled matric water stress, using a solution of defined water potential. An experimentally convenient system can be constructed by placing the cells in an enclosure in close proximity to agar that has been amended with the appropriate concentration of solute [33]. The immobilization of cells on inert supports, such as filter paper or nylon filters, and their desiccation through air drying under sterile air or using chemical desiccants such as silica gel or phosphorous pentoxide afford the means to achieve rapid drying and storage of microorganisms [7,33]. Alternatively, cells can be preserved through freeze-drying, and even though lyophilization does not simulate the stress imposed by air drying, trehalose, unlike other compatible solutes, provides protection to both air drying and freeze drying [12].

3. Desiccation-induced damage

Water is required for the establishment of cellular microenvironments that influence the physiological properties of macromolecular systems and membranes, such as folding and assembly of proteins, regulation of gene expression, conservation of membrane structure and function.

Reduced hydration of proteins and their consequent conformational changes are expected to cause severe dysfunction in enzymes and /or electron transport chains, which leads to the accumulation of free radicals, and subsequently lipid peroxidation, protein denaturation, and mutation of DNA [33]. The effects of reactive oxygen species are exacerbated in organisms capable of oxygenic photosynthesis [34]. Here the removal of water generates reactive forms of oxygen as a consequence of the disruption of the photosystems and an impairment between the reaction initiated by light and the photosynthetic electron transport chain. The removal of the hydration shell from phospholipids of membrane bilayers increases the van der Waal's interactions between adjacent lipids, causing an increase in the phase transition temperature (T_m) of membranes and their transition to the gel phase at environmentally relevant temperatures. Membranes with a higher T_m will pass to the gel phase first and separate from those with lower T_m, which results in the aggregation of proteins as they become excluded from various domains. After rehydration membranes undergo a further phase transition leading to leakage and loss of solutes from cells [12].

Nucleic acids are clearly a prime target of desiccation-induced damage. Damage to DNA may arise through chemical modification (alkylation or oxidation), cross-linking, base removal such as depurination, or damage by ionizing and UV radiation [6,33]. The effects of these stressors are greatest when nucleic acids are in the fully hydrated state, but damage occurs even under reduced water content. For example, the genome of a *Bacillus* spore suffers around 50 breaks during 3 weeks of exposure to vacuum, and even the radiation-resistant *Deinococcus radiodurans* accumulates DNA single-strand breaks during a few days of exposure to vacuum [3].

4. Survival of dried prokaryotes

The ability to revive after air drying is not only crucial for microbial life in soil habitats, but it is also an important issue in human health. *Mycobacterium tuberculosis* remains viable for around 1 week when dried as an aerosol on glass in physiological saline, while in dust, tubercle bacilli remain viable for 120 days and the viability is extended to 2 years if stored under vegetable oil [33]. The maximal longevity of microorganisms in the air-dried state is unknown [33], but there have been controversial [16] reports of ancient, yet viable bacterial cells in 25–35-million-year-old Dominican amber [26]. A database of reports and records of microorganisms revived from the sea-bed, rocks, salt deposits,

permafrost, bricks from ancient temples, herbaria, etc. was established [25]. The collection contains the accounts of over 500 microorganisms being revived after storage greater than 50 years, and many after millions of years. The problem when evaluating such claims is that it is hard to assess whether these microorganisms ever experienced one or more rehydration events during the presumptive period of drying. In comparing the survival of laboratory-dried bacteria, difficulties arise because many factors influence survival after drying, including the modality of drying, the growth conditions, the cell concentration, the physiological state of the cells and the storage conditions [6,33].

Among nonspore-forming bacteria, *D. radiodurans* is well known for its ability to survive not only high doses of ionizing and UV radiation but also extreme desiccation [28]. Indeed, the capacity to resist the lethal effects of ionizing radiation has been proposed to have its evolutionary origin in desiccation tolerance. In cold and hot deserts cyanobacterial communities dominate the bacterial population in different habitats ranging from soil crust, exposed rocked faces or the inner space of porous rock [31,34]. Many desiccation-resistant communities of cyanobacteria include forms that survive high doses of ionizing radiation [5,34]. Air-dried colonies of the filamentous cyanobacterium *Nostoc commune* are conspicuous on the exposed limestone of karst regions, and vegetative cells maintain their viability despite several decades of storage [35]. In the most extreme arid cold and hot deserts the unicellular cyanobacterium *Chroococcidiopsis* dominates the cyanobacterial communities which are found in rocks [13]. Viable cells of *Chroococcidiopsis* spp. were recovered from both quartz flints collected from the Negev desert after storage for 30 years and long-term laboratory-dried cultures [17]. It was calculated that cryptoendolithic communities in the ice-free Ross Desert (Antarctica) are wetted and metabolically active for a total of 500 h per year [31]. Therefore, in these environments the time scales of biological and geological processes overlap, so that communities of *Chroococcidiopsis* might be extant representatives of "eoanhydrobiotes", ancient desiccation-tolerant cells.

The inability of anhydrobiotes to be revived from long-term desiccation is influenced by the accumulation of deleterious reactions of reduced oxygen species, Browning reactions, and cross-linking of proteins which accumulate in the dried state, when repair mechanisms are unlikely to work [6,34]. Damage is due to the production of disulfide bridges from sulfhydryl groups of proteins and cross-links between proteins and DNA [6,34]. In the so-called Maillard or Browning reaction, the carbonyl groups of reducing sugars react with primary amines of nucleic acids and free amino group of proteins to form covalent bonds [6,34]. However, critical experiments to assess how viability changes with time are lacking.

5. Adaptation to anhydrobiosis

A feature shared by eukaryotic anhydrobiotes is the intracellular accumulation of trehalose and sucrose, which may account for as much as 20% of the dried weight [10–12]. Accumulation of these two sugars has been reported in desert cyanobacteria subjected to water stress [21]. Evidence is limited on the presence of trehalose in vegetative cells of bacteria subjected to desiccation [6]. However, the induction of trehalose synthesis by osmotic stress or its addition to the cultures prior to drying can increase desiccation tolerance (see below). According to the water replacement hypothesis, trehalose and sucrose hydrogen bond membrane phospholipids and proteins and thus prevent the transition to gel phase of membranes and inhibit protein denaturation [12]. In addition, the propensity of trehalose and sucrose to form glasses at low water content (vitrification) is relevant to anhydrobiosis [10,11]. In the amorphous state the molecular diffusion is reduced and uncontrolled reactions that would be disastrous over the prolonged desiccation are avoided. Some bacteria elaborate extracellular polysaccharides (EPS) that are so varied in their gel and sol properties that they may actually have properties similar to those described for glass-forming polymers [11,33]. *N. commune* produces large amount of an extracellular glycan, which provides a matrix in which enzymes such as water stress proteins (Wsp), which probably play a role in its synthesis and/or modification, and Fe-superoxide dismutase, are maintained in the active state after long-term storage [20,22,37,38]. The development of thick multilayered envelopes rich in polysaccharides, lipids and proteins was described in the field- and laboratory-desiccated cells of *Chroococcidiopsis* spp. [17]. In *D. radiodurans* the presence of predicted highly expressed (PHX) genes coding for surface structure proteins has been proposed to provide protection against desiccation [24].

It is reasonable to suppose that stress proteins which serve as molecular chaperones to protect proteins from stress-associated denaturation and aid their renaturation would be involved in the ability of organisms to withstand desiccation [10]. The analysis of the genome sequence of *D. radiodurans* suggests a surfeit of chaperones, that may function to repair proteins [24]. At the time a cell is rehydrated a considerable fraction of its protein pool may be damaged. It is not known whether rehydrating cells discriminate between damaged and undamaged proteins. The role of ubiquitin (a protein which regulates selective degradation of proteins in eukaryotic cells) in protein turnover during desiccation and rehydration remains to be determined. A mechanism of repair based on the ubiquitin-mediated protein degradation was assessed in vegetative desiccation tolerance of lower plants [32]. Evidence suggests that there is a great variability in the stability of proteins of dried cells. Active Fe-SOD was detected in 13-year-dried cells of *N. commune*, upon rehydration, while phycobiliprotein complexes are very sensitive to even short-term desiccation in *N. commune* UTEX 584 [33,38]. In view of the oxidative stress associated with

desiccation, proteins involved in oxygen-scavenging mechanisms might have a central role in increasing cell tolerance to air drying. Fe-SOD was also found in desiccated cells of *Chroococcidiopsis* sp [18]. It is not surprising that subareal cyanobacteria reduce photodamage accumulating in the extracellular layers through UV-absorbing pigments which might represent an important strategy to ensure survival after prolonged air drying.

It was estimated that the genome of a *Nostoc* cell would achieve 1% depurination after storage at 37°C for 10 years [33]. Yet, desiccated crusts in situ are exposed to temperatures far in excess of this value and depurination rates would be expected to increase; herbarium specimens remain viable after more than a century of desiccation [35]. It might be that anhydrobiotes employ strategies to prevent extreme DNA degradation. A connection between the ability to repair DNA damage and desiccation tolerance was indicated by the fact that mutants of *D. radiodurans* exhibited a loss of viability after desiccation [28]. The fact that these mutants were also ionizing-radiation-sensitive suggested that cellular responses to ionizing radiation and dehydration may overlap. The remarkable resistance of desert strains of *Chroococcidiopsis* sp. and *D. radiodurans* is thought to be associated with the presence of multiple genome copies, which may facilitate interchromosomal recombination and repair of DNA-induced damage [5,28]. It was speculated that the overrepresentation in the genomes of some cyanobacteria of an octameric palindrome, designed HIP1 (highly iterated palindrome) may reinforce their adaptation to ecological stresses through an enhancement of genome plasticity and an increase in genetic diversity through gene rearrangements [29]. A rigorous investigation of the habitat distribution of HIP1-rich species is needed in order to assess why an high HIP1 content has been selected [36]. However, a different explanation from an enhanced repair system was proffered for the radioresistance of *D. radiodurans*. Compared with other complete prokaryotic genomes *D. radiodurans* contains the greatest number of PHX genes encoding chaperone/degradation, protease, and detoxification genes, while the set of DNA repair proteins are predominantly of low to moderate predicted expression [24]. Comparative genomics analysis pointed out that *D. radiodurans* apparently acquired several genes by horizontal gene transfer from various sources, such as three homologs of plant putative desiccation resistance-associated genes, which may contribute to its extreme resistance phenotype [27].

6. Anhydrobiotic engineering

The understanding of the role of trehalose and sucrose in the stabilization of macromolecules in anhydrobiotes has opened up a whole field of research that is leading to the most interesting applications in the storage of dried biomolecules and development of biosensors. Embedding in trehalose or sucrose was used successfully in the preservation of dry membranes and enzymes [12], as well as intact bacterial cells [14]. The fact that trehalose plays a role in both osmotolerance and in anhydrobiosis suggested that cells loaded with this compound could better stand dehydration. Indeed, the induction of trehalose synthesis by osmotic shock significantly increased the desiccation resistance in *Escherichia coli*, while glycine betaine, accumulated intracellularly, had no influence on a subsequent desiccation challenge [40]. However, trehalose accumulation does not seem to be the only factor associated with the enhancement of desiccation tolerance; stationary phase cells of *E. coli* were found to be more resistant than exponential phase cells, although trehalose further increases their tolerance of desiccation [40].

In view of the simplicity of the biosynthetic pathway of trehalose and sucrose, which is a two-step process involving a synthase and a phosphatase, it was suggested that the engineering of trehalose or sucrose synthesis in cells might provide a means to manipulate desiccation tolerance [12]. The engineering of *E. coli* with a cyanobacterial gene that encodes sucrose-6-phosphate synthase (*sps*A) led to a marked increase in survival after air drying, freeze-drying and chemical desiccation over phosphorus pentoxide [7]. The possibility of using a genetic approach in which desiccation-sensitive mammalian cells are made desiccation-tolerant through the expression of foreign trehalose synthase genes is still controversial. Human fibroblasts transiently expressing the *E. coli otsA* and *otsB* trehalose synthase genes have been reported to accumulate trehalose and survive complete desiccation [19]. Genetically engineered mouse fibroblasts, though able to accumulate trehalose, exhibited improved resistance to partial dehydration resulting from hypertonic shock, but were unable to survive complete desiccation [15].

Novel technologies in anhydrobiotic engineering might derive from the employment of EPS purified from desiccation-tolerant cyanobacteria, as suggested by the fact that the extracellular glycan of *N. commune* inhibits fusion of membranes vesicles during desiccation and freeze drying [9, 23].

7. Conclusions

Although available information on how prokaryotes recover from vegetative dehydration is still relatively sparse it does appear that tolerance of desiccation is a multifactorial trait, involving mechanisms which span from cellular protection to repair of desiccation-induced damage. To date, most studies on desiccation-tolerant forms have dealt with two anhydrobiotic cyanobacterial cells, *N. commune* and *Chroococcidiopsis* spp. The only desiccation-tolerant cyanobacterium currently the subject of genetic manipulation is *Chroococcidiopsis* [4]. This cyanobacterium may provide a model system to approach the genetic analysis of desiccation tolerance; transposon mutagenesis and gene knockout experiments may contribute to highlight some aspects of

its desiccation tolerance. Progress is now anticipated from the analysis of the genome sequence of *D. radiodurans*, which has revealed some of the molecular bases underlying its extreme resistance. Further investigations are necessary not only to fully decipher the mechanisms of desiccation tolerance, but also to assess whether individual desiccation-tolerant species meet the criteria for surviving desiccation by using similar protection-repair strategies. Comparative genome analysis of desiccation-tolerant and desiccation-sensitive microorganisms and experimentation by RNA and DNA microarray chip technologies are now under way and promise to identify the genetic basis of anhydrobiotic adaptation.

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