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Elemental Economy: microbial strategies for optimizing growth in the face of nutrient limitation

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Abstract

Microorganisms play a dominant role in the biogeochemical cycling of nutrients. They are rightly praised for their facility at fixing both carbon and nitrogen into organic matter, and microbial driven processes have tangibly altered the chemical composition of the biosphere and its surrounding atmosphere. Despite their prodigious capacity for molecular transformations, microorganisms are powerless in the face of the immutability of the elements. Limitations for specific elements, either fleeting or persisting over eons, have left an indelible trace on microbial genomes, physiology, and their very atomic composition. We here review the impact of elemental limitation on microbes, with a focus on selected genetic model systems and representative microbes from the ocean ecosystem. Evolutionary adaptations that enhance growth in the face of persistent or recurrent elemental limitations are evident from genome and proteome analyses. These range from the extreme (such as dispensing with a requirement for a hard to obtain element) to the extremely subtle (changes in protein amino acid sequences that slightly, but significantly, reduce cellular carbon, nitrogen, or sulfur demand). One near universal adaptation is the development of sophisticated acclimation programs by which cells adjust their chemical composition in response to a changing environment. When specific elements become limiting, acclimation typically begins with an increased commitment to acquisition and a concomitant mobilization of stored resources. If elemental limitation persists, the cell implements austerity measures including elemental-sparing and elemental-recycling. Insights into these fundamental cellular properties have emerged from studies at many different levels; including ecology, biological oceanography, biogeochemistry, molecular genetics, genomics, and microbial physiology. Here, we present a synthesis of these diverse studies and attempt to discern some overarching themes.

Keywords

metal homeostasis; sparing; phosphorus; sulfur; iron; zinc; copper; cyanobacteria; diatom; Chlamydomonas; *Bacillus*

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I. Overview: Mendeleev meets Darwin

One of the great achievements of 20th century science was the melding of two largely distinct disciplines, chemistry and biology, leading to a renaissance in the molecular life sciences. Chemistry underlies the myriad processes that power and enable cell growth, and an understanding of chemical principles is imperative for the modern biologist. One of the most fundamental principles of chemistry is that elements are immutable, at least under the conditions conducive to life processes. Transformation of one element into another occurs either rarely and stochastically, in the case of radioactive decay and nuclear fission, or under extremes of temperature and pressure, in the case of fusion-based nucleosynthesis.

It is ironic that the very foundations of Chemistry are rooted in the notion of elemental transmutation and the countless years of effort devoted to alchemical pursuits including, most famously, efforts to turn base metals into gold. Isaac Newton was one of the more ardent practitioneers; it is estimated that he devoted far more attention to alchemy than to mathematics and physics combined (Dobbs, 1983). Ultimately, of course, the atomic theory as espoused by John Dalton (1766–1844), with the notion of atoms as indestructible and indivisible, was hailed for its explanatory power and provided a foundation for modern chemistry.

The atomic theory set the stage for an increased understanding of the properties of the elements, which displayed a periodic pattern when arranged, in early versions, relative to their masses. While many scientists contributed to the development of the periodic table, Dmitri Mendeleev (1834–1907) is generally credited with this synthesis (ca. 1869). In biology, a contemporary of Mendeleev, Charles Darwin (1809–1882) published his landmark "Origin of Species" in 1859 with its description of evolution by natural selection. Here, we consider the conceptual intersection of these two great ideas <Figure 1>. Our theme will be the wide range of remarkable adaptations found in the microbial world that have resulted from limitations for elemental nutrients.

A. The Elemental Composition of Life

Living cells rely on only a small, and somewhat variable, subset of the periodic table <Figure 2>. The elements of life can be divided into the macronutrients (C,H,N,O,P,S), major cations (K,Mg,Ca), and the so-called micronutrients (including many metal ions) (Frieden, 1985, Frausto da Silva and Williams, 2001). Many familiar elements are dispensable to living cells and in many cases can be deleterious if present.

It is unexpectedly difficult to define the minimum set of elements absolutely essential for life, although there is little doubt that all cells require the so-called macronutrients of CHNOPS (note that all elements will be referred to by their atomic symbols with ionization state indicated only where relevant). The requirements for metal ions and other elements is more idiosyncratic. It is likely that all cells require Zn and Mg and nearly all also require Fe. The requirements for Mn, Cu, Co, Ni, Ca, K, Na, Mo, Se, and other elements are likely to be variable and are often unestablished. Some elements have very specialized but beneficial or even essential functions in a very select subset of organisms. Examples include the use of Si in plant and diatom cell walls, B in plants, and Cd in certain marine organisms <Table 1>.

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The atomic inventory has been measured for numerous organisms and is relatively constant for the most abundant macronutrients, but remarkably flexible for the much less abundant micronutrients. As an example, the elemental composition of a representative marine cyanobacterium, *Synechococcus* sp. CCMP835, is shown in <Figure 3> (Quigg et al., 2011).

The requirements for macronutrient elements are well established and easily understood. Life is fundamentally based on aqueous chemistry and this requirement alone provides an absolute requirement for H and O, although of course not all organisms require molecular oxygen (O_2). The chemistry of proteins, nucleic acids, carbohydrates, and lipids accounts for the major requirements for C, N, P and S. In no case can cells be assembled in the absence of these crucial elements although, as we will see, there are sophisticated mechanisms for minimizing (to the extent possible) the requirements for these macronutrients when they might otherwise be limiting.

Requirements for the remaining elements are less firmly established and few, if any, are likely to be universal requirements (Table 1). Zn is widely used as a cofactor for protein folding and as a Lewis acid cofactor for several families of enzymes (Andreini et al., 2009). Mg(II) is the major biological cation in many microbial systems. We suggest that both of these are likely essential for all life, at least until a counterexample is identified. Fe is essential for growth of nearly all microbes and is present in heme-cofactored proteins, iron-sulfur cluster (Fe/S) proteins, and di-iron and mononuclear enzymes, amongst others. However, there are organisms, including the lactobacilli and *Borrelia burdorferi*, that have dispensed with an Fe requirement (Posey and Gherardini, 2000). Other cations (e.g. Ca, Cu, Co, Mn, K) are often needed or at are least stimulatory for growth, but a requirement is not necessarily universal. Note that in most cells the major anions are likely organic compounds (acetate, glutamate), so a requirement for Cl^- or other inorganic anions is not universal.

Microorganisms are the consummate experimentalists; over the course of several billion years of evolution single-celled life forms learned to exploit a wide variety of niches that often differed in the availability of the "elements of life." As organisms adapted to new environments where previously accessible elements were now limited in abundance or absent, they had to evolve alternative solutions for life's chemistry. It is the results of these evolutionary experiments, as embodied in the physiology and genetics of contemporary microbes, that form the basis for this review.

B. Reference Systems for Understanding Microbial Responses to Elemental Limitation

We will here explore the molecular mechanisms by which microbial cells adapt and acclimate to elemental limitation. Note that throughout this review, the term **elemental** refers to the atomic elements. We will use **adaptation** to refer specifically to changes in the DNA that occur in response in selection pressures, potentially acting over very long time scales, and **acclimation** to refer to those changes in cellular physiology that allow individual cells to better utilize available nutrients. Adaptations may include, for example, changes in protein sequence or the evolution of isozymes with alternative cofactor dependencies. The conditional expression of alternative enzymes or pathways, in response to changes in elemental availability, is a major feature of the acclimation response for many elemental limitations.

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Access to the elements of life plays a large role in defining the microbial composition of the diverse habitats within the biosphere. For some environments, such as the surface waters of the open ocean, elemental composition (and hence limitations) may be relatively constant, and are therefore a driving force for adaptation. Perhaps two-thirds of the ocean's surface waters have biologically limiting levels of P, and for much of the rest, it is the availability of Fe that limits primary productivity. The most successful organisms in these environments have evolved adaptations that allow them to maximize growth by reducing demand for limiting nutrients. These organisms are specialists, and their genomes and their metabolism reflect an evolutionary optimization for their environment. For other cosmopolitan organisms the environment is ever-changing and a more generalist approach is advantageous. Where elemental availability is variable, cells have evolved genetic mechanisms to acclimate their physiology to the changing local environment. In many cases, these types of acclimation involve changes in gene expression.

1. Genetic model systems for investigating mechanisms of elemental economy—Not surprisingly, many of the best understood mechanisms of adaptation and acclimation have been revealed in well-studied reference or "model" organisms <Table 2>. Here, we will focus on the Gram-negative bacterium *Escherichia coli* and the Gram-positive bacterium *Bacillus subtilis*. Eukaryotic microbes will be represented by the eukaryote *Saccharomyces cerevisiae* and the photosynthetic eukaryote, *Chlamydomonas reinhardtii*, a soil-resident alga. Each of these four organisms displays sophisticated mechanisms for acclimation to changing nutrient availability (Table 2a). Work in the Archaea is still not well developed in this area, although this diverse group of organisms clearly has sophisticated systems for the optimization of nutrient utilization and, in particular, for metal homeostasis (Bini, 2010). Emerging Archaeal model systems include the halophiles *Halobacterium salinarum* and *Haloferax volcanii* and the methanogens *Methanococcus maripaludis* and *Methanosarcina acetivorans* (reviewed in Leigh et al., 2011). Of note, systems level studies of Fe homeostasis have been initiated in *H. salinarum* (Schmid et al., 2011) and methanogenesis is an intensively metal-utilizing process.

2. Phytoplankton and the marine ecosystem: the global impacts of elemental limitation—Life is thought to have evolved in shallow seas and perhaps other surface waters and the atomic composition of cells reflects this archaic environment (Dupont et al., 2010). As life diversified and colonized a wider variety of environments, the ability to adapt to changing elemental availability likely assumed greater importance (Armbrust, 2009, Sohm et al., 2011). The abundance of life enabled by the evolution of mechanisms to access the most abundant forms of C (photosynthetic fixation of atmospheric CO_2) and N (fixation of atmospheric N_2) inevitably led to growth restriction by other nutrients (Morel and Price, 2003, Konhauser et al., 2009). The result has been the evolution of mechanisms to either bypass these restrictions or, minimally, to increase the efficiency of utilization of limiting nutrients. Moreover, elemental availability has changed dramatically on geological timescales (Quigg et al., 2003, Konhauser et al., 2009). One major driving force was the great oxidation event (~2.4 billion years ago) and the subsequent depletion of soluble ferrous Fe from ocean surface waters and other aerobic environments. It is estimated that ~30% of the open ocean is Fe-limited and this left a major imprint on the genetics and

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physiology of the constituent plankton (Armbrust, 2009, Behrenfeld et al., 2009, Sohm et al., 2011).

We will discuss the marine microbial community in some detail, including cyanobacteria and other phytoplankton, since these organisms provide particularly informative examples of adaptations to limiting P, Fe, and Zn (Table 2b). Because of the severity of nutrient limitations in this environment, and the long timescales over which these limitations have persisted, the resident organisms have evolved unique adaptations to bypass elemental restrictions. These adaptations are, in many cases, complemented by acclimation responses.

The dominant microorganisms in the open ocean vary depending on location, light fluxes, and availability of both macro- and micronutrients. In many areas of the open ocean, the numerically dominant microorganisms include members of the cyanobacteria (in particular, *Synechococcus* spp. and *Prochlorococcus* spp.) (Zehr et al., 2007) and diatoms (Bowler et al., 2010, Partensky and Garczarek, 2010). Diatoms are eukaryotic phytoplankton notable for their silica-containing cell walls (frustules) and are responsible for perhaps 20% of total primary productivity globally (reviewed in Armbrust, 2009). Representative organisms which have helped illuminate mechanisms of elemental economy, and where genomics-enabled approaches are now coming to the fore, are summarized in Table 2b.

C. General Strategies for Dealing with Elemental Limitation

Insights into how microbes adapt to elemental limitations emerged in laboratory studies of microbial metabolism on the one hand (e.g. Hutber et al., 1977, Wood, 1978, Schönheit et al., 1979, Bishop et al., 1980, Ragsdale and Ljungdahl, 1984) and the study of the microbial ecology, and in particular the ocean ecosystem, on the other (Morel and Price, 2003). Increasingly, genomic and molecular genetic investigations of model organisms (Table 2a) are revealing mechanisms of adaptation and programs for acclimation to elemental limitation. These same mechanisms likely occur in globally important but less tractable organisms such as the marine phytoplankton. Studies of representative marine microbes, many of which are now amenable to genomics-based investigations (Table 2b), reminds us of the impact of elemental limitations on global ecology and primary productivity. Comparing these two bodies of largely distinct literature reveals that similar strategies for elemental economy have evolved independently in many systems.

With their ability to double their population in as little as 20 minutes or less (under optimal growth conditions), responses to resource limitation are deeply ingrained in the genetics and physiology of microorganisms. The impact of nutrient limitation is apparent both in cellular adaptations and in the processes of acclimation. At the level of adaptation, macronutrient limitation has led microbes to alter the elemental composition of selected constituents through a process of **elemental optimization**. As one example, signatures of macronutrient limitation are recorded in the protein sequences of the elemental acquisition machinery of phylogenetically diverse organisms (Baudouin-Cornu et al., 2001). At the level of acclimation, nutrient limitation leads to several predictable responses that may be activated either simultaneously or sequentially, depending on the system. These responses can be classified as elemental **acquisition, mobilization, sparing**, and **recycling**.

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1. Elemental optimization—Organisms adapt to elemental limitation by altering the atomic composition of their constituents; a trend noted in protein primary sequences. For example, proteins necessary for the acquisition of S are selectively depleted of Cys and Met in their amino acid sequences (Baudouin-Cornu et al., 2001). Clearly, this enhances the ability of the cell to express these proteins under S limiting conditions where translation of proteins requiring high levels of Cys and Met might be impaired. Parenthetically, we note that an analogous selection pressure also operates at higher levels of organization: the amino acids themselves. In the "cognate bias hypothesis" it has been noted that amino acid biosynthetic enzymes are under selection pressure to minimize use of the cognate amino acid in their coding sequences (Alves and Savageau, 2005). Other notable examples of elemental optimization include the elimination of an Fe requirement in organisms that grow in severely Fe-limited environments (Posey and Gherardini, 2000), and the substantial reduction in phospholipid content in cells that have adapted to persistent P limitation (Van Mooy et al., 2009). As defined here, elemental optimization is a fixed adaptation and is therefore most characteristic of specialist organisms that inhabit environments of relatively constant elemental composition. However, analogous changes in elemental composition also contribute to the conditionally expressed programs by which organisms acclimate to nutrient limitation.

2. Acclimation: acquisition and mobilization—Generally, the first major cellular response during acclimation to nutrient limitation is an increased expression of acquisition pathways. Acquisition pathways are dominated by transporters for the limiting element or compounds that are rich in the limiting element. Associated functions include enzymes to help mobilize nutrients, such as proteases, nucleases, and various hydrolases. Acquisition pathways also include, for many organisms, induction of pathways to access resources that are chemically recalcitrant or more energetically prohibitive and therefore only used as a last resort. One well characterized example is the repression of nitrogen fixation by ammonium (Dixon and Kahn, 2004). A second example is the derepression of enzymes to cleave direct C-P bonds (phosphonates) when P (as phosphate) is limiting (Jiang et al., 1995, Baek and Lee, 2007), an adaptation that appears widespread in marine bacteria (Martinez et al., 2010). Phosphonates may comprise up to 25% of the high molecular weight organic P pool in the ocean and uptake and utilization of phosphonates is widespread in the important marine cyanobacterium *Prochlorococcus* as well as in *Synechococcus* spp. in microbial mats in hot springs (Adams et al., 2008, Feingersch et al., 2011).

Concomitant with the expression of acquisition pathways, organisms will mobilize stored resources. Examples of this pathway include release of Fe from ferritin, degradation of polyphosphates to release inorganic P, and mobilization of excess C stored, for example, in lipid bodies. In each of these examples, mobilization is from a source whose primary, if not exclusive, function is elemental (or energy) storage. Once dedicated stores are depleted, cells may resort to more drastic measures that involve the selective consumption of their own organelles (autophagy) which we classify as elemental recycling (see below).

Acquisition and mobilization from stores are both typically activated when nutrients first start to become limiting for growth, a state that can be defined as **elemental deficiency**. If the acquisition and mobilization strategies are ineffective, and the deficiency is not relieved,

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the lack of the specific required element will ultimately lead to a cessation of growth or of key metabolic activities, a state that can be defined as **elemental limitation** (La Fontaine et al., 2002). While interesting in their own right, processes of nutrient acquisition and mobilization have been well reviewed elsewhere and we focus our attention on the elemental sparing and recycling responses.

3. Acclimation by elemental sparing—When nutrients are not accessible in the environment, and intracellular stores are depleted, austerity measures are implemented following the precepts of reduce, reuse, and recycle. **Elemental sparing** refers to responses that serve to **reduce** cellular demand for limiting nutrients by selectively repressing synthesis of non-essential proteins and macromolecules. This is, in essence, a **prioritization** mechanism by which the cell distinguishes between high priority and often essential functions and those that are of lower priority for survival. Elemental sparing responses are common for both macronutrient (P,S) and micronutrient limitations.

To be effective, and therefore a target of evolutionary selection, elemental sparing frequently targets the most abundant macromolecules in the cell. As a result, limitations for some of the key macronutrients (C,N,S) typically lead to changes in protein composition (the proteome) and targets highly abundant proteins. Limitation for P often leads to changes in the membrane and cell envelope, which can contain abundant phospholipids and other P-containing macromolecules. P-limited cells may also recycle P from nucleic acids (either DNA in the case of polyploid plastids or RNA in ribosomes). Limitation for specific metal ions often leads to a shift to enzymes that use alternative metal cofactors. Alternatively, some cells may simply dispense with a requirement for what would otherwise be an essential and hard to access nutrient.

While it is often possible for microorganisms to **reduce** their dependency on particular elements by the elimination of specific proteins or other macromolecules, in many cases these changes must be compensated for by the expression of alternative pathways. As a result, a common feature of many elemental sparing responses is functional **substitution**. For example, in response to metal ion limitation, substitute pathways may take advantage of an alternative, non-metal-based chemistry, or may rely instead on a more abundant metal in place of a scarce one. In response to P limitation, simply repressing the synthesis of phospholipid membrane synthesis would impede cell growth. Expression of alternative biosynthetic pathways for lipids lacking P enables continued cell growth, while sparing P for its more indispensable functions.

Not all elemental substitution processes in cells contribute to elemental sparing. In many organisms, S is substituted with Se, usually at a single active site residue. Since most organisms contain between one and three selenoproteins (Zhang and Gladyshev, 2011), this is not sufficient to affect the overall S budget. In the case of P, there has been a report of its replacement by As with an apparent reduction in cellular P demand (Wolfe-Simon et al., 2011), but this has been widely challenged (see below). Indeed, there is not a single documented example of As replacing P in a biological molecule that retains its normal function in the cell.

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4. Acclimation by elemental recycling—In those systems where it is difficult to dispense with an elemental requirement, and the gains to be obtained by elemental sparing are limited, cells may additionally implement an **elemental recycling** program. Such strategies may include the degradation of key cellular components (proteins, the ribosome, photosynthetic complexes, and even chromosomal DNA in polyploid plastids) for the sole apparent purpose of recovering the nutrients stored therein to supply new biosynthetic processes <Figures 4 and 5>. One notable example is the degradation of ribosomes (ribophagy) upon starvation, which frees up significant stores of C, N, P, and possibly Mg (Martin et al., 1976, Kraft et al., 2008). Similarly, in *C. reinhardtii* and in a marine cyanobacterium, abundant metalloenzymes may be proteolytically degraded simply to release and recycle the valuable copper and iron cofactors (Merchant and Bogorad, 1986a, Saito et al., 2011).

In the following sections, we will highlight numerous examples, in both model organisms and representative environmentally significant microbes, of these major strategies for dealing with elemental limitation. We will begin with some of the best understood processes that enable efficient macronutrient utilization and conclude with studies of metal homeostasis and the evolution of pathways to bypass metal limitations on growth.

II. Microbial Adaptations to Macronutrient Limitation

By weight, cells are mostly water and this accounts for, and provides a source of, both H and O. For all of the remaining macronutrients (C,N,P,S) there are well-documented processes of both adaptation and acclimation in response to limitation. The effects of macronutrient limitation on cell growth have long been appreciated in ecology and were perhaps first formalized in terms of crop production by Professor Carl Sprengel of Göttingen (1839) in what became known as the "law of the minimum" as popularized by Justus von Leibig. The impact of elemental limitations was highlighted by the influential work of the oceanographer Alfred Redfield who noted that the averaged ratio of macronutrients in biomass (largely phytoplankton) from the oceans (C:N:P=106:16:1) was very similar to the elemental composition of the ocean surface waters. This so-called Redfield ratio is a founding concept in the development of "ecological stoichiometry": a discipline which traces the impact of elemental composition and limitations on ecosystem dynamics (Jeyasingh and Weider, 2007).

Despite a long and influential history, ecological stoichiometry has remained a largely descriptive approach. However, insights into the underlying mechanisms by which microorganisms adapt and acclimate to elemental limitation are emerging due to both molecular biological studies and, increasingly, bioinformatic analyses. Genome sequence information, especially when coupled with expression (transcriptome and proteome) data, allows insights into the protein composition of cells from different environments and under different growth regimens. Remarkably, the evolutionary impact of both macro- and micronutrient limitation is often discernable by a careful analysis of genomes: an emerging science for which the name "stoichiogenomics" has been proposed (Elser et al., 2011).

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A. Carbon, Nitrogen, and Sulfur Limitation: adaptation and acclimation mechanisms

The bulk of cellular C, N, and S demand is in support of protein synthesis. While C and N are constituents of all amino acids, these elements are not equally distributed due to variations in the length and composition of the side-chains. Two amino acids, Cys and Met, contain S and together these account for the bulk of cellular S requirements. The ability of changes in protein sequence to spare limiting macronutrients was first hinted at from protein sequence analyses of individual proteins, but has more recently been inferred on a genome-wide basis.

1. Adaptation and acclimation to S limitation

a. Elemental optimization: selective reduction of Cys and Met content in proteins: For any given protein, a small subset of amino acids is typically essential for catalytic function, and many others may contribute to optimal expression, protein folding, or stability. However, at many positions, identified as variable in alignments of homologous proteins, substitutions may occur more or less freely (e.g. Wen et al., 1996). The identity of the amino acids at these variable positions can change and become established in response to a variety of selection pressures, including elemental availability.

One of the first noted examples of such an effect emerged from the protein composition analysis of the abundant sulfate binding protein induced in *E. coli* upon S starvation (Pardee, 1966). Amino acid analysis suggested that this polypeptide, which can constitute 1% of cellular protein when cells are grown under S limitation, was lacking Cys and Met. The lack of these amino acids (in the mature protein) ensures that its synthesis in response to S limitation is not impaired by restrictions on Cys and Met availability. Numerous other examples are now apparent where proteins expressed under S limitation either lack or have a reduced content of Cys and Met <Figure 6>. Indeed, at a proteomic level, proteins that are involved in S assimilation may be selectively depleted in Cys and Met relative to other proteins. For example, analysis of proteins annotated as having likely roles in S assimilation in both *E. coli* (23 proteins) and *S. cerevisiae* (20 proteins) revealed a substantial decrease (up to 2-fold) in the fraction of amino acids that contain S relative to the rest of the proteome (Baudouin-Cornu et al., 2001). No such difference was seen in the orthologous enzymes (where present) from human, which was interpreted as evidence that S limitation has not been a selective pressure in mammals. The selective reduction of S content in S assimilation proteins is just one of many ways in which macronutrient limitations and energy constraints can influence protein sequences. A survey of ~150 different species (mostly Bacteria) suggests that there are also substantial differences (approaching 2-fold) in the computed fractional S content of proteomes amongst species. One notable trend is a slightly higher S content, on average, amongst anaerobes, although the significance of this observation is not yet clear (Bragg et al., 2006).

b. Remodeling of the proteome as a S sparing mechanism: Expression of S depleted protein variants or isozymes is also an important mechanism of acclimation to S limiting growth conditions. For example, in cyanobacteria, the light-harvesting phycobilisome proteins can account for ~50–60% of soluble protein. In the cyanobacterium *Calothrix* sp. PCC7601, the phycocyanins, a key phycobilisome constituent, are encoded by three

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differentially expressed operons (Mazel and Marlière, 1989). The *cpc3* operon is specifically induced under conditions of S limitation. The encoded phycocyanins have been selectively depleted of S-containing amino acids relative to the phycocyanins encoded by the *cpc1* and *cpc2* operons. In over 1000 amino acids of five proteins encoded by *cpc3*, there are only five Met initiation codons and three indispensable Cys used as attachment sites via thioether linkage for phycobilin (Mazel and Marlière, 1989). A similar response has been documented in a freshwater cyanobacterium, *Fremyella diplosiphon* (Gutu et al., 2011). This organism also remodels its abundant phycobilisome complex in response to S depletion to replace proteins relatively rich in Cys and Met with a paralog that is depleted of these S-containing amino acids. The replaced phycobilisome proteins are likely degraded which thereby provides the cell with a source of S (and potentially N) under conditions of nutrient deprivation (elemental recycling).

A comparable acclimation response has been noted in the yeast *S. cerevisiae*. In this organism, intracellular S demand was modified by exposure to Cd, which induces synthesis of the Cys-containing tripeptide glutathione and hence creates a draw on the S metabolite pool. Cd is toxic to cells because of its affinity for intracellular thiols (e.g. at the active site of enzymes) and is detoxified by tight binding to glutathione. The strength of the binding (essentially irreversible), combined with the transport of the glutathione-Cd complexes into vacuoles, results in sequestration of Cd and depletion of intracellular S. Analysis of changes in the proteome of Cd-treated cells revealed the induction of isoforms of three central metabolic enzymes that are selectively depleted in S-containing amino acids (Fauchon et al., 2002). These include an alternative pyruvate decarboxylase, an enolase, and an aldehyde dehydrogenase. These induced isozymes contain only 22 S atoms as compared to 42 for their counterparts. The usual isoforms are among the most abundant proteins in yeast, constituting >6% of the soluble proteome. For pyruvate decarboxylase, down-regulation of the usual isoform was also documented. In addition, several other mRNAs that were induced in Cd-exposed cells were noted to be significantly depleted in Cys- and Met-encoding capacity. These responses are dependent on Met4p, a transcriptional regulator of S assimilation. Interestingly, of the 66 transcripts induced by Cd and dependent on Met4p, just 13 (encoding the most abundant proteins) were significantly depleted of sulfur amino acids. Analysis of the fate of sulfate nutrient in Cd-treated cells indicated a 70% distribution of S in favour of GSH biosynthesis as compared to a 79% distribution towards protein in untreated cells. These S-sparing changes are estimated to lead to an ~30% reduction in cellular S allocation towards protein, which increases S availability for Cd-detoxification pathways (Fauchon et al., 2002).

In the alga *C. reinhardtii*, S-starvation induces massive changes in the proteome and transcriptome (González-Ballester et al., 2010). As expected, many of the changes relate to S acquisition, such as mobilization of S from esterified organic sulfates, utilization of less preferred S sources, and sulfate transport (reviewed in Irihimovitch and Yehudai-Resheff, 2008). There is also evidence for S recycling (González-Ballester et al., 2010). For instance, enzymes involved in S mobilization (including *CDO1, TAUD1* and *TAUD2* encoding cysteine and taurine/α-ketoglutarate dioxygenases, respectively) are strongly up-regulated. These responses are dependent on a plant-specific SNF-related kinase, which is central to

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the S starvation signaling pathway, indicating the direct relevance of these responses to S metabolism.

Down-regulation of transcripts encoding biosynthesis of S-containing vitamins, thiamin and biotin, and the cofactor *S*-adenosyl methionine, is likely to be part of an S sparing response in which the limited pool of intracellular S metabolites is directed towards critical processes. Several extracellular proteins (Ecp56, Ecp61, Ecp76 and Ecp88) and a particular isoform of chlorophyll binding light harvesting protein are induced by S deficiency (Takahashi et al., 2001, Nguyen et al., 2008, González-Ballester et al., 2010). In fact, the mRNA for the light-harvesting chlorophyll-binding protein Lhcb9 increases as much as 10^3-fold to become the 2nd most abundant transcript in the cell. In each case, the induced proteins are depleted for Cys and Met. The induced Ecps have at most one S-containing amino acid out of 500–600 (0 to 0.2%) compared to 4.1 to 7.5% for other cell wall proteins. In the case of Lhcb9, out of five S-containing amino acids that are invariant in the other 8 major Lhcb proteins, this isoform retains only two: Met157, Met213 and Cys101 are replaced by Leu, Ser and Ile, respectively. Interestingly, the depletion is noted only in the mature part of the protein which is the form that accumulates and is hence subject to selective pressure, but not in the signal sequence for export or the transit peptide for thylakoid membrane targeting, both of which are cleaved, with the constituent amino acids recycled. The authors estimate that this form of S sparing reduces from 4.2% to 2.3% the S amino acid content of the *C. reinhardtii* proteome (González-Ballester et al., 2010), reminiscent of the yeast study discussed above.

S sparing has been documented in multicellular organisms as well (Petrucco et al., 1996, Kim et al., 1999). In the soybean seed, the S content of storage proteins in seed is determined by the level of S nutrition: S-amino acid-rich glycinin is the major protein if S is available, but S-amino acid depleted β-conglycinin accumulates in the S-poor situation. The abundance of a key metabolite, *O*-acetyl-Ser, signals the change in gene expression (Kim et al., 1999).

2. Elemental optimization: signatures of C and N limitation in proteomes—Just as selection in the face of S limitation has led to a reduction or even eradication of S-containing amino acids from proteins, both C and N limitation can affect protein composition (Figs. 4 and 5). It has been noted, for example, that a single amino acid change in a protein can add up to three N atoms (Gly to Arg) or nine C atoms (Gly to Trp). When considered in the context of protein abundance, mathematical modeling suggests that a single Arg to Gly change, for example, would have a sufficient effect on N balance to be targeted for selection in the most abundant ~8% of yeast proteins and a single Trp to Gly change would provide sufficient C savings to be visible to selection in ~4% of proteins. The effects of depleting proteins for S (by elimination of one or more Cys or Met residues) is even more dramatic and is potentially subject to selection in more than half of all yeast proteins, although this value depends on the precise parameters used (Bragg and Wagner, 2009). Such changes are particularly apparent when one considers only the subset of proteins expressed in response to a particular elemental limitation, as noted for S assimilation proteins above (Baudouin-Cornu et al., 2001). In both S. *cerevisiae* and *E. coli*, those enzymes specifically involved in processes of C assimilation have, on average, slightly shorter (less C-rich) side chains than the bulk proteome (or S assimilation enzymes).

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Conversely, enzymes for N assimilation are built, on average, using amino acids with fewer N atoms (Baudouin-Cornu et al., 2001). These effects are relatively small (5–10% reduction), but the fact that this signature of selection is visible at the proteomic level is rather remarkable. Elemental composition is not the only factor that affects amino acid selection at otherwise neutral positions in protein sequences. It has been shown, for example, that amino acid selection is also driven by the energetic demands of amino acid biosynthesis (Akashi and Gojobori, 2002) or even periodic fluctuations in the predominant metabolic pathways at different metabolic phases of the yeast cell cycle (de Bivort et al., 2009).

3. Recycling: macromolecular turnover as a way of redistributing C, N, and S—Elemental sparing responses can significantly reduce cellular demand for S, but are relatively ineffective in the face of the ubiquitous use of C and N in cell constituents. While elemental optimization of protein sequences provides some additional efficiency for utilization for all three of these macronutrients (C,N,S), the gains are incremental. Ultimately, when no external sources can be scavenged, and cell growth becomes severely limited for macronutrients, processes of recycling assume great importance.

Recycling of nutrients within and between cells typically involves the degradation of abundant cellular components to release their molecular (e.g. amino acid and nucleotide) and elemental constituents. In multicellular populations, entire cells may serve as nutrients in this way. Even if we restrict ourselves to the microbial world, this type of process is likely widespread as noted, for example, in the cannibalism response of *B. subtilis*. When growing populations of *B. subtilis* become nutrient limited, they synthesize toxins that kill and lyse non-starved cells of the population, which thereby delays entry into sporulation (Gonzalez-Pastor, 2011). Similar processes of fratricide occur in other, genetically homogeneous populations of cells (Claverys and Havarstein, 2007) and it has been suggested that toxin:antitoxin modules may contribute to programmed cell death in a sort of altruistic suicide triggered, at least under some conditions, by nutritional stress (Engelberg-Kulka et al., 2006).

Individual cells recycle nutrients by the selective degradation of surplus or replaceable constituents. This general process is termed autophagy (eating of self), but can be further classified as ribophagy, mitophagy, and so forth, depending on the structure or organelle that is targeted for destruction. The physiological role of autophagy is not always clear, although recycling of nutrients is one obvious benefit. Autophagy is also related to quality control and serves to selectively degrade non-functioning or damaged organelles. Studies in animal models indicate that autophagy helps prevent numerous degenerative diseases and may be a key mediator of the beneficial effects of exercise (He et al., 2012).

a. Recycling of ribosomes (ribophagy): Ribosomes represent a major fraction of cell mass in rapidly growing bacteria and the cell's requirement for ribosomes scales linearly with growth rate. During rapid growth, ribosomes may comprise close to 1/3 of the dry mass of a rapidly growing bacterium, but far smaller numbers are needed for the slow growth during elemental deprivation or for maintanence of the cell during non-growing (stationary) phases that are imposed by elemental limitations. Early studies in *E. coli* revealed that ribosomes

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are degraded in cells presented with C or N limitation (Kaplan and Apirion, 1975, Zundel et al., 2009). Degradation appears to occur during the transition to stationary phase, and is correlated with the formation of free ribosomal subunits (Zundel et al., 2009, Piir et al., 2011).

Recent studies have begun to reveal the pathways of rRNA degradation and their regulation (Deutscher, 2009). Degradation of ribosomal subunits in response to nutrient starvation is initiated by a pathway distinct from the ones operating for quality control during growth (Basturea et al., 2011). Turnover of ribosome subunits begins, in the case of the 30S subunit, with the trimming of the 16S rRNA 3'-end by RNase PH. Since this region contains the anti-Shine-Dalgarno sequence, this functionally inactivates the subunit. Another early step in ribosome degradation is endonucleolytic cleavage which, in both subunits, targets the RNA-rich subunit interface. Although the identity of the relevant endonuclease is not yet clear, this may be part of the mechanism by which un-associated subunits are selectively targeted for destruction. Once cleaved, the rRNA molecules are degraded by processive exonucleases including RNase II, RNase R and polynucleotide phosphorylase. These RNases may work in concert with proteins related to the Ro autoantigen (Wurtmann and Wolin, 2010) and may themselves be regulated. For example, RNase R is regulated by protein acetylation which leads to instability during exponential growth and stabilization during stationary phase (Liang et al., 2011).

It is not clear whether degradation of ribosomes serves primarily to liberate nutrients associated with the abundant rRNA (which is ~50% of the ribosome mass), the ribosomal proteins, or both. In most cases, it is rRNA that is monitored as this is technically easier. However, it has also been noted that macronutrient limitation leads to a starvation-specific proteolysis of ~20–40% of total cell protein in *E. coli* (Nath and Koch, 1971), which would be consistent with degradation of released ribosomal proteins (r-proteins).

Ribophagy is also increasingly appreciated for its role in nutrient cycling in eukaryotic cells. In *C. reinhardtii*, nitrogen starvation triggers a conversion of vegetatively growing cells into gametes and this differentiation process involves extensive remodeling of the ribosome pool (Martin et al., 1976). Indeed, it has been suggested that mobilization of precursors (nucleotides) from rRNA degradation is needed for DNA replication under these conditions. However, this remodeling of the ribosome pool may also have other roles in the cell such as modulation of translational accuracy (Bulté and Bennoun, 1990).

In *S. cerevisiae*, the induction of ribophagy upon nutrient limitation involves de-ubiquitination of ribosomal proteins by the Ubp3p/Bre5p ubiquitin protease that then triggers engulfment of the large ribosomal subunit into a vacuole for degradation (Kraft et al., 2008, Lafontaine, 2010). This process, for which the term ribophagy was first coined, involves the targeted delivery of both ribosome subunits to the vacuole, the presumed degradation of their protein and nucleic acid components, and the recycling of macronutrients. Genetic studies indicate that the process of ribophagy contributes to survival during prolonged periods of starvation (Kraft et al., 2008).

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b. Recycling of other abundant protein components: In addition to ribosomes, other abundant proteins may be targeted for proteolytic destruction upon nutrient limitation. Indeed, in mammalian cells, protein degradation mediated by the proteasome provides an important source of amino acids for ongoing protein synthesis when amino acid availability becomes limiting (Vabulas and Hartl, 2005). Photosynthetic organisms may also take advantage of protein degradation to release macronutrients when they find themselves N limited. In *Synechococcus* strain DC2 the light-harvesting phycobiliproteins may constitute 50% of total protein. Phycobilisome complexes in *Synechococcus* can be targeted for degradation in response to either N or S limitation (Collier and Grossman, 1992) and that degradation appears to be a highly regulated and ordered process (Grossman et al., 1993). It is likely that similar targeted recycling processes will be present in many cell types that devote a large fraction of their biosynthetic resources to one or a few specific proteins.

c. Recycling of sulfolipids as a S source: Many oxygenic photosynthetic organism produce S-containing lipids as part of their chloroplast membrane. For example, sulfoquinovosyl diacylglycerol (SQDG) is a major component of the thylakoid membrane in cyanobacteria and in *C. reinhardtii*. In the latter organism, SQDG accounts for ~13% of total cell S, and up to 85% of this lipid is degraded within 6–12 hours in response to S limitation. This S recycling mechanism, which is regulated as part of the S acclimation response (Sugimoto et al., 2010), provides a large fraction of the S needed for ongoing protein synthesis. It is formally possible that degradation of an abundant protein (such as Rubisco) could provide a comparable amount of S (and in the more convenient form of Cys and Met) (Sugimoto et al., 2007). Indeed, S starvation does lead to a decrease in Rubisco levels, but this response appears to occur on a much longer timescale than the mobilization of S from SQDG, suggesting that this may be a secondary mechanism (Sugimoto et al., 2007).

B. Phosphorous (P): An indispensable element for information and energy transfer

Phosphorous is required for life and exists in cells primarily as the phosphate anion (PO_4^{3-}) in various states of protonation and esterification. At neutral pH, phosphate is predominantly in the HPO_4^{2-} and $H_2PO_4^{-}$ states; phosphate esters also carry a net negative charge in cells. Phosphate forms the linkage unit of nucleic acids and is therefore essential for the storage, transmission, and expression of genetic information <Figure 7>. Nucleoside triphosphates (NTPs) not only serve as precursors for the synthesis of DNA and RNA, but also function as the universal energy currency in the cell with most biosynthetic processes fueled directly or indirectly by NTP hydrolysis. In *E. coli*, the total P content is ~3% of dry weight, making P one of the major macronutrients for cell growth (Neidhardt et al., 1990). The ribonucleotides are present at millimolar concentrations and their γ-phosphoryl groups turnover rapidly during growth. Most ATP is synthesized in respiring cells by ATP synthase using the energy of the proton-motive force. The free energy of hydrolysis of the phosphoanhydride linkages drives anabolic metabolism, while energy yielding catabolic metabolism can be coupled to ATP or GTP synthesis. GTP hydrolysis fuels the process of translation, one of the single most energy intensive processes in growing cells.

Phosphorous can also be used for a variety of other functions including most commonly the synthesis of the phospholipids of the membrane lipid bilayer. Phosphorus containing

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polymers are also abundant in the cell walls of Gram positive bacteria (in teichoic acids). When in excess, phosphate can be polymerized into polyphosphate, which is a potential storage form of P. Although each of these P-containing molecules can be a significant fraction of total cellular P, they are not universally present and may, in some cases, be expressed conditional on P availability (Fig. 7).

While it is generally accepted that P is required for life, it has been speculated that alternative types of biochemistry might be feasible in which P is substituted with As (Wolfe-Simon et al., 2009). This idea grew out of an exercise in trying to imagine what types of alternative biochemical processes might be able to evolve in extreme habitats (or on planets) where the availability of elements is substantially different (Davies et al., 2009). The authors postulate that there might exist on Earth a "shadow biosphere" of alternative chemistries and analysis of these "weird" organisms could shed light on possible lifeforms on other planets. This concept rose to international prominence recently with the publication of an article (Wolfe-Simon et al., 2011) with the unfortunately misleading title of "A Bacterium That Can Grow by Using Arsenic Instead of Phosphorus." In this specific case, a bacterium tolerant to growth in very high concentrations of As was suggested to use As in place of P in some of its macromolecules, and a specific claim was made for incorporation (albeit at a very low level) into DNA. Nevertheless, the evidence presented did not meet biochemical criteria for establishing chemical linkages (see Comments published in response to the article). One of the key concerns is that replacement of a significant amount of P by As is chemically implausible. The phosphoanhydride linkage is special because its hydrolysis has a high activation energy (requiring catalysis *in vivo*) but a negative free energy change, which drives group transfer reactions. Interested readers will find further discussions of these results in numerous online blogs, news commentaries, and several published articles (Danchin, 2010, Rosen et al., 2011, Silver and Phung le, 2011). As described below, there are in fact numerous examples of elegant solutions to the problem of limited P availability, and these can reduce the P requirements for growth significantly, but not eliminate this requirement completely. These responses often target P-rich components of the cell wall or membrane rather than NTPs or nucleic acids.

1. Acclimation to phosphorous limitation: cell wall remodeling in *Bacillus subtilis*—Growth of *B. subtilis* in phosphate limiting conditions activates a complex acclimation process regulated by the PhoPR two-component system (Hulett, 1996). A central feature of this acclimation mechanism is a remodeling of the cell wall as a mechanism to optimize growth. In media containing sufficient phosphate (~2 mM or more), *B. subtilis* cell walls contain an abundant anionic polymer known as teichoic acid that accounts for nearly 50% of cell wall weight (Bhavsar and Brown, 2006). Teichoic acids are alternating copolymers of glycerol and phosphate and can be linked to a glycolipid carrier (in lipoteichoic acid) or to the peptidoglycan cell wall (in wall teichoic acid). Cells grown in P limited medium repress the expression of the wall teichoic acid biosynthetic pathway and activate the expression of an alternative anionic polymer, teichuronic acid (Qi and Hulett, 1998, Lahooti and Harwood, 1999). In teichuronic acid, the carboxylates provide the negative charge that the phosphates provide in the teichoic acids. This is not necessarily a complete replacement, since the synthesis of teichoic acids may continue at a low

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maintenence level even under P limitation (Botella et al., 2011). Nevertheless, this remodeling of the cell wall is found to significantly reduce cellular P demand. Cells grown with limiting P contain only 32–47% as much P as cells grown in P-replete medium and the amount of P in the cell wall fraction was reduced by 25-fold (Lang et al., 1982). The ability of cells to reduce their P demand by a factor of 2 or more, simply by altering the composition of the cell wall (while maintaining a comparable level of anionic polymers in the wall), is an efficient mechanism for optimizing P usage. It is not yet clear to what extent other P-containing macromolecules (including lipoteichoic acid) may be functionally replaced, nor is it clear whether pre-existing teichoic acid is scavenged as a P source, although this seems reasonable.

2. Adaptation and acclimation by membrane phospholipid remodeling in Bacteria—Membrane phospholipids are responsible for a large fraction of the phosphorus content of both prokaryotic and eukaryotic cells. Heterotrophic and phototrophic microorganisms face P deficient growth conditions in unfertilized soils as well as in aquatic environments (Bieleski, 1973). P deficiency has been described for many regions of the open ocean, yet it does not appear to limit productivity because the inhabiting *Prochlorococcus* species have adapted to the low P content of that niche by reducing their P quota to 1:500, which is substantially lower than the Redfield ratio of 1:106 (Bertilsson et al., 2003). This is accomplished by replacement of membrane anionic phospholipids by anionic sulfolipids, specifically sulfoquinovosyl diglyceride (SQDG): less than 1% of the assimilated P is incorporated into membrane lipids, which spares P for nucleic acid and nucleotides, where its function is irreplaceable (Van Mooy et al., 2006). In comparing the fate of P assimilate in the Sargasso sea where P levels are <10 nM to the South Pacific subtropical gyre with 10-fold higher P concentrations, the authors noted 1.3% allocation to phospholipid in the former vs. 17% in the latter (Van Mooy et al., 2009). In parallel, analysis of membrane lipids from these locations showed that S- and N-containing lipids were more abundant in the former vs. the latter, and it was suggested that this P sparing adaptation is important for the success of prokaryotic *Prochlorococcus* and cyanobacterial species as well as eukaryotic phytoplankton in these environments. Measurements of various lipid types in laboratory experiments with P-replete vs. –deplete conditions indicate that the ability of the prokaryotes to synthesize a sulfolipid spares up to 43% of the P quota.

The replacement of phospholipids is a common acclimation response in bacteria faced with P deficiency. It has been documented in rhizobia, where SQDG, ornithine-containing lipids and diacylglyceryl trimethylhomoserine are used as substitutes, in *Pseudomonas*, where acidic glycolipids replace phospholipids, in gram positive *Marinococcus* species, where sulfolipid replaces phosphatidylglycerol, and anoxygenic photosynthetic bacteria where each of these replaces phospholipids in response to P deficiency (e.g. Minnikin et al., 1974, Benning et al., 1995, Geiger et al., 1999, Sprott et al., 2006). Cyanobacterial mutants blocked in sulfolipid synthesis or rhizobium mutants that are unable to synthesize ornithine or betaine lipids are growth compromised only in P-deficiency, indicating the importance of lipid substitution as a P-sparing mechanism (Güler et al., 1996, López-Lara et al., 2005).

Lipid substitution is an effective P-sparing response and degradation of pre-existing phospholipids also provides a P-recycling mechanism. Molecular genetic analysis in

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Rhizobium meliloti indicates that P-recycling is part of the phosphate-deficiency program mediated by the response regulator PhoB. A specific intracellular phospholipase C is induced so that the phospholipids (whose function can be covered by non-P-containing molecules) can be used as a pool of mobilizable P, which is recycled for molecules in which P is essential (like nucleotides) (Geiger et al., 1999, Zavaleta-Pastor et al., 2010).

3. P-sparing and recycling in Chlamydomonas—Because of the importance of P in agriculture (it is one of the major constituents of fertilizer), there is an excellent understanding of P metabolism and its regulation in reference organisms like *C. reinhardtii* (reviewed in Irihimovitch and Yehudai-Resheff, 2008, Moseley and Grossman, 2009). Three phospholipids (phosphatidylglycerol, -ethanolamine and –inositol) are prevalent in membranes of P-replete cells. In P-deficiency, phosphatidylglycerol is reduced by as much as 50%, concomitant with a greater than 2-fold increase in sulfolipids. The importance of this substitution is evident from the phenotype of an *sqd1* sulfolipid biosynthesis mutant, which grows poorly under P starvation (Riekhof et al., 2003). Nevertheless, because of specific binding sites for lipids in membrane protein complexes (e.g. phosphatidylglycerol in photosystem II; PS II), sulfolipid cannot completely replace phospholipid in the thylakoid membrane (Yu et al., 2002).

P sparing via membrane lipid re-modelling is conserved throughout the plant lineage, and has been documented in moss, Arabidopsis and perennial rye grass, species that are separated from Chlamydomonas by a billion years of evolution (e.g. Yu et al., 2002, Wang et al., 2008, Byrne et al., 2011). In Arabidopsis, *SQD1* mRNA and protein are dramatically increased in P-deficient plants and promoter-reporter fusions implicate transcriptional regulation (Essigmann et al., 1998, Hammond et al., 2003). In bacterial, algal, as well as Arabidopsis *sqd* mutants, the level of glycerolipids is maintained (or even increased), recycling of P is precluded, and mutant cells grow poorly in P-deficient conditions. The eukaryotic phytoplankton, such as the diatoms *Thalassiosira pseudonana* and *Chaetoceros affinis* and the coccolithophorid *Emiliana huxleyi*, use both sulfolipids and betaine lipids as substitutes for phospholipids, which spares about 10–30% of the P quota (Van Mooy et al., 2009, Martin et al., 2011).

Nucleic acids represent the other major reservoir of P. In *C. reinhardtii*, where the plastid genome is polyploid with up to 80 copies per cell, there is evidence for copy number reduction, which could release P for recycling to other processes. Interestingly, plastid mRNA abundance increases under P limitation because of down-regulation of a polynucleotide phosphorylase, a phosphorylytic enzyme responsible for cpRNA degradation which requires phosphate as a substrate. The regulation is dependent on Psr1, a Myb-domain transcriptional activator, which also turns on extracellular phosphatases and assimilatory transporters in P-deficient *C. reinhardtii* (Wykoff et al., 1999, Yehudai-Resheff et al., 2007). The up-regulation of nucleases in P-deficient plants has been noted in moss and Arabidopsis, but specific intracellular targets have not yet been identified and therefore whether this nucleic-acid based acclimation response extends beyond *C. reinhardtii* is not known.

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III. Microbial Adaptation and Acclimation to Metal Ion Limitation

Some metal ions are essential or beneficial for life, but others are neutral or even harmful, and some of the beneficial ones can become harmful when they are present in excess. The importance of metals in biology is reflected in the maturation of bioinorganic chemistry as a distinct discipline with its own meetings, societies, and journals including, for example, Bioinorganic Chemistry (Elsevier; initiated 1970), Biometals (Springer; 1997) and, more recently, Metallomics (RSC publishing, 2009).

It is estimated that 30% or more of proteins contain at least one metal cofactor in most organisms (Waldron et al., 2009, Seravalli and Ragsdale, 2010). The Zn metalloproteome is typically 5–6% of proteins in Bacteria to near 10% in Eukaryotes, with Fe-containing proteins having an inverse trend (Andreini et al., 2009). The key chemical properties of metal ions that contribute to their essential roles are their ability to serve as electron carriers and to function as electrophilic centers in catalysis. The midpoint potentials of many biological Fe and Cu centers are well suited for their electron carrier roles, and Fe, Zn, Mn, Co, and Mg are well suited for roles as electrophilic catalysts. Metal ions can also serve as organizing centers for the folding of small protein domains as exemplified by Zn finger proteins or Ca-binding domains, which are abundantly represented in eukaryotes.

Microbial responses to metal ions typically follow a gradient including states that can be defined as metal-limited, metal-deficient, metal-replete, or metal-excess. **Limitation**, also known as starvation, refers to a lack of an essential metal that leads typically to a cessation in the ability of the cell to grow and may lead to a loss of viability. **Deficiency** refers to a sub-optimal level of metal availability that impacts cell physiology and leads to measurable alterations in metabolism. Metal-replete conditions are those that provide enough metal to support growth and the accumulation of the full quota of proteins that require that metal, whereas metal excess refers to conditions where high concentrations of metals begin to negatively impact growth or may lead to cell death. As cells transition from replete to deficient to limited conditions they engage a variety of acclimation responses which include, as for the macronutrients, elemental acquisition, mobilization, sparing, and recycling. Conversely, as cells transition from replete to excess, they engage mechanisms to store excess metals for future use or may efflux the ions from the cell. Although detailed metal requirements vary significantly between species, similar acclimation strategies have evolved in multiple organisms across phylogenetic boundaries.

A. Metal homeostasis across three domains of life

The use of metals within biology differs substantially between the Bacteria and Archaea and the Eukarya. In some cases, these differences reflect adaptations to specific niches. For example, the Archaeon *Ferroplasma acidiphilum* grows in highly acidic environments rich in Fe(II), the more bioavailable oxidation state, and routinely uses Fe in place of Zn to organize metalloprotein domains (Ferrer et al., 2007). More generally, the prevalence of different metal ions in various organisms reflects their evolutionary history. Life evolved in the ancient oceans and, in many ways, cells reflect the chemical composition of this environment (Dupont et al., 2010). The ancient (Archaen) ocean in which life evolved (beginning ca. ~4.5 billion years ago; GYA) was anoxic and chemically reducing with

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relatively high concentrations of Fe, Mn, Ni, and Co. The evolution of oxygenic photosynthesis contributed to an increase in atmospheric oxygen (~2.4 GYA) leading to an eventual transition to an ocean with oxygen-rich surface waters. As the ocean waters became more oxidizing (by ~0.8 to 0.5 GYA), Cu, Zn, and Mo became abundant whereas Fe, Mn, Ni, and Co were greatly reduced in abundance. Thus, Fe-containing proteins like cytochromes and ferredoxins and many Ni-containing enzymes are ancient, while Cu proteins are a more recent innovation (Crichton and Pierre, 2001).

With the availability of thousands of microbial genomes, and an ever improving ability to discern metal specificity from protein sequence, it has been possible to distinguish several trends in microbial metal ion utilization (Dupont et al., 2010). Bacteria and Archaea contain a core group of Zn proteins, often involved in central metabolism (transcription, translation), and comparatively more Fe, Mn, and Mo proteins. Assigning metal specificity to protein domains is not trivial, but it is notable that many of the earliest evolving protein domains appear to be cambialistic (able to use or function with multiple metal cofactors). Later evolving Eukaryotes make much greater use of Ca, Cu, and Zn, with the latter represented by the proliferation of Zn finger proteins. Whereas many non-heme Fe enzymes are conserved in all Domains of life, consistent with their early appearance in evolution, many Cu enzymes are specific to the eukaryotes and likely evolved after the great oxidation event when the bioavailability of Cu as cupric ion increased (Crichton and Pierre, 2001, Andreini et al., 2009). Conversely, the use of Ni as an enzyme cofactor appears to have declined over time (Zhang et al., 2009).

Metal ion deficiencies are also widely appreciated in both medicine and agriculture. For instance, Fe deficiency anemia alone is estimated to affect nearly 1.7 billion people (25% of the population) (Benoist et al., 2008). Similarly, Fe-deficiency chlorosis is a common problem in agriculture, especially in alkaline soils. The consequences of excess exposure can also be severe, even for those metals required for life. Sophisticated metal ion homeostasis systems are therefore operative in cells to prevent the adverse effects of either deficiency or excess. Defects in metal ion homeostasis pathways, often involving proteins first defined in model systems, are increasingly recognized as a source of genetic disorders (Bleackley and Macgillivray, 2011).

Competition for limiting metal ions can be a determining factor for the outcome of host-pathogen interactions. The Fe-withholding response is an important feature of the innate immune system in mammals (Ganz, 2009). Bacterial pathogens have responded to the low levels of available Fe in the human host by elaboration of very high affinity siderophores such as enterobactin made by *E. coli* and its relatives (Fischbach et al., 2006). Humans have responded by the synthesis of a high affinity siderophore binding protein known as lipocalin or siderocalin. As this evolutionary arms race has continued, *Salmonella* has evolved the ability to decorate enterobactin with glycosyl groups (generating a family of compounds known as Salmochelins), thereby rendering lipocalin ineffective (Fischbach et al., 2006, Muller et al., 2009). The success of macrophages in killing engulfed bacteria can also depend on metal ion competition: the NRAMP family of divalent metal ion transporters were named for their role as "natural resistance associated macrophage proteins." Subsequent to engulfment, NRAMP proteins deplete the phagocytic vacuole of Mn (and

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possibly Fe) thereby limiting bacterial growth (Cellier et al., 2007). Many bacteria, in turn, also have NRAMP family transporters working to import these very same cations. Neutrophils also play a role in the competition for metals by secreting a protein, calprotectin, that sequesters Mn and Zn (Corbin et al., 2008, Kehl-Fie and Skaar, 2010, Kehl-Fie et al., 2011). Metal toxicity is also employed as part of the macrophage killing arsenal by the delivery of redox active Cu to the phagosomal compartment (White et al., 2009, Wakeman and Skaar, 2011). Genetic studies indicate that, individually and collectively, these mechanisms can have a large impact on the outcome of infection (White et al., 2009, Haley and Skaar, 2011, Hammer and Skaar, 2011).

B. Challenges in defining the roles of metals in biology

The concept of the metallome, as first coined by RJP Williams (Williams, 2001), refers to the quantitative description of the metal contents of cells. Ideally, metallomics seeks to describe both the amounts of each metal required for life and their distribution within the cell and its sub-cellular compartments. Clearly, the metallome differs between organisms and depends on the precise growth conditions studied. For many metals, the major fraction of the metallome is bound to proteins (the metalloproteome). However, efforts to define the nature of the metalloproteome are still in their early stages and there are many surprises still in store (Cvetkovic et al., 2010, Seravalli and Ragsdale, 2010).

The application of high sensitivity techniques for elemental analysis, such as inductively-coupled plasma mass spectroscopy (ICP-MS), allows the quantitation of elements down to at least the 1 part-per-billion (ppb) range. It is thereby possible to determine the elemental composition of cells with very high sensitivity and over many orders of magnitude of concentration. The amount of metal ion per cell is sometimes referred to as the metal quota. The very sensitivity of this technique, however, leads to additional challenges because of the ease with which samples can be contaminated. Analyses of trace elements in environmental samples, and in particular ocean waters (Sohrin and Bruland, 2011), have required the development of sophisticated sampling methods and the corollary laboratory studies typically require extensive efforts to prevent contamination including the use of clean-rooms (e.g. Shiller and Boyle, 1985, Trefry et al., 1985, Tortell and Price, 1996).

While elemental analyses of cells can define metal ion quotas, these measurements do not distinguish metal ions bound to proteins or other macromolecules relative to those that are hydrated or bound to low molecular weight ligands. Nor do these measurements, by themselves, distinguish between cell compartment and organelles or between metal that is required for metabolism or stored for future use. When grown under replete conditions, much of the metal may be in storage compartments rather than in active use. Conversely, the metal remaining in metal-limited cells is likely to define a lower limit capable of supporting growth under the tested conditions. The metal quota of a replete cell was used recently in an approach to devise a trace element mix for *C. reinhardtii* (Kropat et al., 2011). This resulted in removal of non-beneficial elements (Co, B) and reduction of others (Zn, Mn) to provide only 3-fold the quota of a healthy cell, which allows room for accommodating an increased quota in situations of altered physiology.

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The distribution of metals between their various possible coordination environments (referred to as speciation) varies enormously between metals, but is often quite similar between cells, governed as it is by the fundamental properties of the ions themselves (Waldron and Robinson, 2009). For example, Mg(II) is very soluble as a hydrated ion and is often present at high concentrations in cells where it is frequently complexed with phosphoryl groups in nucleic acids and other metabolites. Conversely, metals like Zn and Cu are tightly bound either to proteins or other chelating groups within the cell (Colvin et al., 2010, Robinson and Winge, 2010). As a result, the equilibrium concentration of free ions is sub-picomolar, although there is nevertheless a substantial pool of ions within the cell that is kinetically accessible for incorporation into nascent metalloproteins (Finney and O'Halloran, 2003). For some metals, specific protein "chaperones" ferry metals from their sites of uptake to their target proteins (O'Halloran and Culotta, 2000). Such metallochaperones play important roles in the delivery of Cu to specific proteins (Robinson and Winge, 2010), in the assembly of Fe/S clusters (Subramanian et al., 2011), in the insertion of Fe into protoporphyrin to generate heme, and the insertion of Ni into urease (Carter et al., 2009).

The speciation of metals in cells is governed by both kinetics and thermodynamics. The thermodynamics is described by the Irving-Williams series (Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II)). The ability of Cu to bind more tightly to ligands than other ions leads to toxicity, in part owing to the disruption of Fe/S centers in enzymes (Macomber and Imlay, 2009, Chillappagari et al., 2010). Kinetics is also important, since the ease with which metal-ligand bonds can be exchanged can determine the availability of the metal, both environmentally and within the cell. Intracellular movement of Cu is controlled by protein-protein interactions involving metal binding domains on target proteins and metallochaperones (Tottey et al., 2005, Boal and Rosenzweig, 2009). Proteins that require Cu for function are therefore metallated only by a specific Cu chaperone or after export from the cytosol (Tottey et al., 2008).

Defining which metals are absolutely essential for growth is experimentally challenging even in the best understood model systems. Since many microorganisms can be grown in chemically defined media, it would appear to be easy to define the minimal elemental requirements for growth. Indeed, in the simplest cases the requirements are sufficiently high that they can be determined by monitoring growth as a function of added metal ion. However, in other cases the low level requirements, contamination of reagents and growth chambers, and the presence of high affinity acquisition systems make this approach problematic. Indeed, the impressive ability of cells to scavenge miniscule quantities of essential metals from their environment is a recurring challenge in metal ion nutrition studies. High affinity transport systems are likely present for all essential metals and may involve the secretion of high affinity chelators such as siderophores for Fe. Researchers sometimes rely on chelators to impose metal limitation, but chelation reduces but does not eliminate the bioavailability of the metal and has the added complication that chelators are generally not specific for a single element. The use of high purity chemicals and acid-washed glassware is important for reproducibility in laboratory experiments (e.g. Cox, 1994, Quinn and Merchant, 1998). However, metal ions tend to slowly leach even from acid-washed glassware and for some studies more stringent conditions are needed.

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In one notable example, experiments to study the effects of Zn depletion in *E. coli* required the pretreatment of all non-metal media components with a solid-phase metal chelator (Chelex-100) and the use of a chemostat made from non-metal components (Graham et al., 2009). Cells were pregrown in Zn free chemically defined medium and yet, when the cells were recovered they contained more Zn than was added to the entire volume of growth medium used for their culture. This anomaly can be accounted for by the ability of cells to leach Zn from within the glass walls of the culture flask, despite the prior removal of bound metals by acid-washing. Indeed, the extent of Zn limitation increased with subsequent uses of the same flask, suggesting that *E. coli* is more efficient at removal of excess Zn from the culture flask than chemical chelation and acid-washing (Graham et al., 2009). Similar observations were made in laboratory experiments to generate Mn-deficiency in *C. reinhardtii* (Allen et al., 2007b).

Ultimately, the goal of metallome studies is to define both the metals needed for cellular functions, and to identify the enzymes that require metals for their activity. While the study of metalloenzymes *in vitro* has a long history, there are now several examples where the cofactor required for *in vivo* function was initially misassigned (Jain et al., 2005, Chai et al., 2008). This reflects the fact that metalloenzymes are often assayed under conditions that may not mimic the levels of metal ion availability in the cell. Moreover, many Fe-containing enzymes must be assayed anaerobically and, when assays are done aerobically, the strongest activation will often be provided by a different ion (Tripp et al., 2004).

An additional complexity in defining the metals essential for life is that, for some functions, metal ions play redundant roles. For example, two enzymes may each use a different metal ion to catalyze the same reaction and thereby be functionally redundant. This is the case, for example, with the multiple SOD isozymes in many organisms. In other cases, a single protein may be able to function with more than one ion. Such cambialistic enzymes include a subset of SODs (Priya et al., 2007), some carbonic anhydrases (Lane and Morel, 2000b), and a lipid A biosynthetic enzyme (Gattis et al., 2010). More generally, it has been suggested that many *E. coli* enzymes that may normally function with a non-heme Fe as cofactor may, under conditions of oxidative stress, use Mn instead (Anjem et al., 2009). Although there are relatively few well defined examples to date, these studies suggest that functional redundancy is likely to be more widespread than generally appreciated. One result is that the required levels of each metal may be interdependent, especially for abundant metalloenzymes. For example, for some phytoplankton limitation for Zn can be partially alleviated by Co or Cd (Morel, 2008). In marine systems, low Zn levels are also correlated with low P availability, which is problematic since alkaline phosphatase, a key P acquisition enzyme, is itself a Zn enzyme. To circumvent this Zn-P colimitation, many *Prochlorococcus* species contain a distinct Ca-dependent phosphatase, PhoX (Kathuria and Martiny, 2011). These complex interactions between elements emphasize the challenges inherent in defining a minimal or representative metallome for any organism.

Recent years have seen the development of powerful new techniques for monitoring metal ion speciation in cells, including both genetically encoded and chemical sensors to monitor metal availability in living cells (reviewed in Cook et al., 2008, Domaille et al., 2008, McRae et al., 2009, Vinkenborg et al., 2010, Palmer et al., 2011), nanometer-scale imaging

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Secondary Ion Mass Spectrometry (nanoSIMS) and related techniques to monitor spatial distributions of metals on a sub-micron scale (e.g. Orphan and House, 2009, Byrne et al., 2010), and fractionation techniques for monitoring metalloproteomes (Cvetkovic et al., 2010). These analytical techniques, particularly when applied to genetically amenable model organisms, will likely enable new insights into the diverse roles of metal ions in cells and, as a corollary, increase our understanding of the corresponding acclimation mechanism when metals become limiting.

C. Iron (Fe): A near universal transition metal and redox center

Iron is required for the growth of nearly all cells. In cells, Fe is found in heme proteins such as catalase and cytochromes, iron-sulfur cluster (Fe/S)-containing electron carriers and enzymes, and non-heme Fe enzymes. In most cell types, Fe has multiple essential roles in the cell. For instance, both heme and Fe/S clusters are commonly essential for cell growth <Figure 8>.

The only known exceptions to this general requirement are certain Bacteria that grow in severely Fe-limited environments. For example, the Lactobacilli and the spirochete *Borrelia burgdorferi* (causative agent of Lyme disease) are thought not to require Fe (Weinberg, 1997, Posey and Gherardini, 2000). In the case of *B. burgdorferi*, there are at most a handful of Fe atoms present in the cell and no known or demonstrable requirement for Fe for growth (Posey and Gherardini, 2000). In addition to dispensing with heme and Fe/S-containing proteins, enzymes that in other organisms often require Fe for catalytic activity use a different metal in these systems. For example, in both *L. plantarum* and *B. burgdorferi* peptide deformylase contains a catalytically essential Zn in place of what would normally be Fe (Nguyen et al., 2007). This is an illustration of the general principle that one way in which cells adapt or acclimate to metal limitation is to replace one metal cofactor with another.

1. Overview of molecular mechanisms of adaptation and acclimation to Fe-limitation—Most bacteria, and all known eukaryotes, do not have the luxury of simply dispensing with a requirement for Fe. For these organisms, Fe is essential for growth and the response to Fe limitation is to reduce, to the maximal extent possible, the burden placed on the cell by the need to obtain Fe. In many different microbial systems, acclimation to Fe limitation involves Fe-sparing and Fe-recycling mechanisms analogous to those discussed above for macronutrients. The Fe-sparing response reduces the requirement for Fe-containing proteins by synthesizing alternative enzymes using either organic cofactors or other metal ions in place of Fe, and concurrently shutting off the synthesis of low-priority Fe proteins. In this context, low priority refers to those functions that are not absolutely essential for growth or which can be functionally replaced by other proteins and pathways. In some cases, as we will see, cells may also recycle Fe: they actively degrade pre-existing Fe-containing protein complexes simply to recover the valuable sequestered Fe.

Acclimation to Fe limitation requires, first, that cells have mechanisms to monitor their Fe status and alter gene expression appropriately. In Bacteria, iron homeostasis is regulated by specific Fe-sensing metalloregulatory proteins, such as the ferric uptake repressor (Fur) and

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the diphtheria toxin repressor (DtxR), which serve to sense the cytosolic availability of Fe(II) (Hantke, 2001, Andrews et al., 2003). In *S. cerevisiae* and other fungi, Fe-acclimation responses are coordinated by functionally equivalent metalloregulatory proteins such as Aft1 and Aft2 (Philpott and Protchenko, 2008), whereas *C. reinhardtii* may use orthologs of the regulatory proteins found in Arabidopsis (Long et al., 2010).

Fur is representative of a large group of metalloregulators that sense metal ions such as Fe, Zn, Mn, and Ni to regulate metal homeostasis (Lee and Helmann, 2007). Typically, Fur family regulators act as metal-dependent transcriptional repressors, although there are exceptional organisms where Fur proteins can also act as direct activators by binding upstream of target genes and enhancing the recruitment or activity of RNA polymerase (Delany et al., 2004, Danielli et al., 2006). Under Fe replete conditions, Fur binds Fe(II) and represses the expression of uptake function and activates the expression of Fe storage proteins. Conversely, when Fe levels drop, iron uptake functions are derepressed and pathways contributing to acclimation are induced.

In response to Fe deficiency, induction of the Fur regulon leads to the expression of iron acquisition systems. Fur directly regulates the synthesis of high affinity uptake systems and chelators (siderophores) for import of Fe. This can potentially relieve the shortage, but only if accessible Fe is present in the environment. Fe may also be mobilized from intracellular stores including ferritins, bacterioferritins, and mini-ferritin/Dps family proteins. Ferritins can sequester up to ~5000 atoms of Fe while the smaller miniferritins can sequester 500 atoms within their spherical protein cores (Bevers and Theil, 2011). Although the pathways of Fe mineralization into these storage proteins are well studied, the processes by which this Fe is mobilized upon starvation are less clear (Smith, 2004). Fe mobilization likely involves the gating of specific pores (Liu et al., 2003, Bevers and Theil, 2011), and presumably also requires either a specific or non-specific reduction of the oxo-Fe core to release soluble Fe(II). In mammals, autophagy by degradation in the lysosome also plays a role in Fe recovery (De Domenico et al., 2009). As cells transition from iron deficient to iron limited conditions, they additionally engage programs of Fe-sparing and, in some systems, Fe-recycling.

Insights into mechanisms of adaptation and acclimation to Fe limitation emerged early in ecological studies of Fe-limited systems and, in particular, studies of the marine ecosystem and its constituent microorganisms. These studies are complemented by detailed analyses of molecular mechanisms in reference organisms (including the Bacteria *E. coli* and *B. subtilis* and the Eukarya *S. cerevisiae* and *C. reinhardtii*) that have provided insights into how cells acclimate to changes in Fe availability.

2. Fe-sparing and Fe-recycling: insights from marine picoplankton—Primary productivity of about 30% of the world's ocean is Fe-limited, due in part to the high Fe-demand for photosynthesis (Raven et al., 1999, Behrenfeld et al., 2009). In addition, Fe-containing proteins are widely used for various redox reactions in cellular metabolism. Ocean surface waters often contain very low levels of Fe, with measurements indicating between 0.01 and 2 nM (Sandy and Butler, 2009). Marine bacteria, like many other Fe-limited organisms, synthesize and secrete siderophores (Sandy and Butler, 2009), express

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high affinity transport systems, and implement complex acclimation strategies to maximize growth in the face of elemental limitations. Here, we focus specifically on adaptations that have emerged in this environment and on documented examples of acclimation by Fe-sparing and Fe-recycling.

a. Fe-sparing by substitution of ferredoxins with flavodoxins: Perhaps the most widespread acclimation strategy for Fe-limitation is replacement of the electron carrier protein ferredoxin with an Fe-free alternative, flavodoxin. Ferredoxins are Fe/S proteins with negative midpoint potentials and they serve as electron donors in many biosynthetic reactions and, accordingly, are often abundant. In 1966, a protein with ferredoxin activity was isolated from *Clostridium pasteuranium* cultured in iron-poor medium (Knight et al., 1966). This protein had a bound flavin and was named flavodoxin. Subsequent studies revealed that the substitution of ferredoxin with flavodoxin allows the former to be actively degraded in order to release and recycle Fe for maintenance of pyruvate synthase (another Fe/S protein) (Schönheit et al., 1979).

The loss of ferredoxins appears to have emerged as a genome adaptation for a clade of *Prochlorococcus* that is associated with very low Fe (estimated at <0.5 nM) in high nutrient, low chlorophyll (HNLC) regions of the ocean (Rusch et al., 2010). Metagenomic analyses suggest that *Prochlorococcus* strains from this environment have lost ~10% of the estimated 60 or so predicted Fe-containing proteins found in similar strains from more Fe-rich environments. The missing genes encode two ferredoxins, a plastoquinol terminal oxidase, and a cytochrome. Thus, one mechanism for adapting to a chronic limitation for Fe is to reduce the number of Fe requiring proteins encoded in the genome (Rusch et al., 2010).

A more common evolutionary strategy is to retain the genes for both ferredoxins and flavodoxins, but only express the former when Fe is relatively abundant. The Fe-regulated substitution of ferredoxin with flavodoxin as a major electron carrier has been noted in numerous organisms including a chlorophyte alga, several cyanobacterial species, cryptomonads, and diatoms (e.g. Zumft and Spiller, 1971, Hutber et al., 1977, Sandmann and Malkin, 1983, Ragsdale and Ljungdahl, 1984, McKay et al., 1997, Li et al., 2004). The reciprocal pattern of expression is consistent with an Fe-sparing mechanism, since the substitution will decrease the Fe quota of the cell. The phenomenon is so widespread in nature that flavodoxin abundance is now the accepted standard biomarker for assessing Fe-status in the marine environment (La Roche et al., 1996). Down-regulation of ferredoxin in Fe-deficiency occurs even in organisms that do not have genetic information for flavodoxin. In these situations ferredoxin is essential and Fe-deficient cells reduce the abundance of ferredoxin, but do not eliminate it entirely (e.g. Pardo et al., 1990, Terauchi et al., 2010).

In *Synechococcus* sp. PCC7942, the regulation of ferredoxin occurs by Fe-dependent stabilization of the *petF* transcript. Induction of flavodoxin, encoded by the iron-starvation inducible gene *isiB*, occurs at the level of transcription (Leonhardt and Straus, 1992, Bovy et al., 1993). In many cyanobacteria, *isiB* is part of an Fe-deficiency stress operon that also encodes a modified antenna for the Fe-rich photosystem I (PS I) (discussed below).

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In a cyanobacterial strain, *Nostoc* sp., Fe deficiency had a different impact on two ferredoxins of different midpoint potentials, leading to the suggestion that flavodoxin may not substitute for all the reactions of ferredoxin. Accordingly, the ferredoxin whose activity could be replaced by flavodoxin was more rapidly lost upon transition to Fe deficiency. Flavodoxins can replace ferredoxin in photosynthesis, where the proteins are acceptors from PS I and donors to $NADP^+$ via a ferredoxin $NADP^+$ oxidoreductase, and as substrates for a number of enzymes like ribonucleotide reductase, nitrate reductase, pyruvate formate oxidoreductase, hydrogenase, nitrogenase and the fatty acid desaturation reactions (e.g. Sandmann et al., 1990, Gangeswaran and Eady, 1996, Cotruvo and Stubbe, 2008, Chazarreta-Cifre et al., 2011). Biochemical comparison of ferredoxin and flavodoxin from *Synechocystis* sp. 6803 indicates very similar physical properties of the two proteins (with respect to isoelectric point and midpoint potentials) compatible with co-evolution in response to common reaction partners (Bottin and Lagoutte, 1992).

Curiously, although this Fe-sparing mechanism is widespread in microbes, it appears to have been lost in land plants, even though Fe can be a limiting nutrient in the soil environment (Yi and Guerinot, 1996). Transgenic expression of a chloroplast-targeted flavodoxin complements a ferredoxin mutant, indicating that the protein can function in the plant chloroplast, so its absence in plant genomes remains a mystery (Blanco et al., 2011). Most plants, including algae, express multiple ferredoxin isoforms with specificity for a subset of the many ferredoxin-dependent reactions in the chloroplast (e.g. Hanke et al., 2004, Terauchi et al., 2009). The slightly reduced specificity of flavodoxin interaction with reaction partners compared to that of ferredoxin may have led to its loss in the land plant lineage in the absence of selective pressure for Fe sparing (Zurbriggen et al., 2007, Goñi et al., 2008).

b. Fe-sparing by substitution of FeSOD with MnSOD and NiSOD: The replacement of FeSOD by MnSOD is another widespread Fe-sparing mechanism. It has been noted in bacteria, algae, as well as diatoms (Privalle and Fridovich, 1993, Wolfe-Simon et al., 2006, Allen et al., 2007b). In organisms adapted to the low Fe content of the open ocean, the use of MnSOD may contribute to their higher Mn quota (Peers and Price, 2004). The two SODs evolved from a common ancestor and are structurally highly similar, with specificity for Fe vs. Mn determined by the second shell ligands (Wintjens et al., 2004). Accordingly, in bacteria with an Fe-sparing genetic program, there are separate Fur-regulated genes, *sodA* and *sodB*, for each form (see below). Interestingly, it was reported that Cu can replace Fe or Mn, but the resulting enzyme is less active (Meier et al., 1994). This type of substitution may be adventitious rather than programmed.

An adaptation that has emerged in some organisms faced with chronic Fe limitation is the evolution of NiSOD (SodN). SodN is a structurally distinct protein that can functionally replace FeSOD. It has been noted that genes encoding SodN are widespread in the marine cyanobacteria and are correlated with the presence of urease, another (typically) Ni-requiring enzyme. Together, these two enzymes contribute to a Ni-requirement for bacteria in the ocean (Dupont et al., 2008b) while concomittantly reducing the cellular Fe quota. *Streptomyces griseus* and *S. coelicolor* also use a NiSOD to replace an Fe/ZnSOD and an

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FeSOD, respectively, but its use is controlled by Ni availability rather than Fe unavailability (see below).

c. Fe-sparing and Fe-recycling by remodeling of PS I: In aerobic photosynthetic organisms, about half the Fe in the photosynthetic apparatus is found in PS I, which has three Fe_4S_4 centers. Accordingly, down-regulation of PS I is a common Fe-sparing acclimation response to Fe-deficiency in cyanobacteria, diatoms, and algae (Moseley et al., 2002, Strzepek and Harrison, 2004). In cyanobacteria, the down-regulation of PS I is accompanied by a complete re-modelling of the PS I-associated peripheral antenna to change the supply of excitation energy to that photosystem (Park et al., 1999, Ivanov et al., 2000, Havaux et al., 2005). The new antenna protein is encoded by the *isiA* (for iron stress induced) gene and is often co-transcribed with the previously-mentioned *isiB* gene encoding flavodoxin (Laudenbach et al., 1988, and see above). Indeed, *isiA* sequences are retrieved with the highest frequencies in metatranscriptome surveys of plankton communities in the oligotrophic open ocean. It ranked in the top 4 during a bloom of *C. watsonii* in the SW Pacific (Hewson et al., 2009). In *Synechocystis* 6803, the transcription of *isiA* is repressed by Fur in the Fe replete situation. When Fe is limited, the *isiA* operon is derepressed, although expression is limited, at least initially, by an antisense RNA (*isrR*) encoded on the opposite strand (Dühring et al., 2006). Diatoms adapted to low Fe content in the ocean appear to have lowered the ratio of PS I to PS II and also reduced the abundance of cytochrome-containing complexes to reduce the cellular Fe quota (Strzepek and Harrison, 2004).

A fascinating example of Fe-sparing and recycling to reduce the cellular Fe quota was recently described in a marine cyanobacterium (Saito et al., 2011). The constitutive expression of flavodoxin in this organism suggests that it is adapted to persistent Fe-limitation. In many photosynthetic diazotrophs, photosynthesis and N_2-fixation are separated either in space or time because of the incompatibility of oxygenic photosynthesis with the O_2-sensitive metalloclusters in dinitrogenase (e.g. Schneegurt et al., 1994, Steunou et al., 2008). Temporal separation in *Crocosphaera watsonii* offers an opportunity for reducing the Fe quota. Proteomic analysis showed that nitrogenase metalloproteins changed from being the most abundant in the dark phase to being undetectable in the light phase while the Psa proteins of the Fe/S containing PS I complex and cytochromes showed the opposite pattern. The authors calculate that the “sharing” of intracellular Fe by daily degradation of the iron proteins and recycling of the released Fe reduces the Fe requirement by 40%, which would clearly be advantageous in a low Fe environment.

When an organism is never in a state of Fe luxury owing to persistent (over evolutionary time scales) poor Fe supply in its niche, it may simply dispense with the gene for an Fe-containing protein, as suggested by the constitutive use of Cu-containing plastocyanin in place of cytochrome (Cyt) c_6 in the diatom *T. oceanica* (Peers and Price, 2006). The trade-off is that this organism is now more sensitive to Cu deficiency due to an increased requirement for Cu for photosynthesis. However, since Cu levels in the open ocean (0.4–1 nM) are often higher than Fe levels, this is favorable adaptation (Peers and Price, 2006). A converse example is offered by a subset of the chlorophyte (green) algae, which appear to have dispensed with the genes encoding CuZnSODs in favour of Cu allocation to plastocyanin and Cyt oxidase, owing perhaps to persistent Cu-deficiency in their

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environments (see below) (Asada et al., 1977). In these organisms, SOD function is provided by Fe- and Mn-containing enzymes.

3. Fe homeostasis in *E. coli*—Bacterial iron homeostasis is exceptionally well understood in *E. coli*, which is the reference organism for many different aspects of bacterial metabolism (Andrews et al., 2003). *E. coli* normally accumulates Fe to a level corresponding to ~1 mM averaged over the cell volume, with the majority of this Fe bound to proteins. The acclimation of *E. coli* to Fe limitation is regulated by Fur, which senses the cytosolic availability of Fe(II) (Hantke, 2001).

E. coli mutants unable to maintain Fe homeostasis, due to inactivation of the *fur* gene, have a number of striking phenotypes. They display an elevated sensitivity to reactive oxygen species, such as hydrogen peroxide (Touati et al., 1995), are unable to grow on succinate, and are relatively resistant to elevated levels of Mn which presumably acts as a Fur agonist and inappropriately represses Fe uptake (Hantke, 1987). Although essential for the function of numerous enzymes, Fe(II) in excess is toxic due to its ability to catalyze (via Fenton chemistry) the formation of destructive hydroxyl radicals (Imlay and Linn, 1988). This problem is exacerbacted in *E. coli*, since even low levels of endogenously produced oxidants have the potential to inactivate the Fur:Fe(II) complex leading to further elevation of intracellular Fe levels (Varghese et al., 2007).

The peroxide-sensitivity of *fur* mutant cells provided early evidence that cytosolic Fe levels are elevated (Touati et al., 1995), as might be expected from the constitutive expression of Fe import functions. Evidence for elevated cytosolic Fe was obtained using a whole cell electron paramagnetic resonance (EPR) approach in which desferrioxamine is added to stabilize chelatable (bioavailable) Fe as Fe(III) (Keyer and Imlay, 1996). This analysis led to an initial estimate of the level of Fe in the *E. coli* cytosol as ~10 μM with this increasing to ~70 μM in the *fur* mutant strain (Keyer and Imlay, 1996), although subsequent studies have measured values as much as 10-times higher (Jacques et al., 2006). This chelatable Fe pool represents an approximation of the labile Fe pool (presumably Fe in transit from uptake systems to protein targets) and may also include some Fe stripped by the chelator from enzymes with a relatively weak Fe affinity. The nature of the labile Fe pool is poorly understood, and indeed the whole issue of metal speciation in the cell is largely unexplored. The development of whole cell spectroscopic approaches, which is particularly amenable to genetically-tractable microorganisms, is promising in this regard (reviewed by Lindahl and Holmes-Hampton, 2011).

Although the labile Fe pool is elevated in *fur* mutants, S. Andrews and coworkers have noted that *fur* mutant cells are actually Fe-deficient with total Fe levels reduced 2.5-fold relative to wild-type (WT) (Abdul-Tehrani et al., 1999). These observations can be reconciled, since the labile Fe pool measured by EPR is a small subset (routinely less than 10%) of total cellular Fe: although *fur* mutants have an elevated chelatable Fe pool, their total Fe content is significantly reduced due to repression (mediated by RyhB, see below) of numerous Fe-containing enzymes. The same type of remodeling of the proteome, to efficiently utilize Fe, occurs during the acclimation of *E. coli* to Fe limitation. Indeed, sudden imposition of Fe starvation leads to large changes in gene expression which can be

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modeled as resulting from transient changes in a labile Fe pool that represents ~1% of the total Fe needed by the cell and that directly controls Fur activity (Amir et al., 2010).

Fur contributes to Fe homeostasis in numerous ways. First, Fur directly represses, under replete conditions, Fe acquisition pathways. Second, Fur indirectly activates synthesis of the Fe-storge protein ferritin under Fe replete conditions. Fur binds to the region upstream of the *ftnA* gene and acts to prevent gene repression by the H-NS nucleoid protein (Nandal et al., 2010). Third, Fur regulates RyhB, a small, non-coding RNA (sRNA) with wide-ranging effects that serves to remodel the proteome as part of a global Fe-sparing response (see below). Fourth, Fur regulates the expression of enzymes that can substitute for what would otherwise be Fe-dependent pathways. The synthesis of deoxyribonucleotides (from their ribonucleotide precursors) is essential for DNA replication, and most bacterial ribonucleotide reductases contain Fe as an essential catalytic cofactor. An elegant example of a Fur-regulated substitution pathway is the utilization in Fe-deficient cells of an alternative, Mn-utilizing ribonucleotide reductase (RNR) encoded by the *nrdEF* operon (Andrews, 2011, Cotruvo and Stubbe, 2011, Martin and Imlay, 2011). The ability of the NrdEF enzymes to be derepressed under Fe limitation provides a mechanism to sustain growth. Consistent with its expression under Fe replete conditions, the class Ia Fe-dependent RNR requires an Fe_2S_2 ferredoxin for maintenence and likely assembly of the diferric-tyrosyl radical at the heart of the enzyme (Wu et al., 2007). In contrast, assembly of the class Ib Mn-dependent enzymes (which contains a dimanganese(III)-tyrosyl radical) requires a flavodoxin (NrdI) as oxidant (Cotruvo and Stubbe, 2008, Boal et al., 2010). Iron limitation alone is rather inefficient at supporting active NrdEF, since *E. coli* normally does not actively accumulate significant amounts of Mn. Uptake of Mn, through the MntH proton-coupled importer, is required for efficient activation of NrdEF. Activation of the Mn-dependent RNR is more efficient under mild oxidative stress conditions which inactivate Fur and thereby induce *mntH* transcription (Martin and Imlay, 2011).

An additional, and earlier, example of substitution of an Fe-containing enzyme by its Mn counterpart is the inverse regulation of the *sodB* and *sodA* genes encoding, respectively, an Fe-superoxide dismutase (FeSOD) and MnSOD (e.g. Niederhoffer et al., 1990, Privalle and Fridovich, 1993). The *sodA* gene is repressed by Fur in response to Fe and this gene is preferentially expressed when Fe is limited. Conversely, the *sodB* gene is expressed when Fe is available and is positively regulated by Fur. Although *sodA* can be expressed in response to Fe limitation, up to 95% of the enzyme is inactive under standard growth conditions since, as noted above, *E. coli* does not normally import sufficient Mn for SodA metallation unless oxidative stress is imposed (Anjem et al., 2009).

The mechanism of Fe-dependent activation of *sodB* expression was initially quite puzzling. Early experiments to test the hypothesis that Fur might bind to the *sodB* promoter region to activate transcription were inconclusive (Fee, 1991, Dubrac and Touati, 2000) and subsequent studies suggested that much of the regulation was likely post-transcriptional (Dubrac and Touati, 2000, Dubrac and Touati, 2002). This work set the stage for the description of RyhB, the sRNA that serves as the key mediator of the Fe sparing response in *E. coli* (Masse and Gottesman, 2002).

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a. Fe-sparing by RyhB-dependent proteome remodeling: The RyhB sRNA was originally discovered in a survey of *E. coli* for small, non-coding RNAs (sRNA). Fur links the synthesis of the RyhB sRNA to Fe availability: when cells are Fe-limited the Fur repressor is inactive and RyhB accumulates (Masse and Gottesman, 2002). RyhB, in turn, down-regulates the synthesis of several abundant Fe-containing proteins. The initial characterization of RyhB noted that this sRNA has extended complementarity to a region near the end of the first gene of the *sdhCDAB* operon encoding succinate dehydrogenase (SDH). As predicted for an antisense RNA-based mechanism, the effect of RyhB on expression of SDH is post-transcriptional and relies on Hfq, an RNA chaperone that facilitates the annealing of sRNAs with their mRNA targets, often leading to degradation (Masse and Gottesman, 2002). The destruction of the *sdhCDAB* mRNA under Fe-limiting conditions can therefore account for the inability of *fur* mutants to grow on succinate as a carbon source. Repression of SDH synthesis reduces Fe demand since this enzyme complex contains ten Fe atoms including one heme and a complex electron transfer chain ([Fe_2S_2], [Fe_4S_4], and [Fe_3S_4]) within the SdhB subunit (Cecchini, 2003).

In addition to *sdh*, several other target operons share a similar pattern of positive regulation by Fur in the presence of Fe (Hantke, 2001, Masse and Gottesman, 2002). These have in common the fact that they encode abundant proteins that bind Fe as cofactor (aconitase, fumarase A, FeSOD) or store Fe (bacterioferritin and ferritin). It is interesting to note that in some cases (aconitase, fumarase, and SOD), the Fe-regulation targets only one of mutiple paralogs. In most cases, this apparent positive regulation is attributed to RyhB.

The regulation of gene expression by RyhB is one of the best understood sRNA regulons and serves as a paradigm for thinking about Fe-sparing mechanisms in Bacteria (Masse et al., 2007). Homologs of RyhB are found in several related bacterial species and the overall outlines of the response are largely similar, although there are unique aspects to each system. Altogether, RyhB targets at least 18 distinct mRNA targets leading to the down-regulation of >50 proteins (Masse et al., 2005). In addition, RyhB targets several other sites leading to more nuanced effects on gene expression (Salvail and Masse, 2012). RyhB is complementary to an upstream open reading frame that is translationally coupled to *fur* and thereby down-regulates Fur expression. RyhB targets an intergenic region in the *iscRSUA* mRNA (encoding proteins for iron sulfur cluster assembly and regulation) leading to degradation of the *iscSUA* portion of the mRNA and stabilization of the *iscR* portion which encodes an operon-specific repressor (Desnoyers et al., 2009). Finally, RyhB can activate gene expression in the case of targets required for the optimal synthesis of enterobactin, the major *E. coli* siderophore (Prevost et al., 2007, Salvail et al., 2010).

The physiological impact of the RyhB-mediated Fe sparing response on *E. coli* is quantitatively significant and amounts to a greater than 2-fold reduction in Fe demand. This process allows *E. coli* to make the most efficient use of a scarce resource and direct the available Fe to the most high priority enzymes. These are presumably those essential functions that can not be replaced by alternative enzymes or pathways, although the enzymes that constitute these essential functions will likely vary depending on the precise growth conditions. In other cases, such as for RNR, *E. coli* has evolved a backup Fe-independent pathway that allows growth even when the Fe-cofactored enzyme is not able to

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be metallated. The analysis of the *E. coli* Fe sparing response also illustrates another recurring theme: discovery of the transcription factors that respond to elemental limitations, and thereby the regulons that they control, often points directly to key players in the acclimation process.

b. Fe-sparing in other Gram-negative bacterial systems: Iron-sparing responses are widespread in the Bacteria (Salvail and Masse, 2012), although the molecular details are not as well defined as for *E. coli*. For those organisms more closely related to *E. coli*, RyhB orthologs are common and a similar overall pathway is likely present. For example, RyhB orthologs have been described for *Shigella* (Africa et al., 2011) and *Vibrio* spp. (Davis et al., 2005, Mey et al., 2005). A more divergent system is found in *Pseudomonas aeruginosa* that requires two, tandemly encoded sRNAs designated PrrF1 and PrrF2. These two sRNAs (116 and 114 nt) are nearly identical in sequence and, as far as is known, completely redundant in function (Oglesby-Sherrouse and Vasil, 2010). Functionally, they are analogs of RyhB and target the mRNAs for some of the same types of Fe-utilizing proteins including SDH, SodB, and a bacterioferritin (Wilderman et al., 2004). Curiously, in response to combined heme and iron limitation a longer RNA (*prrH*) is produced, perhaps by a regulated antitermination mechanism, that spans the *prrF1* and *prrF2* genes, and includes what would otherwise be the intergenic region. This additional sequence information may allow this longer sRNA to additionally target heme biosynthesis functions (Oglesby-Sherrouse and Vasil, 2010). PrrF-like loci are also involved in controlling Fe-sparing in other bacteria including ArrF in *Azotobacter vinelandii* (Jung and Kwon, 2008) and NrrF in *Neisseria meningitidis* (Mellin et al., 2007). The regulons for each of these sRNAs are likely distinct, although they also target abundant, Fe-using proteins such as SOD or SDH.

4. Fe homeostasis in *B. subtilis*—*B. subtilis* also responds to Fe limitation with derepression of a large and complex regulon of genes controlled by the Fe-sensing repressor, Fur (Baichoo et al., 2002). The induced operons lead to the synthesis of siderophore (bacillibactin) and expression of transport systems for ferric-bacillibactin and other siderophores (Ollinger et al., 2006). Clearly, the major function of these operons is Fe acquisition. In addition, other operons function in Fe-sparing by encoding proteins that can substitute for functions that might be disabled under Fe limitation.

One function likely to be compromised in Fe-limited cells is electron transfer, and *B. subtilis*, like the marine phytoplankton discussed previously, substitutes ferredoxin with flavodoxins. Recently, a ferredoxin has been described as the single most abundant Fe-containing protein in the soluble fraction of *B. anthracis* cells (Tu et al., 2011), which makes this protein a logical target for an Fe-sparing response. When *B. subtilis* is Fe-limited, the *ykuNOP* operon is strongly derepressed, which leads to the synthesis of two flavodoxins (YkuN and YkuP) that are proposed to functionally substitute for at least some functions of ferredoxin. These flavodoxins have been biochemically characterized and can function with BioI, a P_{450} enzyme (Lawson et al., 2004), nitric oxide synthase (Wang et al., 2007), and the Fe-dependent -5-acyl lipid desaturase for remodeling membrane lipids (Chazarreta-Cifre et al., 2011). In the latter case, both the ferredoxin and flavodoxin proteins are catalytically active, and it can be imagined that when Fe is limiting, the flavin-containing protein may

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functionally replace the Fe-containing protein for at least this and possibly other functions. The fate of previously synthesized ferredoxin is not known in *B. subtilis*, but in *Clostridium pasteurianum* it has been found to be degraded in response to Fe limitation to serve as an endogenous source of Fe (Schönheit et al., 1979).

a. The FsrA-dependent Fe-sparing response: *B. subtilis* has a robust Fe-sparing response that is activated when Fe is limiting for growth (Gaballa et al., 2008). In this organism, Fur represses the synthesis of an sRNA designated FsrA which is distinct in sequence, but similar in function, to RyhB. The FsrA sRNA is highly complementary to the leader region of the *sdhCAB* operon and, as for *E. coli, fur* mutants are unable to grow on succinate. This growth defect is due to FsrA since a *fur fsrA* double mutant regains the ability to grow on succinate (Gaballa et al., 2008). A number of other FsrA targets have been identified using proteomic and transcriptomic approaches and in at least one case the predicted RNA:RNA pairing interactions have been confirmed by genetic analysis. Some of the notable FsrA-regulated targets include SDH, and several other Fe/S-containing enzymes including aconitase, a lactate oxidase complex, glutamate synthase, and dehydratases involved in branched chain amino acid biosynthesis (Gaballa et al., 2008). As a result of the wide range of metabolic activities targeted by FsrA, a *fur* mutant strain is significantly impaired in growth even when Fe is sufficient. This is due to the inappropriate (under these conditions) repression of metabolic enzymes. One notable consequence of this is a significant reduction in the activity of the TCA cycle as monitored using metabolomics (Fischer and Sauer, 2005).

The *B. subtilis* Fe-sparing response differs from that of the enteric bacteria in that it also involves three small, basic (positively charged) proteins named FbpA, B, and C (for Fur-regulated basic proteins). The ability of FsrA to target mRNAs for regulation is independent of a presumed Hfq homolog in *B. subtilis*, but is at least partially dependent on the FbpABC proteins, which are postulated to function as Fur-regulated RNA chaperones (Gaballa et al., 2008). However, direct evidence for chaperone activity (the ability to increase the rate of RNA:RNA annealing) is still lacking. Transcriptome and proteome studies of mutant strains lacking Fur, FsrA, and/or one or more Fbp proteins indicate that these regulators overlap in their specificity, and that several FsrA targets are co-regulated by one or more Fbp. Mutations have been introduced into the start codons of the *fbpA* and *fbpB* genes that prevent protein expression while having negligible effect on RNA sequence stability. Using these constructs, and complementation studies, it has been demonstrated that the requirement for the *fbpAB* operon for repression of lactate utilization (*lutABC*) genes during Fe limitation requires the FbpB protein, but not FbpA (Smaldone et al., 2012). In contrast, for some targets (e.g. *sdh*) there is no apparent requirement for Fbp proteins (even a triple *fbpABC* mutant still regulates SDH normally).

The *B. subtilis* Fe sparing response has drastic effects on cell physiology due to extensive remodeling of the proteome as visualized using gel-based proteomics (Gaballa et al., 2008). Comparable to *E. coli*, the Fe content of the *B. subtilis* cell corresponds to ~1 mM averaged over the cell volume and it is likely that only a very small fraction of this corresponds to the labile Fe pool sensed by Fur. Ultimately, a complete metallomics analysis of *B. subtilis* is needed to quantify the distribution of Fe between heme-, Fe/S- and non-heme-Fe-containing

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proteins and to better understand how this distribution is remodeled by the Fe-sparing response. Fur itself is quite abundant (~10,000 molecules per cell) and elevation of this level by even two-fold grossly perturbs Fe homeostasis (Faulkner et al., 2011). The *fur* gene is repressed by a paralogous metalloregulatory protein, PerR, and in the absence of *perR*, Fur levels are elevated. As a result, *perR* mutants are severely Fe starved. This is due, in part, to an abundant catalase (KatA) which is highly expressed in *perR* mutants and likely accounts for ~10% of the total Fe quota in these cells (Faulkner et al., 2011).

5. A protein-mediated Fe-sparing response in *Corynebacterium glutamicum*— Iron sparing responses are also likely to have evolved in other Gram positive bacteria although these are, in general, not well defined. Of note, *Corynebacterium glutamicum* has a protein rather than an sRNA-based Fe-sparing response (Wennerhold et al., 2005). In this organism, the DtxR protein functions as a global regulator of Fe homeostasis (analogous to Fur) and upon Fe limitation an AraC-type repressor named RipA (regulator of iron proteins A) is derepressed (Wennerhold and Bott, 2006). RipA, in turn, binds directly to the promoter regions of numerous Fe-utilizing enzymes to block their expression. The RipA targets include aconitase, succinate dehydrogenase (*sdhCAB*), nitrate/nitrite transporter and nitrate reductase (*narKGHJI*), isopropylmalate dehydratase (*leuCD*), catechol 1,2-dioxygenase (*catA*), and phosphotransacetylase (*pta*). The DtxR-RipA regulatory circuit is thereby analogous in function to the Fur-RyhB (*E. coli*) or Fur-FsrA (*B. subtilis*) systems (Wennerhold et al., 2005). Repression of aconitase and SDH in response to Fe limitation has also been noted in one of two studied strains of *Mycobacterium avium* (Janagama et al., 2010), although the mechanism remains to be defined.

6. Fe homeostasis in *S. cerevisiae*— *S. cerevisiae* is an exceptionally well understood model system for defining processes of Fe homeostasis in a model Eukaryote (reviewed in Kaplan et al., 2006, Bird, 2008, Philpott and Protchenko, 2008). Under Fe replete conditions, most of the Fe that enters cells is transported by low affinity pathways including the Fet4 and Smf1 proteins. Under these conditions, aerobic respiration pathways are active, the cellular Fe quota is relatively high, and the Ccc1 protein imports Fe into vacuoles (Li et al., 2001).

The primary response to Fe deprivation is the activation of the Aft1 and Aft2 transcription factors, which coordinate a multifaceted acclimation response that can reduce the cellular Fe quota by two-fold or more. Most genes activated by Fe-deprivation are under the transcriptional control of Aft1, which is translocated to the nucleus in response to declining Fe status. Conversely, in the presence of Fe, Aft1 is oxidized to form mixed disulfides with a monothiol glutaredoxin and interacts with an exporter that mediates Aft1 translocation to the cytosol (Kaplan et al., 2006, Philpott and Protchenko, 2008). Aft2, an Aft1 paralog, may activate many of the same genes as Aft1 does, but appears to play a secondary role most apparent in strains lacking Aft1 (Philpott and Protchenko, 2008). Note that homologues of Aft1/2 are found only in the fungi, and therefore other Fe-sensing regulators function in animals and plants (Long et al., 2010, Wang and Pantopoulos, 2011).

Aft1 coordinates the activation of a complex and multilayered Fe-sparing response. First, Aft1 activates the transcription of genes for Fe acquisition and mobilization (Philpott and

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Protchenko, 2008). Second, Aft1 activates transcription of two RNA-binding proteins, Cth1 and Cth2, that target numerous mRNAs for functional inactivation (Puig et al., 2005, Puig et al., 2008). Third, the metabolic changes resulting from both the direct and indirect effects of Aft1 alter the activity of other regulons in the cell that may also impact Fe homeostasis (Ihrig et al., 2010).

Genes for Fe acquisition and mobilization functions, including many encoding high affinity transporters for Fe-chelates and elemental Fe, are under the direct transcriptional control of Aft1. In yeast, elemental Fe uptake is mediated by a reductive system that depends on a copper-containing oxidase (Fet3p) and a specific permease (Ftr1) (Kosman, 2003). Aft1 also activates the expression of an Fe transporter (Fet5/Fth1) homologous to the Fet3/Ftr1 system that localizes to the vacuolar member for Fe mobilization from this site of storage. In addition, Aft2 activates expression of an NRAMP family divalent cation transporter, Smf3, that also functions in Fe mobilization from the vacuole (Singh et al., 2007).

The mitochondria are the sites for both heme and Fe/S biosynthesis and this can be a significant drain on cellular Fe pools. Aft1 activates the expression of the heme oxygenase Hmx1 that degrades heme. This can serve dual roles in the cell. First, heme degradation can release Fe, and thereby allow its recycling for other purposes. Second, heme normally serves as a signal molecule to activate the expression of respiratory cytochromes. Thus, degradation of heme also serves to down-regulate the expression of a major Fe-consuming pathway in the cell (Kaplan et al., 2006). As an additional Fe-sparing mechanism, Aft1 up-regulates a biotin importer which compensates for the transcriptional repression of biotin biosynthetic functions (including the Fe/S containing Bio2 protein) under Fe deprivation.

Aft1 also activates synthesis of Cth2 that coordinates a large-scale Fe-sparing and prioritization response in which numerous Fe-consuming proteins are post-transcriptionally down-regulated (Vergara and Thiele, 2008). Conceptually, this is analogous to the RyhB-mediated Fe-sparing response of *E. coli* and the similar FsrA-mediated response of *B. subtilis*. However, rather than relying on a small RNA, the yeast Cth2 RNA-binding protein recognizes AU-rich elements in the 3'-untranslated regions of target mRNAs, recruits the Dhh1 helicase, and activates an mRNA decay pathway (Pedro-Segura et al., 2008). Yeast also contain a Cth2 paralog, Cth1, that preferentially down-regulates mitochondrial functions (Puig et al., 2008). Both Cth1 and Cth2 contain two tandem Zn-finger domains involved in nucleic acid binding.

Cth2 is transcriptionally induced ~200-fold in response to Fe deficiency. Studies with deletion mutants lacking Cth2 revealed an increase in the level of mRNAs for numerous Fe-consuming processes including TCA cycle enzymes (e.g. SDH), mitochondrial respiration, fatty acid synthesis, heme biosynthesis, and Fe/S proteins (Puig et al., 2005). The down-regulation of these many Fe-consuming processes presumably functions to spare Fe for more essential processes, likely including the synthesis of Fe/S cluster enzymes and an Fe-dependent RNR. Cth2, together with Cth1, ensures that limited Fe is directed towards RNR by targeting the nuclear tethering protein Wtm1 for degradation (Sanvisens et al., 2011, Seguin et al., 2011). Wtm1 binds to the R2 subunit of RNR thereby limiting its translocation to the cytosol where it is active. Thus, Fe deprivation leads to Cth2-mediated down-

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regulation of numerous Fe-consuming reactions while simultaneously stimulating the translocation and thereby activity of an essential, Fe-requiring enzyme (Sanvisens et al., 2011, Seguin et al., 2011).

Global studies have revealed that Fe deprivation, and in particular the Aft1-mediated pathways noted, lead to many pleiotropic effects on transcription. In addition to the direct effects of Aft1 on gene expression, and the indirect effects of Cth2-mediated RNA destabilization, there are additional effects that result from changes in metabolites that themselves have regulatory roles. As noted above, Aft1 induces heme oxygenase, which leads to a decrease in heme levels. Heme is sensed by the Hap1 transcription factor and decreasing heme availability leads to down-regulation of cytochrome *c* (Ihrig et al., 2010). Similarly, decreased activity of Fe/S containing biosynthetic enzymes can lead to changes in metabolite pools that alter gene expression (Ihrig et al., 2010).

7. Fe homeostasis in Chlamydomonas—Fe homeostasis in *C. reinhardtii* is regulated at multiple levels. The iron assimilation pathway consists of a cell surface ferrireductase, a high affinity Fe-selective transporter consisting of a multicopper oxidase (Fox1) and a trivalent cation specific permease Ftr1 analogous to the *S. cerevisiae* Fet3/Ftr1, as well as Irt1/Irt2, members of the ZIP family of divalent cation transporters, and a periplasmic presumed Fe binding protein (Allen et al., 2007a, Blaby-Haas and Merchant, 2012). Each of the corresponding genes is under transcriptional control, although the Fe sensor and transcription factor remain to be discovered. Iron status in photosynthetic cells is readily visualized as “chlorosis” or loss of chlorophyll (Chl) pigment. Merchant and co-workers used chlorosis and growth rate to define “stages” of poor Fe nutrition in *C. reinhardtii*: the replete situation where all Fe-containing proteins are satisfied, Fe-deficiency where chlorosis is not evident and the growth rate is not impacted but where the genes for assimilation are fully turned on, and Fe-limitation where cells are chlorotic and growth inhibited because there is insufficient Fe for maintaining activities of all essential enzymes (La Fontaine et al., 2002). In the absence of known transcription factors, these operational definitions help to distinguish primary (and perhaps direct) responses to Fe-deficiency from secondary responses resulting from physiological stress caused by Fe-limitation.

Fe is required for both photosynthesis and respiration in *C. reinhardtii*, with PS I contributing to 50% of the Fe quota in the photosynthetic apparatus and NADH-dehydrogenase/complex I contributing to most of the Fe demand of the respiratory electron transfer chain. In a situation of respiratory growth (acetate as a carbon source), a program of Fe sparing in the chloroplast is activated (Moseley et al., 2002). This involves loss of PS I (with its 3 Fe_4S_4 centers) and cytochrome complexes (containing heme and an Fe_2S_2 Rieske center) so that their abundance is reduced to <1% of that noted in Fe-replete cultures. In contrast, the abundance of ferredoxin is decreased but not as drastically. The different impact on PS I and the Cyt b_6f complex as compared to ferredoxin is consistent with the fact that on acetate-containing medium, photosynthesis is non-essential. On the other hand, ferredoxin is used also as a reaction partner for O_2-utilizing oxidations in various biosynthetic reactions, including fatty acid desaturation, N-assimilation and dNTP synthesis. Since there is no evidence for a flavodoxin encoded in the *C. reinhardtii* genome, ferredoxin is an essential (high priority) function.

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Interestingly, the activity and abundance of FeSOD is also maintained under Fe deficiency, further pointing to prioritization of Fe utilization in the chloroplast (Page et al., 2012). Five MnSOD isoforms are encoded in the *C. reinhardtii* genome by *MSD1* through *MSD5*. The individual gene products are differently localized with one isoform assigned to the plastid. The corresponding gene, *MSD3*, is dramatically up-regulated (10^{2}–10^{3}-fold) in Fe-starved cells at the transcriptional level, with the response initiated already in a situation of deficiency rather than limitation, suggestive of a direct response to Fe status. An increased demand for SOD is expected in photosynthetic cells with compromised PS I Fe_4S_4 clusters, since the midpoint potentials of the PS I acceptors are sufficiently negative to reduce O_2 to superoxide (Herbert et al., 1992, Asada, 2006). The use of a Mn-isoform for this protective response would spare Fe for use in other pathways. Another photoprotective mechanism that operates in this situation is the disconnection of the light harvesting Chl-binding proteins from the PS I complex (Moseley et al., 2002). This process is a selective program that responds to the activity of a di-iron enzyme in the Chl biosynthetic pathway.

When cells are shifted from phototrophic to heterotrophic growth, Fe is recycled by degradation of the photosynthetic apparatus. Released Fe is buffered by ferritin in the chloroplast stroma and can presumably be mobilized as needed for maintenance of respiratory complexes in the mitochondria (Naumann et al., 2007, Busch et al., 2008). In contrast, when cells are grown phototrophically (on CO_2 and light), the photosynthetic apparatus is essential and is accordingly maintained (Terauchi et al., 2010). Other Fe-sparing and recycling mechanisms are also presumed to operate, but these are not yet well-investigated.

D. Zinc (Zn): an essential metal for life

Zinc is an essential element for life and, unlike Fe, there are no known examples of organisms that have completely dispensed with a Zn requirement. Zn is not redox active under biological conditions, and serves as an electrophilic catalyst (Lewis acid) in numerous enzymes and as a scaffold for organizing protein domains. It is this latter function that has expanded tremendously in eukaryotes leading to the proliferation of Zn-finger proteins encoded in many plants and animal genomes. Zn deficiency is considered the most common mineral deficiency of plants (Assuncao et al., 2010) and is one of the major health threats in the developing world where it contributes to the death of 800,000 children annually (Black, 2003, King, 2011).

Although Zn is required for all cells, distinguishing between its essential and its dispensable roles is not easy. *E. coli* is estimated to contain perhaps 100 distinct Zn-containing enzymes. A survey of the *M. tuberculosis* genome for likely essential Zn functions highlights the involvement of this metal in the synthesis of DNA, leucine, inositol derivatives, and mycothiol and in key metabolic reactions such as aldolase in glycolysis and methionine aminopeptidase (Riccardi et al., 2008). Zn is also found as a component in RNAP, some ribosomal proteins, and some aminoacyl tRNA synthetases that together may account for its essential nature. Because Zn has so many different roles, cells have evolved complex responses to acclimate to Zn deprivation. The major common mechanisms involve increased

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uptake of Zn, the synthesis of alternative, Zn-independent enzymes and proteins (where possible), and the mobilization of intracellular Zn <Figure 9>.

1. Zn homeostasis in *E. coli*—*E. coli* contains a total of ~0.2 mM Zn when averaged over the cell volume. However, only a tiny fraction of this total is in a labile Zn pool. The labile Zn pool has been estimated at ~20 pM using a genetically-encoded fluorescent biosensor based on carbonic anydrase (Wang et al., 2011a). This small, labile Zn pool is bound to small molecules in the cytosol and there is no "free" Zn in the cell at equilibrium (Outten and O'Halloran, 2001). Zn homeostasis in *E. coli* is governed by the Fur-like metalloregulatory protein, Zur, which binds to DNA target sites when Zn levels in the cell are sufficient. Since Zur binds Zn with an affinity in the femtomolar range, it can be concluded that Zur and other molecular ligands in the cell maintain the small labile Zn pool in a largely bound, but kinetically labile state. When Zn levels rise, the ZntR metalloregulator senses this excess and activates efflux.

Insights into the processes of acclimation to Zn deficiency can be obtained from the analysis of the Zur regulon (Graham et al., 2009). In *E. coli*, the primary function of the Zur regulon appears to be increased Zn uptake. Zur also regulates at least one alternative ribosomal protein, which presumably functions to replace a normally Zn-containing one (Hensley et al., 2011), as described in detail for *B. subtilis* (see below). Unlike the RyhB Fe-sparing response, there is no evidence for a global Zn-sparing response in *E. coli*, which may simply mean that Zn limitation is not a stress commonly encountered in the natural habitats of this organism.

2. Zn homeostasis in *B. subtilis*—Zn homeostasis in *B. subtilis* is also regulated by Zur which was discovered as a Fur-like regulator that represses the synthesis of an ABC transporter for Zn uptake (Gaballa and Helmann, 1998). *B. subtilis* Zur, like that from *E. coli*, binds Zn with sub-picomolar affinity (Ma et al., 2011) and monitors a small, labile pool of Zn to coordinate responses to Zn limitation. In response to sufficient cellular Zn, Zur represses the synthesis of a high affinity Zn uptake ABC transporter (YcdHI-YceA) and a putative metallochaperone (YciC) (Gaballa et al., 2002), a GTP cyclohydrolase (FolE2) (Sankaran et al., 2009), and three alternative ribosomal proteins (Gabriel and Helmann, 2009).

a. Expression of Zn-independent isozymes: The Zur-regulated *yciABC* genes form a complex operon that is transcribed from Zur-regulated promoters upstream of both the *yciA* and *yciC* genes (Gabriel et al., 2008). YciC is a representative of a widely distributed family of putative metallochaperones that belong to the COG0523 (conserved orthologous group) proteins found in Bacteria, Archaea, and Eukarya (Haas et al., 2009). YciC itself is a highly abundant protein and was originally identified by SDS-PAGE analysis of *zur* mutant cells (Gaballa and Helmann, 1998). Based on homology, it is proposed that YciC is a GTP-dependent metal insertase, although neither the relevant metal nor the relevant client proteins are known. Since YciC is expressed under Zn limitation, one hypothesis is that this protein serves to direct Zn to the most essential enzymes in the cell. However, it remains possible that YciC may function with a different metal(s) to activate enzymes that replace Zn-dependent functions (Haas et al., 2009). Indeed, some cells have more than one

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COG0523 family member, raising the possibility that they may have different metal selectivities.

The YciA protein was assigned as an alternative GTP cyclohydrolase based on the original observation that some cells contain a complete folate biosynthetic pathway except for the apparent absence of a *folE* gene encoding GTP cyclohydrolase (GCYH-IA), the first step in folate biosynthesis. Phylogenomic comparisons revealed that cells lacking *folE* contained instead a member of the COG1469 family, which was therefore suggested to be a non-orthologous replacement designated GCYH-IB (El Yacoubi et al., 2006). Most Bacteria (65%) encode a GCYH-IA protein, a subset (14%) encode only GCYH-IB, and others (12%) encode both (the remainder are symbionts or other organisms that rely on uptake of folate from their environment) (Sankaran et al., 2009). For those organisms that encode both types of GTP cyclohydrolase (such as *B. subtilis*), the GCYH-IB isozyme is typically regulated by Zur (or its functional equivalent) and is induced by Zn limitation. Biochemical analyses have revealed that GCYH-IA proteins are Zn-dependent enzymes whereas GCYH-IB can function with various other divalent metal ions, including Mg (Sankaran et al., 2009). Experiments with *B. subtilis* confirmed that the derepression of YciA in a *zur* mutant strain can compensate for the absence of the Zn-dependent FolE enzyme (Sankaran et al., 2009). Collectively, these results support a model where Zn activation of GTP cyclohydrolase is sensitive to cellular Zn depletion and the resulting inability to synthesize folate, which is essential for synthesis of dTMP and other key metabolites, imposes a growth restriction. One evolutionary solution to this elemental limitation has been the emergence of an alternative isozyme that relies instead on a different metal ion. Other candidates for this type of response in *B. subtilis* include peptide deformylase and methionine aminopeptidase encoded by paralogous genes. Although both enzymes are known to be metal-dependent, in this case there is no evidence for metal-dependent regulation that might provide hints as to the adaptive value of the gene duplication.

b. Expression of alternative ribosomal proteins and acclimation to Zn limitation: Bacteria have a remarkable but under-appreciated mechanism for acclimating to Zn limitation. Several ribosomomal proteins (r-proteins) contain a bound Zn ion and these proteins can be replaced by paralogs that lack Zn, thereby freeing Zn for use in other proteins (Panina et al., 2003). Ribosomes are highly abundant in growing cells and it is estimated that they contain 50% or more of the cellular Zn quota (Gabriel and Helmann, 2009, Hensley et al., 2011). This represents a physiologically significant form of stored Zn that can be re-partitioned, as needed, to allow assembly of Zn-cofactored enzymes.

The role of the ribosome in Zn homeostasis emerged from two independent lines of investigation. In the first, Panina and coworkers performed a comparative genomics analysis of Zn-regulated genes and noted that, in several organisms, there were r-proteins that were predicted to be up-regulated under conditions of Zn limitation (Panina et al., 2003). In each case, the corresponding r-protein was encoded by a duplicated gene with one copy under Zn-regulation and the other apparently constitutively expressed. r-proteins are also known to exist in variants that either have (C^{+}) or lack (C^{-}) potential Zn-binding motifs (CxxC motifs) (Makarova et al., 2001). This led Panina to propose that, under conditions of Zn limitation,

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Zn-containing (C^+) r-proteins could be replaced by their non-Zn-containing (C^-) counterparts (Panina et al., 2003).

The second set of experiments focused on the proteomic composition of the *B. subtilis* ribosome as a function of growth conditions (Nanamiya et al., 2004). When *B. subtilis* cells entered stationary phase the large subunit r-protein L31 disappeared and was replaced with a highly similar (C^-) variant, encoded by the *ytiA* gene. The molecular basis of this protein switch was traced to derepression of the *ytiA* gene, which was shown to be a member of the Zur regulon (Nanamiya et al., 2004). In retrospect, the medium used for these growth studies was noted to lack added Zn which led, serendipitously, to the discovery of r-protein displacement. Since L31 is surface exposed on *B. subtilis* ribosomes, this r-protein can be displaced by newly synthesized YtiA protein and the released L31 thereby provides a potential source of Zn (Akanuma et al., 2006). A second large subunit protein, L33, is likely to be regulated similarly although the C^- paralog (*rpmGc*) has a frameshift mutation in laboratory strains of *B. subtilis* (Gabriel and Helmann, 2009). Interestingly, neither L31 nor L33 is required for growth since mutants lacking all genes for either protein are still viable (Gabriel and Helmann, 2009).

A related substitution mechanism also occurs for r-protein S14 in *B. subtilis* (Natori et al., 2007). Unlike L31, the S14 protein assembles early into the ribosome and can not be replaced once the ribosome is synthesized. Under conditions of Zn limitation, the inability to metallate new S14 protein would thereby block the synthesis of new ribosomes. The cell has evolved a "fail-safe" mechanism in which derepression of a Zur-regulated paralog (YhzA) can functionally substitute for S14 and allow new ribosomes to be synthesized. Both the L31:YtiA and S14:YhzA protein pairs are nearly identical in sequence, but differ in the selective removal of Zn-binding Cys ligands from the C^- partner.

Regulation of the YtiA(L31) and YhzA(S14) r-protein paralogs by Zur facilitates growth under Zn limitation by two distinct mechanisms. The displacement of surface-exposed large subunit r-proteins (L31 and potentially L33) releases substantial Zn for recycling into other target proteins (Nanamiya and Kawamura, 2010). The derepression of YhzA enables the continued synthesis of new ribosomes. The physiological impact of these acclimation mechanisms has been confirmed using strains lacking high affinity Zn uptake, which can thereby more readily be rendered Zn-limited (Gabriel and Helmann, 2009).

Acclimation to Zn limitation by derepression of C- family r-proteins is widespread, although the proteins induced, and the potential number of Zn atoms mobilized per ribosome, varies (Panina et al., 2003, Chen et al., 2009). Paralogous pairs of C^+ and C^- r-proteins are found for S4, S14, and S18 in the small subunit and L28, L31, L32, L36 in the large subunit (Chen et al., 2009). Note that the large subunit proteins were originally numbered in order of decreasing molecular mass and those that have paralogs are amongst the smallest subunits. Indeed, these r-proteins are little more than Zn-finger peptides and they are, at least in some cases, dispensable for growth (Gabriel and Helmann, 2009). One view is that these are simply small, positively charged Zn-finger peptides with a primary function in Zn storage and mobilization, and that binding to the surface of the ribosome is expedient for stabilizing

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the peptides against degradation. Alternatively, these may be dual function proteins with legitimate roles in translation or even in co-translational metal-loading into nascent proteins.

Paralogous pairs of r-proteins likely involved in Zn homeostasis have been documented in *E. coli* (Hensley et al., 2011), *S. coelicolor* (Owen et al., 2007, Shin et al., 2007), and *M. tuberculosis* (Maciag et al., 2007). The record for the most pairs of C^+/C^- r-proteins in a single organism is *S. coelicolor* with seven. Most of these are regulated by Zn mediated by Zur, but in at least one case the induction may be regulated instead by the σ^R oxidative stress response (Owen et al., 2007). Although an analogous Zn mobilization mechanism has yet to be reported in eukaryotes, the presence of duplicated genes for selected r-proteins suggests that such a mechanism may be present in some species.

3. Zn homeostasis in *S. cerevisiae*—*S. cerevisiae* is an outstanding model for the study of eukaryotic metal ion metabolism (Bird, 2008, Bleackley and Macgillivray, 2011), and Zn homeostasis in particular (Eide, 2009). When Zn is relatively abundant, Zn uptake is mediated by the Fet4 transporter (which also imports Fe) and Zrt2, a representative of the ZIP family of transporters that are conserved throughout all three domains of life. Under Zn replete conditions, import of excess Zn into the vacuole (up to 10^9 ions) provides a store for future use (Simm et al., 2007).

When cells encounter Zn deficiency, high affinity uptake systems are induced as is Zn mobilization from the vacuole (Bird, 2008, Eide, 2009). The key regulator of Zn homeostasis in yeast is the metalloregulatory protein Zap1 which contains seven carboxyl-terminal Zn finger motifs implicated in both DNA-binding and Zn sensing (Bird et al., 2000). Zap1 is thought to sense Zn directly by binding to a centrally located activation domain and a second activation domain that includes at least the first two Zn fingers, but the detailed mechanism has yet to be resolved (Frey et al., 2011).

Under Zn deficient conditions, Zap1 activates a regulon of ~80 genes (De Nicola et al., 2007, Eide, 2009). To facilitate Zn acquisition, Zap1 activates the expression of a high affinity importer, Zrt1. To enable mobilization of stored Zn, Zap1 activates expression of another ZIP transporter, Zrt3, that exports Zn stored in the vacuole. Paradoxically, Zap1 also activates expression of Zrc1 which can import Zn into the vacuole. This is proposed to protect yeast expressing high affinity uptake systems from Zn overload should Zn suddenly become available (Eide, 2009). Presumably, the affinity of the vacuolar Zn importer is poised such that import is only active when cytosolic Zn is relatively abundant.

Zap1 also coordinates a Zn-sparing response by differential regulation of isozymes of alcohol dehydrogenase. Under Zn replete conditions, yeast expresses the Adh1 and Adh3 isozymes which can bind a total of 1.5×10^6 atoms of Zn (Eide, 2009). Under conditions of Zn limitation, Zap1 induces the synthesis of RNA that traverses the promoter regions of the *ADH1* and *ADH3* genes and displaces factors needed for promoter activity (Bird et al., 2006). In addition to repression of Adh1 and Adh3, Zap1 induces synthesis of Adh4 which is suspected, based on sequence similarity, of being an Fe-containing alcohol dehydrogenase. Thus, this is likely a Zn-sparing mechanism (Bird, 2008, Eide, 2009). The transcription factors involved in Zn-sensing in other (non-fungal) microbial eukaryotes are

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not known. They are likely to be distinct, since orthologs of Zap1 have not been found in algae and diatoms, where regulatory responses to Zn-limitation have been described (see below).

4. Zn sparing by substitution of carbonic anhydrase with non-Zn alternatives —Aquatic photosynthetic organisms have carbon concentrating mechanisms for providing CO_2 to the active site of Rubisco, the enzyme that initiates the Calvin cycle (reviewed in Badger and Price, 2003, Giordano et al., 2005, Roberts et al., 2007, Wang et al., 2011b). The importance of the carbon concentrating mechanism is evident from the phenotype of cyanobacterial and algal *ccm* mutants which are unable to grow at air levels (0.04%) of CO_2. There are various types of independently-evolved carbon concentrating mechanisms. One type, common in aquatic microbes, relies on a series of bicarbonate transporters that move CO_2 from the environment into Rubisco-containing compartments.

Carbonic anhydrases, which catalyze the inter-conversion of CO_2 and bicarbonate, are critical enzymes in this mode of carbon acquisition, and accordingly, important for productivity of aquatic phototrophs (Cannon et al., 2010). Zn ion is the usual catalyst in carbonic anhydrases. In a Zn-deficient environment, the usual enzyme in the diatom *Thalassiosira weissflogii* (TWCA1) is replaced by a Cd-containing isoform, CDCA1 (Lane and Morel, 2000a). This novel enzyme, a product of convergent evolution, is found in other diatoms, based on sequence data, and its expression is stimulated by Cd availability (Lane et al., 2005, Park et al., 2007). This enzyme represents the first identification of a beneficial role for Cd in biology.

In laboratory experiments, Co was found to stimulate the growth of Zn-deficient *T. weissflogii* cells and this was determined to result from replacement of Zn with Co in the TWCA1 isoform (Yee and Morel, 1996, Lane and Morel, 2000c). In the coccolithophore *Emiliania huxleyi*, Co may be even more effective than Zn as a cofactor in carbonic anhydrase (Xu et al., 2007). Thus, Co may also support photosynthesis in a Zn-deficient natural environment. Because of limited molecular analyses in the diatoms, it is not known yet whether alternate carbonic anhydrases represent a Zn-sparing acclimation mechanism (i.e. both isoforms exist but their individual expression is reciprocally controlled by Zn nutrition) or whether, in some cases, the alternate forms are evolutionary adaptations to long term Zn limitation in their niche.

The carbon concentrating mechanism is well-studied in *C. reinhardtii* (reviewed by Wang et al., 2011). There are as many as 12 carbonic anhydrases encoded in the genome with isoforms distributed in multiple compartments, including the periplasm, mitochondria, cytosol, chloroplast, stroma, and thylakoid lumen. The functions of some of these are documented by genetic studies in which individual *cah* mutants were shown to be growth compromised for phototrophic growth at air levels of CO_2. The abundance of several carbonic anhydrases is decreased in Zn-deficient *C. reinhardtii* cells and the cells are accordingly growth compromised in phototrophic conditions but not heterotrophic conditions (growth on acetate) where the carbonic anhydrases are less relevant (Malasarn and Merchant, unpublished). As in yeast, there is a strong transcriptional response in *C. reinhardtii* to Zn-deficiency, involving up-regulation of ZIP-family transporters encoded by

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the *ZRT* genes (Hanikenne et al., 2009). In parallel, the expression of two genes encoding proteins with a COG0523 domain (see above) is also dramatically increased (Haas et al., 2009). Whether these proteins are involved in Zn-sparing/recycling pathways remains to be tested.

5. Synthesis of Zn-independent isozymes as an adaptation to Zn limitation— The development of Zn-independent alternatives is also likely prevalent in those environments where Zn is present at low levels and deficiency is common. As noted above, much of the open ocean is P-limited and many of these areas also have low Zn availability, which exacerbates P-limitation, since alkaline phosphatases (active in liberating phosphate from dissolved organic matter) are often Zn enzymes. In such environments, many bacteria encode alternative Ca-dependent alkaline phosphatases, PhoX and/or PhoD, instead of the more familiar Zn-dependent enzyme, PhoA (Luo et al., 2009, White, 2009). PhoX is widely distributed in marine bacteria (Sebastian and Ammerman, 2009). *Prochlorococcus* species from P-limited regions commonly substitute PhoX for PhoA (Kathuria and Martiny, 2011) as do many other marine and freshwater organisms, but it is rare for an organism to encode both types (Zaheer et al., 2009). Somewhat more than half of all *phoX* genes are predicted to encode secreted or periplasmically localized proteins (Luo et al., 2009). In *Campylobacter jejuni*, PhoX activity depends on the twin-arginine transport (TAT) export system, suggesting that metallation of this enzyme likely occurs in the cytosol prior to transport (Drozd et al., 2011).

Both metagenomic and metatranscriptomic studies indicate that induction of PhoX in response to P-depletion is a widespread acclimation mechanism in the marine system (Sebastian and Ammerman, 2009). Unexpectedly, a bioinformatics approach revealed that up to 40% of alkaline phosphatases in the marine system may be cytoplasmically located and that the import of organic phosphates, followed by hydrolysis within the cytosol, may be a widespread strategy for P acquisition (Luo et al., 2009). Many marine organisms appear to contain both a PhoX and a PhoD type alkaline phosphatase. Unlike PhoX, PhoD is thought to be predominantly cytosolic, consistent with the presence of organic phosphate uptake systems in this environment (Luo et al., 2009). Note that in these systems, the induction of the PhoD and PhoX enzymes is an acclimation strategy for P-limitation, whereas the use of these Ca-dependent enzymes as substitutes for the corresponding Zn-dependent enzymes appears to be a Zn-sparing adaptation.

Another example of adaptation to Zn limitation is offered by the occurrence of a novel membrane-associated copper containing SOD in *Mycobacterium tuberculosis* (Spagnolo et al., 2004). In this organism, the structure of a prototypical CuZnSOD is modified so that the protein is stable without zinc. The authors speculate that this may be an adaptation to the metal competitive environment of the host macrophage phagosome where these pathogenic bacteria reside.

6. Synthesis of Zn-independent isozymes during acclimation to Zn limitation —As noted above, *B. subtilis* can acclimate to Zn limiting growth conditions by derepression of alternative proteins that lack Zn, but can functionally replace Zn-dependent homologs. These include a Zn-independent GTP cyclohydrolase and ribosomal proteins.

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The presence of two or more differentially expressed isozymes with different metal ion requirements may in fact be a widespread mechanism for acclimation to Zn limitation.

Hints to the roles of alternative forms of a protein can often be obtained from their regulation. Analysis of predicted Zur regulons in various proteobacterial species has identified nine Zn-dependent enzymes with paralogs likely to be regulated by Zur (Haas et al., 2009). These include HisI, PyrC, HemB, CysRS, ThrRS, QueD, carbonic anhydrase, an *N*-acetylmuramoyl-L-alanine amidase, and DksA. While the adaptive role of these various paralogs can be envisioned, the model has only been tested (to date) for DksA. *P. aeruginosa* encodes two genes encoding DksA, an RNA polymerase-binding transcription factor involved in the stringent response. One, DksA1, contains an essential Zn ion bound to four Cys residues. Under conditions of Zn limitation, induction of a Zur-regulated isozyme (DksA2) lacking associated Zn enables the cell to replace DksA function with a non-Zn-dependent protein (Blaby-Haas et al., 2011).

Further evidence for the prevalence of this substitution strategy has emerged from a bioinformatic analysis of Zn-binding proteins in the Protein Data Bank to identify apparent orthologs that lack the known or presumed Zn-binding ligands (Zhang and Gladyshev, 2011). This analysis identified C^+/C^- pairs of isozymes for eight different r-proteins, four subunits of DNA polymerase III holoenzyme, peptide deformylase, three different aminoacyl tRNA-synthetases (Met, Ile, Leu), methionine-*R*-sulfoxide reductase, DnaJ, 5-aminolevulanic acid dehydratase, and adenylate kinase (Zhang and Gladyshev, 2011). In many cases, organisms have either the presumed Zn-dependent or the Zn-independent version. However, where both forms co-occur in a single organism, their regulated expression may serve as a mechanism of acclimation analogous to those noted above for *B. subtilis* (Sankaran et al., 2009) and *P. aeruginosa* (Blaby-Haas et al., 2011). A related example is the heme biosynthesis enzyme porphobilinogen synthase, which exists in both Zn-dependent and Zn-independent forms (Jaffe, 2003, Frere et al., 2005). In this case there is no evidence of regulated expression as an acclimation mechanism, so this is perhaps better considered as an adaptation strategy.

E. Copper (Cu): a versatile redox cofactor

Cu is found as a cofactor in all kingdoms of life where it is particularly useful as a catalyst of redox reactions, such as in electron transfer proteins azurin and plastocyanin, and reactions involving O_2 chemistry, such as in hemocyanin for binding of O_2 for transport (Crichton and Pierre, 2001). A recent (2008) survey of 450 sequenced bacterial genomes showed that most (72%) have at least one Cu enzyme (Ridge et al., 2008). Two oxidation states are relevant in biology, Cu(I) and Cu(II), with different active site geometries and ligands stabilizing one or the other, and hence allowing a range of redox potentials in cuproproteins. Most Cu proteins are classified into one of three groups – the type I proteins include structurally well-characterized plastocyanin and azurin as prototypes with a tightly bound mononuclear Cu (Redinbo et al., 1994, Malmström and Leckner, 1998, Gray et al., 2000, Choi and Davidson, 2011). The sites are often referred to as "blue" copper because of the intense absorption around 600 nm from the thiolate ligand to Cu(II) charge transfer transition. The type 2 proteins, also mononuclear, are exemplified by CuZnSOD, galactose

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oxidase and amine oxidase, and these lack intense absorption bands from the metal center. Type 3 proteins are binuclear and are represented by hemocyanin and tyrosinase. Some enzymes like ascorbate oxidase and ceruloplasmin, which are in a group referred to as "multicopper oxidases," have multiple Cu binding sites (Messerschmidt and Huber, 1990).

The catalytic potential of Cu is similar to that of Fe, except that more positive midpoint potentials are possible with Cu, compatible with reactions involving molecular O_2. The evolution of cuproenzymes is believed to have occurred more recently than Fe-containing enzymes. As mentioned above, Fe was readily available at the origin of life both because of its abundance and its solubility in water in the ferrous form. By contrast Cu(I) was less bioavailable, especially in the presence of sulfide. With the build-up of molecular O_2, Cu became more readily accessible to life as the more soluble Cu(II) ion. Consistent with the notion of Cu proteins being newer to the protein landscape is the observation that *Firmicutes*, which are considered to be relatively ancient organisms, tend not to use Cu enzymes, and among the 35 archaeal genomes analyzed, 69% showed no evidence of Cu enzymes (Ridge et al., 2008). The most commonly occurring Cu proteins are cytochrome oxidase and CuZnSOD, both of which are important only in an aerobic world. Cytochrome oxidase enables energy generation through use of O_2 as an electron acceptor, and SOD is essential for detoxifying superoxide, one of the harmful side products of O_2 chemistry. Among the bacteria, Cu non-users tend to be anaerobic (73% of those surveyed) while Cu users are aerobic (94%), and in the Archaea, the correlation is complete, with all nonusers being anaerobic and all users being aerobic (Ridge et al., 2008). Accordingly, the Cu-utilizing and sparing regulatory pathways are focused on enzymes involved in bioenergetics (Fig. 10).

1. Cu homeostasis in methanotrophs—Methane is the most inert hydrocarbon but there are bacteria, referred to as methanotrophs, that can use methane as their sole source of carbon and energy. These bacteria are a subset of a group known as methylotrophs, which are aerobic bacteria that use one carbon compounds that are more reduced than formate as sources of carbon and energy, and they assimilate formaldehyde as a major source of cellular carbon (Hanson and Hanson, 1996). The first step in the aerobic methane oxidation pathway in methanotrophs, the generation of methanol, is catalyzed by methane monooxygenase. This enzyme comes in two flavors – a Cu containing form associated with membranes and an Fe-containing form that is soluble. The multi-subunit Cu form has mononuclear and dinuclear Cu sites in the extramembrane region of the complex (analogous to subunit II of cytochrome oxidase, see below) (Nguyen et al., 1998, Lieberman and Rosenzweig, 2005). The soluble Fe form is a carboxylate-bridged di-iron enzyme (Elango et al., 1997, Merkx et al., 2001). The Cu form is the more prevalent enzyme found in methanotrophs in both the α and γ proteobacteria clades, but a few methanotrophs (including marine and freshwater species) have both forms (Murrell et al., 2000b, Nakamura et al., 2007). The expression of one or the other is dependent on the Cu nutrition status (Murrell et al., 2000a, Hakemian and Rosenzweig, 2007). The di-iron enzyme is expressed when there is low Cu in the growth medium while the Cu-enzyme is expressed when there is an adequate supply of Cu. An $NADP^+$-linked formaldehyde dehydrogenase is co-expressed with the soluble di-iron enzyme while a dye-linked formaldehyde dehydrogenase is co-

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expressed with the membrane-associated Cu form, indicating that the choice of metal cofactor for the first step dictates the subsequent route of electron flow for the entire pathway (Zahn et al., 2001).

This regulated enzyme substitution has been documented in many different species, including *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b, representing both the γ and α proteobacterial clades, respectively. The Cu enzyme is presumed to be the preferred form because of the higher redox potential of Cu, with the Fe enzyme serving as a backup for this critical step in methane metabolism. The methanotrophs that express the backup Fe form have lower Cu requirements for growth (Graham et al., 1993, Hanson and Hanson, 1996). The Cu enzyme appears to offer an advantage based on the higher growth yields and higher affinity for methane of cells expressing this type. The importance of Cu to these organisms is underscored by the occurrence of two independent Cu acquisition pathways, one involving synthesis of a peptide based chelator, methanobactin, followed by energy-dependent uptake of the Cu-methanobactin complex, and a second route of passive transport of unchelated Cu (Kim et al., 2004, Balasubramanian et al., 2011). The former route may facilitate Cu mobilization from mineralized sources (Knapp et al., 2007).

In *M. capsulatus* and *M. trichosporium*, the expression of the gene cluster encoding the di-iron enzyme is negatively regulated by Cu ions while the expression of the *pmo* genes encoding the Cu enzyme is concomitantly increased, implicating a common regulator in the Cu-responsive switch between one or the other (Nielsen et al., 1997). In both organisms, the MmoR and MmoG proteins have DNA binding activity and are involved in regulation of the *mmo* genes (encoding the soluble Fe enzyme), but the identity of the Cu sensor is not yet known (Csáki et al., 2003, Scanlan et al., 2009).

2. Cu homeostasis and bacterial respiratory pathways—Although Cu is stimulatory or even required for growth for numerous other bacterial species, the responses to Cu deprivation have been studied in only a few systems. Just as Mn is key for oxygenic photosynthesis, Cu is a key element for aerobic respiration. In *P. aeruginosa* it has been noted that aerobic respiration requires one of four different terminal oxidases (Frangipani et al., 2008). Three operons encode paralogous cytochrome *c* oxidases of the heme-Cu superfamily. A fourth cytochrome *bd*-type cyanide-insensitive oxidase lacks Cu in its active site. When *P. aeruginosa* is grown in the absence of Cu (through repeated sub-cultures) or in the presence of strong Cu chelators, growth is only possible if the Cu-free oxidase is present. Moreover, synthesis of the Cu-free oxidase is strongly induced by Cu depletion, although this seems to be an indirect effect mediated by the failure of the Cu-dependent enzymes to function (Frangipani et al., 2008). Growth of *P. aeruginosa* therefore seems not to absolutely require Cu, and this organism does not scavenge Cu using chelators analogous to methanobactin. It does, however, synthesize a periplasmic protein of the ScoC family postulated to function in Cu acquisition and trafficking (Frangipani and Haas, 2009). The effects of Cu deprivation have also been reported for *Synechocystis* sp. where it prevents respiration while allowing continued photoautotrophic growth (Duran et al., 2004). In the marine bacterium *Pseudomonas stutzeri*, Cu deprivation leads to a blockage in denitrification (Matsubara et al., 1982). This is due to the presence, in nitrous oxide

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reductase, of both a binuclear Cu center and a novel [Cu_4S_2] cluster (Pomowski et al., 2011).

3. Cu homeostasis in fungi—Copper homeostasis has been studied in various fungi including *S. cerevisiae, Schizosaccharomyces pombe, Dactylium dendroides, Cryptococcus neoformans, Candida* spp., and *Podospora anserina* (Shatzman and Kosman, 1978, Bird, 2008). Copper sensors that respond to both low and high Cu were discovered by genetic approaches in *S. cerevisiae* and then identified by homology in other fungi. In *S. cerevisiae*, Mac1 controls transcription of copper assimilators in the deficiency situation, and a related protein Ace1 controls the copper toxicity response (Winge, 1998). In *S. pombe* the regulator Cuf1 is a hybrid between Mac1 and Ace1, in *P. anserina* a Mac1 orthologue is called Grisea, and in *C. neoformans* it is called Oxy2 (Borghouts and Osiewacz, 1998, Labbé et al., 1999, Nyhus and Jacobson, 2004). Each of these organisms has a mitochondrial Cyt oxidase, a cytosolic CuZnSOD and multicopper oxidases (ferroxidases) required for high affinity Fe transport (Gralla and Kosman, 1992, Askwith and Kaplan, 1997, Carr and Winge, 2003). In addition, there are other Cu enzymes like galactose oxidases, tyrosinase, amine oxidases and laccases, whose functions are not essential or only required for a particular physiological program (such as the laccase requirement for virulence of *C. neoformans*, Zhu et al., 2003).

In a pioneering early study with *D. dendroides*, researchers noted the phenomenon of Cu-sparing and the prioritized use of Cu (when its supply was limited), and they proposed the concept of recycling (Shatzman and Kosman, 1978). They noted that galactose oxidase was synthesized and excreted but not metallated in Cu-deficient (<10 nM Cu) cells and it accumulated as the apoform. Clearly this enzyme does not compete effectively for intracellular Cu. CuZnSOD synthesis on the other hand was reduced, and its activity was replaced by induced synthesis of a non-mitochondrial MnSOD (Shatzman and Kosman, 1979). Comparison of cyanide-sensitive (i.e. Cu-dependent) SOD activity in deficient (10–30 nM) vs. replete (5–10 μM) cells indicated that 83% of total SOD activity is attributed to the CuZn enzyme in replete cells vs. 17% in the deficient cells. This Cu-sparing mechanism allowed maintenance of the Cyt oxidase levels independent of Cu nutrition status. As the deficient cells divided and further depleted the Cu pools, only Cyt oxidase is maintained, and the authors proposed that this might occur by degradation of CuZnSOD and recycling of the constituent Cu. At the time, the genes and regulators were not known, but a similar Cu sparing response in *P. anserina*, where apoforms of laccase and tyrosinase accumulate and MnSOD replaces a CuZnSOD in Cu-deficiency, is controlled by Grisea (Osiewacz and Nuber, 1996, Borghouts et al., 2001).

S. cerevisiae does not replace CuZnSOD with a Mn-enzyme, which may explain the loss of Cyt oxidase in Cu-deficiency resulting in poor growth (Giorgio et al., 1963). Nevertheless, gene expression and phenotype analysis of *S. cerevisiae* in response to variation in Cu nutrition suggested that Cu-independent Fe assimilation pathways (involving Fe-siderophore uptake) were up-regulated, presumably to compensate for loss of the Fet3 multicopper oxidase route for Fe uptake (van Bakel et al., 2005). In *S. pombe*, Cuf1 down-regulates components of the Cu-dependent Fe uptake pathway, perhaps as a Cu-sparing strategy (Labbé et al., 1999). In *S. cerevisiae*, under conditions of fermentative growth (where respiration is no longer essential) Cyt oxidase and the respiratory chain is down-regulated

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(van Bakel et al., 2005). The loss of Cyt oxidase in Cu-limited cultures of bacteria and fungi is well-documented (Hubbard et al., 1989, Gabel et al., 1994).

In some fungi, Cu limitation activates the expression of an alternative oxidase, which is a Cu-independent di-iron enzyme (Downie and Garland, 1973, Scheckhuber et al., 2009). This pathway bypasses two of the three sites of proton pumping in the respiratory chain and is therefore less effective than the Cu-dependent pathway, which explains the prioritization of Cyt oxidase in organisms that rely on respiration. The replacement of Cyt oxidase with an alternative oxidase is not viewed as a Cu-sparing mechanism, since it is not directly regulated by Cu nutrition and the Cu sensor, but rather by the redox state of the respiratory chain. It is likely that the alternative oxidase functions as an electron valve to reduce the generation of reactive oxygen species resulting from loss of Cyt oxidase function.

4. Cu homeostasis in algae and cyanobacteria—Plastocyanin is a blue copper protein that was isolated from plant leaves because of its abundance and spectroscopic properties (Katoh and Takamiya, 1961). Its function in the Z-scheme of photosynthesis, where it catalyzes the transfer of electrons from the Cyt b_6f complex to PS I, was established in 1963 by analysis of a plastocyanin-deficient mutant of *C. reinhardtii* (Gorman and Levine, 1965). Plastocyanin was biochemically characterized from many land plants, and it was the first Cu protein to have its structure solved (Boulter et al., 1977, Guss and Freeman, 1983). When researchers analyzed the algae and cyanobacteria on the other hand, they were unable to isolate plastocyanin from some of them (Wildner and Hauska, 1974, Kunert and Böger, 1975), which led to the finding that a soluble *c*-type cytochrome could cover its function in many green algae and cyanobacteria (Bohner et al., 1980a, Bohner et al., 1980b, Sandmann and Böger, 1980, Sandmann et al., 1983, Briggs et al., 1990, Zhang et al., 1992). This cytochrome, originally named for its α-absorption maximum (hence *c-552* or *c-553*), was classified on the basis of its function and eventually named Cyt c_6 (Pettigrew and Moore, 1987).

The synthesis of plastocyanin (Cu) and Cyt c_6 (Fe) was found to be reciprocally dependent on Cu nutrition in both cyanobacteria (*Prochlorothrix hollandica, Anabaena*, and *Synechocystis* spp.) and green algae (*C. reinhardtii*, various *Scenedesmus* spp., and *Pediastrium boryanum*) (Merchant and Bogorad, 1986b, Bovy et al., 1992, Li and Merchant, 1992, Nakamura et al., 1992, Ghassemian et al., 1994, Arudchandran and Bullerjahn, 1996, Miramar et al., 2003). The phenomenon has been observed also in the natural environment. When soluble photosynthetic electron transfer catalysts were isolated from a cyanobacterial bloom in the Potomac, Cyt c_6 was abundant, but when the organisms were cultured in the laboratory (in medium amended with Cu), plastocyanin could be isolated (D. Krogmann, pers. comm.). Functional equivalence, suggested from biochemistry, was firmly established in the plastocyanin-less mutant *pcy1-ac208* of *C. reinhardtii* (Wood, 1978, Merchant and Bogorad, 1987a). In brief, the acetate-requiring phenotype of *pcy1-ac208* could be suppressed by growth on medium lacking Cu, which induced the expression of the heme-containing cytochrome. Biochemical characterization of the two proteins purified from several cyanobacteria indicated that their pIs (which ranged from 4 to 9) co-varied suggesting that they were co-evolving in response to changes in common reaction partners (Ho and Krogmann, 1984).

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The signal transduction pathway responsible for the switch between plastocyanin and Cyt c_6 was shown in *C. reinhardtii* to respond directly to Cu rather than to feedback from the redox state of the chloroplast electron transfer chain (Merchant and Bogorad, 1987b). Since then, *C. reinhardtii* has become a premier reference organism for understanding mechanisms of Cu sensing and sparing in photosynthetic systems (Merchant, 1998). *C. reinhardtii* has 3 abundant Cu proteins, plastocyanin, Cyt oxidase and a multicopper oxidase involved in Fe assimilation, plus a number of other less abundant ones (Li et al., 1996, Chen et al., 2008, Remacle et al., 2010, Castruita et al., 2011). In an Fe-replete situation (when ferroxidase expression is repressed), the Cu quota is determined largely by plastocyanin and Cyt oxidase. The Cu-requirements of *pcy1* mutants gives an indication of the large contribution of plastocyanin to the cellular Cu quota (Hill and Merchant, 1992). Regulation of plastocyanin in *C. reinhardtii* occurs post-translationally by regulated degradation of the protein (Merchant and Bogorad, 1986a, Li and Merchant, 1995) while the *CYC6* gene encoding Cyt c_6 is transcriptionally regulated by a Cu-sensing transcription factor (named CRR1 for copper response regulator) that binds Cu response elements (Quinn and Merchant, 1995, Quinn et al., 2000, Kropat et al., 2005, Sommer et al., 2010). In *C. reinhardtii*, CRR1 controls all known responses to Cu-deficiency, including plastocyanin degradation (Eriksson et al., 2004). The replacement of plastocyanin by Cyt c_6 serves to spare Cu. Degradation of plastocyanin is important for Cu recycling as evidenced by the growth phenotype of *crr1* mutants, which cannot maintain Cyt oxidase because they cannot recycle Cu from plastocyanin (J. Kropat and SM, unpublished).

The amount of Cu required to repress the *CYC6* gene is precisely dependent on the abundance of intracellular Cu proteins (Merchant et al., 1991). For instance, if Cu in the medium is sufficient to support the synthesis of 50% of the cellular plastocyanin quota (~8×10^6 molecules per cell), the *CYC6* gene is expressed to 50% of its maximal level. When the cells divide and hence reduce available Cu so that the plastocyanin quota is now reduced to 25% of its quota, *CYC6* gene expression is increased to 75% of its maximum level, presumably to allow synthesis of just enough more Cyt c_6 to compensate for the deficit.

A recent transcriptome study identified an additional example of Cu-sparing in *C. reinhardtii* (Castruita et al., 2011). A gene encoding a flavin-utilizing amine oxidase is a CRR1 target and is up-regulated in Cu-deficiency. It was suggested that the flavoenzyme may serve as a backup to copper amine oxidases, which function in the marine environment to mobilize N from primary amines (Palenik and Morel, 1991). Indeed, in *C. reinhardtii*, genes encoding amine oxidases are up-regulated in N-deficiency (Boyle and Merchant, unpublished). There are a number of other metabolic modifications in Cu-deficient *C. reinhardtii*, including a change in the level of desaturation of thylakoid membrane galactolipids and increase in the expression of genes encoding O_2-dependent enzymes (Castruita et al., 2011). These responses are dependent on CRR1 and confirmed at the level of the proteome in every case where it was queried. The purpose of these modifications is not understood yet, but one view is that they may be required to accommodate the change from use of plastocyanin in photosynthesis to use of a structurally distinct Cyt c_6. The cold sensitivity and reduced photosynthetic electron transport of a cyanobacterial plastocyanin mutant that compensates by increased expression of *petJ* encoding Cyt c_6 is not inconsistent

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with this view (Clarke and Campbell, 1996). The electron transfer reactions in which they participate rely on complex diffusion steps in a spatially restricted intracellular compartment (the thylakoid lumen) and this may require membrane reorganization (Hervás et al., 1998).

Besides the sparing and recycling responses described above, *C. reinhardtii* also changes expression of two O_2-dependent enzymes in the tetrapyrrole biosynthetic pathway in Cu-depleted conditions. The changes are dramatic and physiologically relevant, since mutants in one of the target genes display Cu-conditional chlorosis, but the underlying rationale remains a puzzle (Hill and Merchant, 1995, Moseley et al., 2000, Quinn et al., 2002). Evidently, there is a connection to anaerobiosis because CRR1 and its target genes are required also in situations of low O_2 tension in *C. reinhardtii* (Quinn et al., 2002, Eriksson et al., 2004). Perhaps the connection persists from ancestral mechanisms where Cu(II) availability was dependent on O_2 levels.

Cyt c_6 has been lost in the land plant lineage, clearly pointing to an evolutionary advantage of plastocyanin. One thought is that commitment to plastocyanin, as noted in a diatom, is an adaptation to economize on Fe (Peers and Price, 2006). This may be true in the marine environment but other more prevalent and more effective Fe-sparing mechanisms (discussed above), like the replacement of ferredoxin by flavodoxin, have not been found yet in the Viridiplantae lineage, suggesting that Fe limitation is not likely to be the driving force for a commitment to plastocyanin in land plants. The evolution of plastocyanin therefore facilitated photosynthesis but increased the dependence on Cu. Although Cyt c_6 is absent, CRR1 homologs have been identified (Yamasaki et al., 2009, Bernal et al., 2012). The Arabidopsis orthologue of *C. reinhardtii* CRR1, SPL7, functions to spare Cu for plastocyanin by replacing CuZnSOD with an FeSOD (Pilon et al., 2009). Interestingly, the green algae that express Cyt c_6 as a plastocyanin replacement have also lost the otherwise ubiquitous gene(s) encoding CuZnSOD (Asada et al., 1977). This is likely a permanent adaptation to long term Cu-deficiency experienced by these organisms. *C. reinhardtii* uses an FeSOD in the chloroplast, which can be supplemented with a MnSOD in Fe-deficient cells, plus MnSODs in the mitochondria and cytosol instead of the Cu enzyme (Allen et al., 2007b). CuZnSOD has also been abandoned in favor of MnSOD in crustaceans where Cu is in demand as the O_2-carrier in hemocyanin (Brouwer et al., 2003).

Cu sparing by replacement of plastocyanin with Cyt c_6 is widespread in the cyanobacteria (cited above). Since the b_6f complex in these organisms transfers electrons to both PS I and Cyt oxidase, the proteins serve both respiration and photosynthesis (Sandmann and Malkin, 1984). The regulatory pathways (.i.e. Cu sensor, cis-acting sequences and DNA binding proteins) are not yet elucidated, but the *petJ* gene (encoding the Cyt) responds transcriptionally to Cu nutrition status while the *petE* gene (encoding plastocyanin) responds transcriptionally in some cases and post-transcriptionally in others (Briggs et al., 1990, Bovy et al., 1992). In the alga *P. boryanum*, a 5' truncated form of the plastocyanin-encoding mRNA (which lacks the initiation codon) is generated in Cu-depleted cells, which prevents synthesis of the polypeptide (Nakamura et al., 2000). This would serve a Cu-sparing function but whether pre-existing plastocyanin is also degraded for Cu recycling is not known.

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F. Elemental substitution: a widespread adaptation for bypassing limitation

Although the regulatory pathways and mechanisms may not always be well understood, the strategy of developing alternative enzymes or pathways that can functionally substitute for a limiting metal extends beyond the Fe, Zn, and Cu homeostasis systems reviewed above. Here, we provide a brief survey of those systems where selection has led to the presence of substitutions that help cells maintain function in response to limitation for Mn, Ni, Co, Mo, V, W, or Se. Remarkably, for nearly all elements for which a biological function as been ascertained, those cells that face limitation have either adapted, in some instance by dispensing with a requirement completely, or developed substitute pathways that are either constitutively expressed or expressed conditional on elemental depletion of the preferred nutrient. The existence of these mechanisms allows life to exploit more diverse ecological niches, which is a powerful driving force.

1. Manganese (Mn): a key to oxygenic photosynthesis and cofactor for SOD— Mn is widely used in biology and is often critical for growth, but the processes that require Mn are often not well understood. The single most critical role for Mn, considered globally, is for its role in the water-splitting complex of PS II in oxygenic photosynthesis. As a result, the Mn quota for the cyanobacterium, *Synechocystis* sp. strain PCC 6803, is >100-fold higher that of the purple bacterium *Rhodobacter capsulatus* (Keren et al., 2002). *Synechocystis* cells grow well at levels of Mn as low as 100 nM; at lower levels they remodel their photosynthetic complexes to prevent photooxidative damage (Salomon and Keren, 2011). Mn appears to be concentrated from the environment and stored associated with the cell envelope (Keren et al., 2002), but the details of this storage and mobilization process are not well understood. However, there is little evidence for an obvious Mn-sparing response, perhaps because no other single component of the cell has such a high demand for Mn.

Phototrophic growth has a similarly high Mn requirement in the Eukaryote, *C. reinhardtii* (Allen et al., 2007b). In this organism, Mn deficiency is elicited by growth with <0.5 μM Mn; lower levels lead to defects in photosynthesis. The first response to declining Mn availability is the expression of acquisition pathways including a member of the NRAMP family (*NRAMP1*). Mn deficiency also led to induction of two *PHO84* family transporters thought to import phosphate:Mn complexes. Possibly as a consequence, Mn deficient cells are secondarily P deficient. Further reduction in Mn availability leads to a loss of MnSOD activity which, in general, precedes the eventual loss of photosytem II activity. This suggests a prioritization of Mn usage, although the corresponding mechanisms are not well understood (Allen et al., 2007b).

Mn is also required for growth of many non-photosynthetic bacteria, although a universal requirement is by no means established. *B. subtilis* requires ~20 nM Mn for growth and sporulation requires higher levels still. In *Lactobacillus plantarum*, mutants defective in Mn uptake were only able to grow when the medium was supplemented with >10 mM Mn (Hao et al., 1999), orders of magnitude higher than for wild-type cells. This is consistent with the high intracellular levels of Mn present in this genus, which is notable for its lack of an apparent Fe requirement (Weinberg, 1997). The highly radioresistant bacterium

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Deinococcus radiodurans also accumulates high intracellular levels of Mn, which in this and other organisms is correlated with greater resistance to damage by reactive oxygen species (Daly, 2009).

Although the Mn-deprivation responses have been characterized in many different organisms, and the corresponding regulatory proteins have often been defined, there is surprising little evidence for a Mn-sparing response. The *B. subtilis* MntR regulator senses Mn directly and represses acquisition systems, but little else (Que and Helmann, 2000). Similarly, *S. aureus* up-regulates Mn import and this helps resist the inhibitory effects of the divalent metal sequestration protein calprotectin (Hammer and Skaar, 2011). Despite the fact that cells fail to grow in the absence of Mn, the essential processes requiring this metal have not been identified. One possibility is suggested by the presence of a single, Mn-dependent RNR in *B. subtilis* and related *Firmicutes* (Zhang and Stubbe, 2011).

Mn also plays a critical role in *S. cerevisiae*, where it serves as cofactor for a mitochondrial SOD (Sod2), but there is no evidence to suggest the presence of a specific metallochaperone (Reddi et al., 2009). Since Sod2 also can bind Fe, which leads to an inactive enzyme, mechanisms that ensure proper metallation are thought to be present in cells but are, as yet, poorly understood (Aguirre and Culotta, 2012). For these and related systems there is a relatively large Mn requirement and conditions of deprivation are easily achieved in the laboratory. We can therefore anticipate that ongoing studies will resolve the key Mn-dependent pathways required to support growth and may reveal sparing and recycling mechanisms analogous to those noted for other elements.

2. Nickel (Ni): a key cofactor for both C and N cycling—Ni is an essential trace nutrient for many Bacteria and Archaea and for many plants, but its roles are generally limited to, at most, a handful of enzymes (Ragsdale, 2009, Zhang and Gladyshev, 2010). The single most widely distributed Ni-dependent enzyme, and the only known Ni enzyme in eukaryotes (Zhang et al., 2009), is urease, which plays a key role in N-cycling <Figure 11>. Interestingly, *C. reinhardtii*, which can use urea as the sole N source, uses a Ni-independent enzyme for urea metabolism, although other organisms in the plant lineage use the Ni enzyme. Other Ni-dependent proteins include [NiFe] hydrogenase, a Ni form of SOD, and CO dehydrogenase (Kaluarachchi et al., 2010). Ni was relatively abundant in the ancient oceans in which life evolved and anaerobic bacteria and Archaea tend to have the most Ni-dependent enzymes (Zhang et al., 2009).

Many bacteria appear to have only one or two Ni-dependent proteins, but nevertheless have dedicated Ni transport and regulation systems. For example, *E. coli* has a [NiFe] hydrogenase and a Ni urease, and encodes the NikR-regulated NikABCDE transport system (reviewed in Li and Zamble, 2009). In contrast, *B. subtilis* has only a single predicted Ni enzyme (urease) and no obvious Ni uptake system. Indeed, efforts to demonstrate a dependence on Ni for urease activity, or a requirement for Ni for growth on urea, have been unsuccessful, raising the possibility that this enzyme is, in fact, not Ni dependent (SE Gabriel and JDH, unpublished studies). This suggests that *B. subtilis* may have dispensed with a requirement for Ni.

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a. Ni, methanogenesis, and the great oxidation event: In the oceans of the Archaean era (before 2.5 GYA), Ni was relatively abundant with estimated concentrations as high as 400 nM. Although cause and effect are uncertain, a large decline in oceanic Ni accompanied the great oxidation event (Konhauser et al., 2009) leading, eventually, to Ni concentrations in the surface waters of the present day ocean of ~1–2 nM (Saito, 2009). Despite this low level, laboratory studies combined with genomic analyses suggest that Ni is a required nutrient for many marine *Synechococcus* and all *Prochlorococcus* strains (Dupont et al., 2008a). While quite low, it should be remembered that Fe, which is used by many more organisms, may be even less available (<0.05 nM) (Saito, 2009).

Early evolving lineages, including the Archaea, have comparatively more Ni-dependent enzymes whereas some Bacteria, and many Eukaryotes (including vertebrates, insects and yeasts), may have dispensed with a Ni requirement as they lack obvious Ni transporters and urease (Zhang et al., 2009). It has been reported that mammals have a Ni requirement, but no Ni-dependent enzymes have been identified. It is notable, however, that human blood contains ~0.5 nM Ni, similar to the ocean in which our (distant) vertebrate ancestors evolved (Ragsdale, 2009).

i. Substitutions for F_{420}-reducing [NiFe]-hydrogenase in Methanobacteria: The methanobacteria are noteworthy for having a relatively large number of Ni-dependent enzymes including some of the key enzymes in methanogenesis itself. The Ni-dependent methyl-CoM reductase is responsible for the production of all biogenic methane and thereby has implications for the global climate both in the Archaean era and as a contributor to global warming today (Singh et al., 2010).

Methanogens use H_2 as a reductant to convert CO_2 to methane (CH_4) in a complex reaction involving several Ni enzymes. As a result, methanogens grow optimally with >1 μM Ni and become limited at lower levels (Thauer et al., 2010). Methanogenesis uses a flavin-based cofactor (F_{420}) that is reduced by a hydrogenase. Under Ni replete conditions, reduction of F_{420} is catalyzed by a multi-subunit flavoprotein containing both Ni and several Fe/S centers (the [NiFe] hydrogenase). Under Ni limiting conditions, the specific activity of this enzyme declines at least 100-fold and it is functionally replaced by two other enzymes that do not require Ni and use a tetrahydromethanopterin cofactor. These two enzymes (an [Fe] hydrogenase and an F_{420}-dependent methylenetetrahydromethanopterin dehydrogenase) are up-regulated several fold in response to Ni deficiency (Afting et al., 2000, Thauer et al., 2010). The [Fe] hydrogenase can functionally replace the [NiFe] enzyme for H_2 oxidation, but it is catalytically inferior with a K_m for H_2 of 0.2 mM vs. 0.01 mM for the [NiFe] enzyme. As a result, Ni-limited cells must synthesize relatively more [Fe] hydrogenase to maintain the same catalytic efficiency as for the [NiFe] enzyme (Thauer et al., 2010). *C. reinhardtii*, which also has an active hydrogenase, uses only the [Fe] type of enzyme (Happe et al., 1994), and given its Ni-independent urease has thereby dispensed with any requirement for Ni as a nutrient.

b. Substitution of Ni-urease with an Fe-variant in *Helicobacter mustelae*: An interesting Ni-sparing response has been identified in an organism that is highly reliant on urease for survival, *Helicobacter mustalae. H. mustalae* is a gastric pathogen of ferrets and, like its

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human counterpart *H. pylori*, relies on NH_3 released by urease to function as a base and locally neutralize the highly acidic pH of the stomach (Stoof et al., 2008). Urease can constitute up to 10% of soluble protein in *H. pylori*, which creates a significant Ni demand. Since ferrets are carnivores, and meat is much lower than vegetable matter in Ni content, *H. mustalae* may commonly face Ni limitation. The result is the presence of two, differentially regulated operons encoding urease. When Ni is limiting, and Fe is abundant, expression of the *ureA2B2* operon is induced and this Fe-containing urease functionally replaces the Ni-dependent isozyme (Stoof et al., 2008, Carter et al., 2011). The structure of the Fe urease reveals a dinuclear metal center. Although this enzyme is not as active as the Ni isozyme, it is sufficient to provide acid resistance (Carter et al., 2011).

c. Recycling of Ni from urease for SodN in marine *Synechococcus*: Ni is present at low levels (ca. 2 nM) in the open ocean, but in comparison to other metals, many of which are present in even lower concentration and/or in greater demand, Ni is a comparatively available resource. *Synechococcus* spp. actively transport Ni from their environment and use this metal as a cofactor for both urease and NiSOD. In laboratory studies, the former is required for growth on urea as a nitrogen source, but for one studied ocean isolate (*Synechococcus* WH8102) Ni is required even when NH_4^+ is provided as N source (Dupont et al., 2008b). This Ni requirement was ascribed to the fact that this organism encodes only a single SOD, which is a Ni enzyme and essential for phototrophic growth. When transferred from urea to NH_4^+ as nitrogen source, Ni-limited cells were able to grow for several doublings before becoming Ni limited, suggesting that Ni was likely reallocated from urease (which was no longer needed) to NiSOD. Phylogenomic comparisons indicate that NiSOD and FeSOD are often mutually exclusive which leads to the hypothesis that all marine *Prochlorococcus* strains have an obligate Ni requirement (they only encode a NiSOD), whereas most *Synechococcus, Trichodesmium*, and *Crocosphaera* also encode a NiSOD and are therefore at least partially dependent upon Ni for growth (Dupont et al., 2008b).

d. Substitutions for Ni-dependent enzymes: Despite the relatively low number of Ni-dependent enzymes in most organisms (Zhang et al., 2009), the presence of alternative, non-Ni-dependent alternatives appears to be common. For example, *Streptomyces griseus* encodes both a NiSOD and an FeZnSOD. These two isozymes are reported to be under reciprocal regulation by Ni: elevated levels of Ni stimulate the production of NiSOD and repress the FeZnSOD. The latter is due to Ni-dependent metalloregulation that involves a complex of an ArsR-family repressor (SrnR) and a Ni-binding sensor protein (SrnQ) (Kim et al., 2003). A similar reciprocal regulation is observed between NiSOD and FeSOD in *S. coelicolor*, but this regulation is mediated by a Ni-sensing Fur family protein designated as Nur. Nur, when bound to Ni, directly represses the transcription of the FeSOD-encoding gene while indirectly activating transcription of the gene for NiSOD (Ahn et al., 2006). A final example of alternatives to Ni-dependent enzymes is provided by the glyoxalases, widely distributed enzymes that detoxify methylglyoxal. Most eukaryotic glyoxalases use Zn as cofacter, whereas many bacterial enzymes use Ni or Co. While most organisms have one or the other, in *Pseudomonas aeruginosa* both types are present (Sukdeo and Honek, 2007). This redundancy presumably allows efficient methylglyoxal detoxification over a wider range of growth conditions relative to organisms with only a single isozyme.

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3. Cobalt (Co): vitamin B_{12} dependent and independent enzymes—Co, most commonly in the form of the corrinoid-based cofactor known as vitamin B_{12}, is required by most prokaryotes (Bacteria and Archaea) and many Eukaryotes (Figure 11). The major exceptions appear to be plants and fungi (Rodionov et al., 2003). Many organisms (including humans) that do not synthesize B_{12} nevertheless have a dietary requirement for it. In humans, B_{12} is needed for methionine synthesis and as a cofactor for propionate metabolism. Organisms that require B_{12} but lack the potential for synthesis have B_{12}-transport systems for acquiring this key nutrient. The expression of B_{12} uptake and synthesis functions is often translationally repressed by cytosolic B_1, which can be sensed by an RNA-based (riboswitch) mechanism (Nahvi et al., 2004).

A common adaptation to B_{12} limitation is the substitution of B_{12}-dependent enzymes with B_{12}-independent isozymes. For example, *E. coli* encodes two forms of methionine synthase (Drummond and Matthews, 1993). In the absence of exogenous B_{12} (which *E. coli* is unable to synthesize de novo), the Zn-dependent MetE protein is required for methionine synthesis (Hondorp and Matthews, 2009). Conversely, in the presence of B_{12}, synthesis of MetE is reduced (Wu et al., 1992). Reciprocal regulation of the two methionine synthase isozymes is also noted in *M. tuberculosis*. In this organism, a B_{12}-sensing riboswitch selectively represses the transcription of the B_{12}-independent isozyme (Warner et al., 2007). We previously noted the functional redundancy between the *E. coli* Fe and Mn forms of ribonucleotide reductase, an enzyme required for DNA synthesis. In *S. coelicolor*, two RNR enzymes are used. The class 1a enzyme is oxygen dependent while the class II enzyme requires B_{12}. When B_{12} is available, the class 1a enzyme is repressed (Borovok et al., 2006).

Functional redundancy of methionine synthases is also widespread in the algae. Amongst the algae, B_{12} is obtained from bacterial symbionts. When present, B_{12}-represses the synthesis of the B_{12}-independent (*METE*) isozyme and functionally activates the B_{12}-dependent enzyme (*METH*). Phylogenetic comparisons combined with growth assays reveal that many algal species require B_{12} for growth due to loss of *METE*, which appears to have occurred multiple times in algal evolution (Helliwell et al., 2011). Algae are estimated to be responsible for fixation of 50% of C worldwide (Field et al., 1998). Since many algae must acquire B_{12} from their environment, B_{12} limitation can influence primary productivity (Bertrand et al., 2011a, Bertrand et al., 2011b, King et al., 2011). B_{12} production by bacteria, in turn, can be limited by the availability of Co.

4. Metal and cofactor substitutions involving Molybdenum (Mo), Vanadium (V) and Tungsten (W)—Mo is a required element for many organisms throughout all three domains of life <Figure 12>. The high solubility of molybdate salts and their abundance in the oceans likely contributed to the adoption of Mo early in evolution as a redox cofactor. Mo often functions as part of a metal-binding pterin (MPT)-based cofactor known as molybdopterin (Moco), which is a required redox cofactor in as many as 50 different enzymes, mostly in bacteria, from four different families (Zhang and Gladyshev, 2008). In some enzymes, MPT functions instead with tungsten (W) to generate Wco (or Tuco), which is analogous to Moco.

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Mo is very broadly required for life (Zhang and Gladyshev, 2008).The major Moco-dependent enzyme families are sulfite oxidase, xanthine oxidase, dimethylsulfoxide reductase, and aldehyde:ferredoxin oxidoreductase (Schwarz et al., 2009). Genomic surveys reveal that ~72% of bacteria encode Moco enzymes with the major exceptions being members of the *Firmicutes* and *Chlamydia*. Moco enzymes are found in ~95% of Archaeal and ~63% of Eukarya genomes. A requirement for Moco appears to have been lost in many parasites, yeast, and ciliates (Zhang and Gladyshev, 2008). The biosynthetic pathway for Moco is also conserved and most organisms that synthesize Moco have dedicated Mo uptake systems, which have been shown in some systems to be inducible by Mo deficiency.

Unlike Mo, tungsten (W) and vanadium (V) have very restricted uses in biology and those enzymes that use these metals often do so in place of Mo (Hille, 2002). The chemical properties of W and Mo are, in many ways, quite similar (W is immediately below Mo in the periodic table; Figure 2) and they can catalyze many of the same reactions. It has been suggested that ancient life may have evolved to use W, which would have been more available in the anaerobic, sulfidic conditions of the Archean era. Subsequent to the great oxidation event, increased solubility of Mo oxides may have favored a displacement of W with Mo. Today, tungstoenzymes are restricted to obligate, usually thermophilic, anaerobes. V also has restricted uses in biology and can functionally substitute, in some bacteria, for Mo by serving as cofactor for an alternative nitrogenase. Here, we focus specifically on those examples where enzyme substitutions appear to play a possible role in metal-sparing responses.

The sulfate reducing bacterium *Desulfovibrio alaskensis* encodes two formate dehydrogenases (members of the dimethylsulfate reductase family of enzymes). One isozyme (W-FDH) requires W, whereas the other can function with either W or Mo (Mo/W-FDH) (Mota et al., 2011). Genome analysis suggested the presence of uptake and cofactor assembly pathways for both Moco and Wco, consistent with the ability of this organism to synthesize both cofactors. Growth in the presence of Mo leads to the up-regulation of the Mo/W isozyme whereas addition of 10 μM W to the medium led to the exclusive synthesis of the W-FDH isozyme. These results suggest that this organism modulates its synthesis of these two enzymes in response to metal availability for cofactor synthesis (Mota et al., 2011). Other organisms may also have functionally redundant pathways that can utilize either Mo or W cofactored enzymes. This has been suggested, for example, in the Archaean *Methanosarcina acetivorans* where genome analysis suggests the presence of paralogous gene clusters encoding Mo-dependent and W-dependent forms of formylmethanofuran dehydrogenases (Rohlin and Gunsalus, 2010). It is presently unknown whether this apparent redundancy is an elemental sparing response and confers an advantage when either Mo or W is limiting in the growth medium.

Insights into acclimation mechanisms can often be obtained by noting the genes that are induced in response to elemental limitation. As noted above for both Fe and Zn, defining the regulons controlled by metalloregulatory proteins led to the identification of numerous sparing responses. Similarly, it can be anticipated that defining the regulatory consequences of Mo or W deficiency may lead to new insights. One powerful tool is provided by the observation that riboswitch elements are associated with the regulation of MPT synthesis

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genes in many different Bacteria (Regulski et al., 2008). To date, however, this regulatory element seems to exclusively regulate uptake and synthesis functions (acquisition), rather than controlling alternative isozymes (e.g. Mo-sparing).

a. Alternative nitrogenases and acclimation to Mo limitation: Mo is also required in nitrogenase, the key enzyme of nitrogen fixation (Hernandez et al., 2009, Schwarz et al., 2009). In the absence of fixed forms of N, diazotrophic (N_2-fixing) organisms synthesize nitrogenase at levels up to 10% of total cell protein, which places a high demand for the corresponding metal ion cofactors (Dingler et al., 1988). The soil bacterium *Azotobacter vinelandii* has emerged as one of the premier model systems for investigating nitrogenase enzymology and regulation. This organism secretes a variety of catechols (metallophores) originally identified as siderophores but which are thought to also help mobilize Mo and other metals to support nitrogenase synthesis (Kraepiel et al., 2009). When Mo is available, and there is no fixed nitrogen, *A. vinelandii* induces the synthesis of a Mo-nitrogenase and, conversely, if Mo is unavailable alternative nitrogenases are synthesized instead.

In the Mo-nitrogenase, Mo does not bind as a complex with MPT, but instead forms a complex polynuclear cluster with Fe. The resulting cofactor (FeMoco, with formula $MoFe_7S_9X$-homocitrate; where X=C,O, or N), together with the $[Fe_8S_7]$ P cluster, is part of the MoFe subunit of nitrogenase. The MoFe subunit functions together with an Fe protein (a homodimeric protein with a bridging $[Fe_4S_4]$ cluster) to catalyze what is ultimately the eight electron reduction of N_2 to 2 NH_3. Two alternative nitrogenases are expressed under conditions of Mo limitation (Joerger and Bishop, 1988, Dos Santos and Dean, 2011). Nitrogenase-2 contains an Fe protein very similar to the Fe protein of the Mo-nitrogenase and a vanadium-containing VFe subunit that functionally replaces the MoFe subunit. When both Mo and V are limiting, nitrogenase 3 is expressed which is sometimes referred to as an Fe-only enzyme. Similarly, *Rhodobacter capsulatus* expresses an alternative Fe-only nitrogenase (containing a cofactor designated FeFe-co) in response to Mo limitation. In general, these alternative nitrogenases are not as efficient as is the MoFe enzyme (Hernandez et al., 2009).

The expression of alternative nitrogenases is regulated by metal ion availability (Bellenger et al., 2011). In *A. vinelandii* grown in the presence of both Mo (7 nM) and V (6.5 nM), the cells first import Mo until this metal is exhausted and then they import V such that the total (Mo + V) maintains a near constant quota in the growing cells. Since both Mo and V can be complexed by metallophores, it appears that the metallophore:Mo complex is selectively recognized and transported prior to expression of a presumed transporter specific for the metallophore:V complex (Bellenger et al., 2011). The Mo nitrogenase is the most active of the three nitrogenase forms and cells hyperaccumulate Mo in excess of their immediate needs to maintain synthesis of this preferred enzyme. Cytosolic Mo activates expression of the Mo-nitrogenase while repressing expression of activator proteins needed for expression of both the V-nitrogenase and Fe-nitrogenase (Masepohl and Hallenbeck, 2010). Although not defined for *A. vinelandii*, in *R. capsulatus* Mo is sensed by two molybdate-binding metalloregulatory proteins (MopA and MopB) that are related to the *E. coli* ModE protein (Masepohl et al., 2002). The V-nitrogenase is only slightly less active, but V does not appear to be stored within the cells and may be toxic in excess (Bellenger et al., 2011).

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Studies in these model organisms, together with metagenomic surveys of diverse environments (Zehr et al., 2003, Gaby and Buckley, 2011), indicate that many organisms contain alternative nitrogenase systems to maintain diazotrophy even when Mo is not readily available. The major N_2 fixing species in the open ocean are likely cyanobacteria such as *Trichodesmium*, but a newly identified small, unicellular cyanobacterium group (UCYN-A) (Moisander et al., 2010, Zehr, 2011), as well as proteobacteria (Farnelid et al., 2011) may also be a major contributors. The impact of metal availability on N_2 fixation is not well understood, and is further complicated by the fact that some N_2-fixing ocean organisms likely exist in still poorly defined symbiotic relationships (Tripp et al., 2010, Zehr and Kudela, 2011).

5. Selenium (Se): substitutions for acclimation to Se deprivation—Selenium is an essential trace element for many organisms where it is cotranslationally inserted into proteins as the 21st amino acid, selenocysteine (Sec) (Figure 12). Insertion of Sec occurs at UGA stop codons within the specific context of mRNAs with a selenocysteine insertion sequence (SECIS). Most selenoproteins have a single Sec residue, which typically serves a catalytic role as in, for example, Se-containing glutathione peroxidases. However, once an mRNA is targeted for Sec insertion, it is possible for other Sec-encoding UGA codons to be tolerated and additional, non-catalytic Sec residues have also been identified (Lee et al., 2011), although these are rare.

Selenoproteins are found throughout all three domains of life although there are exceptions including, notably, yeast and higher plants (Kryukov and Gladyshev, 2004, Lobanov et al., 2009). Sec insertion can be readily inferred from the occurrence of UGA codons within reading frames, typically in place of a Cys codon in orthologs, which (in combination with SECIS elements) is diagnostic of Sec. Bioinformatic analyses (ca. 2003) identified Sec-containing proteins in ~20% of completed Bacterial and ~14% of Archaeal genomes (Kryukov and Gladyshev, 2004). For those organisms that lack Sec, the corresponding selenoproteins may still be present but typically have a Cys residue in place of Sec. Substitution of Sec for Cys has occurred many times in evolution, as has the converse. At least two rationales, not mutually exclusive, have been advanced for the substitution of Cys with Sec. First, selenoenzymes may be more catalytically efficient than their Cys counterparts. Increased enzymatic efficiencies of the Sec variant range from four-fold to perhaps several hundred-fold (Stadtman, 1996, Hazebrouck et al., 2000, Kim et al., 2006). Second, the Sec variants may be significantly more resistant to oxidative inactivation (Hondal and Ruggles, 2011, Nauser et al., 2012).

Selenoproteins are especially prominent in methanogens. For example, *Methanococcus maripaludis* encodes ten Sec proteins and eight of these are involved in methanogenesis. These include subunits of both the F_{420}-dependent and F_{420}-independent hydrogenases, W-containing formyl-methanofuran dehydrogenase, Mo-containing formylmethanofuran dehydrogenase, heterodisulfide reductase and two formate dehydrogenases (Hohn et al., 2011). Despite this abundance of Sec-containing proteins, *M. maripaludis* is able to grow in the absence of Sec by the expression of alternate enzymes containing Cys in place of Sec. The only exception is the formate dehydrogenase, which requires Se for both isozymes. As a

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result, inactivation of Sec incorporation prevented growth on formate, but not growth by methanogenesis (Rother et al., 2003).

The substitute Cys-containing methanogenesis proteins are transcriptionally regulated by a Se-sensing regulatory protein, HrsM (Sun and Klein, 2004). This LysR family regulator represses expression in the presence of Se. Since growth of *M. maripaludis* in the absence of Sec synthesis requires deletion of either the *selD* gene (encoding selenophosphate synthase) or *hrsM* (encoding the repressor), it is hypothesized that selenophosphate may be the co-repressor for HrsM-mediated transcriptional repression (Hohn et al., 2011). The use of selenophosphate as corepressor is expedient, since synthesis of this intermediate is catalyzed by SelD, which is, remarkably, itself a selenoprotein (Stock et al., 2010). Thus, selenophosphate synthesis requires both Se and a functional Sec synthesis and incorporation pathway.

It is interesting that many, but not all, SelD homologs are Sec enzymes, which has previously led to the suggestion that this might serve a positive feedback mechanism (Guimaraes et al., 1996). We suggest instead that the Sec in SelD may be present not for its catalytic advantages, but as an indicator of the cell's ability to synthesize selenoproteins. Although the mechanisms are not yet defined, it has also been proposed that *M. maripaludis* may have a hierarchy for Sec-insertion into proteins with formate hydrogenase (which lacks a Cys-containing alternate) given priority under conditions of limited Sec synthetic capacity (Stock et al., 2011).

IV. Major Themes - Microbial Adaptations to Elemental Limitation

We noted at the outset the explosion of knowledge in the molecular life sciences occasioned by the convergence of biology and chemistry. The periodic table is, arguably, the greatest organizing theme of chemistry, while the theory of evolution similarly pervades biological thought (Figure 1). We have here considered several different microbial systems and the remarkable ways in which they have adapted to elemental limitations. Conceptually similar evolutionary responses have emerged in systems as distinct as: methanogens facing a Ni famine at the end of the Archean era; phytoplankton in the contemporary ocean's surface waters facing chronic limitations for P, Fe or Zn; and mammalian pathogens facing metal ion deprivation imposed by the innate immune system. Our scope has been very broad, and many of these same topics are reviewed (in cited references) in greater detail but with an emphasis on a single element, a single organism, or both. As is often the case in biology, comparisons between systems help reveal common themes, which we hope we have accomplished in this work.

Elemental limitations may be chronic in the environment, imposed by a proliferation of neighboring cells (of the same or a different species), or imposed by a host organism. The corresponding adaptations and acclimations include alterations of the elemental composition of macromolecules to reduce or eliminate the elemental requirement. Elimination of a requirement has occurred repeatedly in evolution: cells have learned to thrive in the absence of elements like Ni, Co, and even Fe (in rare examples). More commonly, cells develop strategies for reducing demand, particularly under times of duress, by altering the

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composition of abundant constituents such as proteins or membrane lipids. Often, eliminating or reducing the demand for one element increases demand for another. The process of substitution, in which one form of an enzyme or other macromolecule is replaced by an alternate form, is a widespread feature of elemental economy. In many cells, these changes are now fixed and the corresponding evolutionary pressures can only be inferred.

Perhaps the most widespread adaptation to elemental restriction is the development of acclimation mechanisms. Acclimation typically involves several complementary strategies that include: (i) high affinity acquisition mechanisms and pathways to access recalcitrant resources that may not be available to all organisms, (ii) mobilization of elements stored during times of relative abundance, (iii) elemental sparing responses, and (iv) elemental recycling. Elemental sparing often involves the synthesis of substitute enzymes that rely on different elements, but these are often catalytically inferior or less robust than the preferred isozymes. Collectively, these responses help to redirect elemental resources to the highest priority targets.

These same strategies, implemented daily by microbes, have numerous parallels throughout society. When a yeast cell is challenged by Cd, S is preferentially directed towards synthesis of the defensive molecule glutathione, while a widespread S-sparing response is implemented to suppress now lower priority uses. Analogously, when the United States entered World War II, the massive shift to synthesis of armaments required the introduction of widespread recycling programs (scrap drives) to recover valuable metals, while the "lower priority" needs of the civilian population were accommodated by substitution (a shift from metal-based consumer goods to those based on wood and other renewables). We are still in the early stages of dealing with our dependency on non-renewable resources including, in many cases, elemental resources. For example, much of modern computer-based technology relies on a family of rare earth metals with ~97% of today's world supply controlled by a single country (Service, 2010) and our agricultural supply of phosphate is expected to run out by the end of this century (Gilbert, 2009). Ultimately, our dependence on elemental resources will require an ever increasing reliance on recycling rather than new acquisitions.

While work in microbial systems highlights these mechanisms of elemental economy, there is still much to be learned. The mechanisms that regulate the processes of sparing, recycling, and substitution are often poorly understood. There are numerous hints that organisms prioritize targets for synthesis as elemental availability declines, but the mechanisms are still obscure. A major challenge for the future is to define the complete elemental requirements for cell growth and how this varies with the environment and form of metabolism. This is presently a focus of numerous metallomics studies which seek to define the elemental quotas for various model organisms. It is remarkable that we, in most cases, are still unable to provide a satisfying answer to the seeming simple questions: which elements, and in which amounts, are required for life, and perhaps more important, why are they needed?

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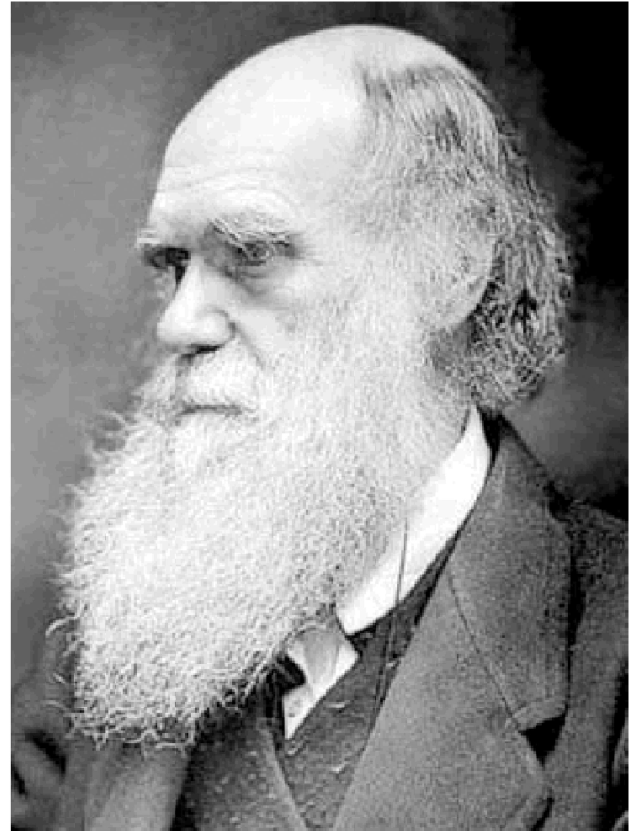

Figure 1.
Mendeleev meets Darwin. Dmitri Mendeleev (1834–1907) (*left*) developed the periodic table of the elements (ca. 1869). Charles Darwin (1809–1882) (*right*) developed the theory of evolution by natural selection.

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1 H																	2 He
3 Li	4 Be											5 B	6 C	7 N	8 O	9 F	10 Ne
11 Na	12 Mg											13 Al	14 Si	15 P	16 S	17 Cl	18 Ar
19 K	20 Ca	21 Sc	22 Ti	23 V	24 Cr	25 Mn	26 Fe	27 Co	28 Ni	29 Cu	30 Zn	31 Ga	32 Ge	33 As	34 Se	35 Br	36 Kr
37 Rb	38 Sr	39 Y	40 Zr	41 Nb	42 Mo	43 Tc	44 Ru	45 Rh	46 Pd	47 Ag	48 Cd	49 In	50 Sn	51 Sb	52 Te	53 I	54 Xe
55 Cs	56 Ba	(La)	72 Hf	73 Ta	74 W	75 Re	76 Os	77 Ir	78 Pt	79 Au	80 Hg	81 Tl	82 Pb	83 Bi	84 Po	85 At	86 Rn

Figure 2.

A cellular perspective on the periodic table. Essential macronutrients are in white against a black background and universally essential cations (Zn, Mg) in white against a grey background. Elements that have important biological roles in many but likely not all cells are shown in boldface against a dark grey background. These include the key transition metals (Mn, Fe, Co, Cu, Mo) and cations (K). Elements that are used for specialized purposes in some microbes are shown against a light grey background. These include a requirement for boron (B) in plants for cell wall structure and in some bacteria for quorum sensing. Elements that may have specialized uses, but are not known to contribute to growth are in large font against a white background (Cr,Cl, I).

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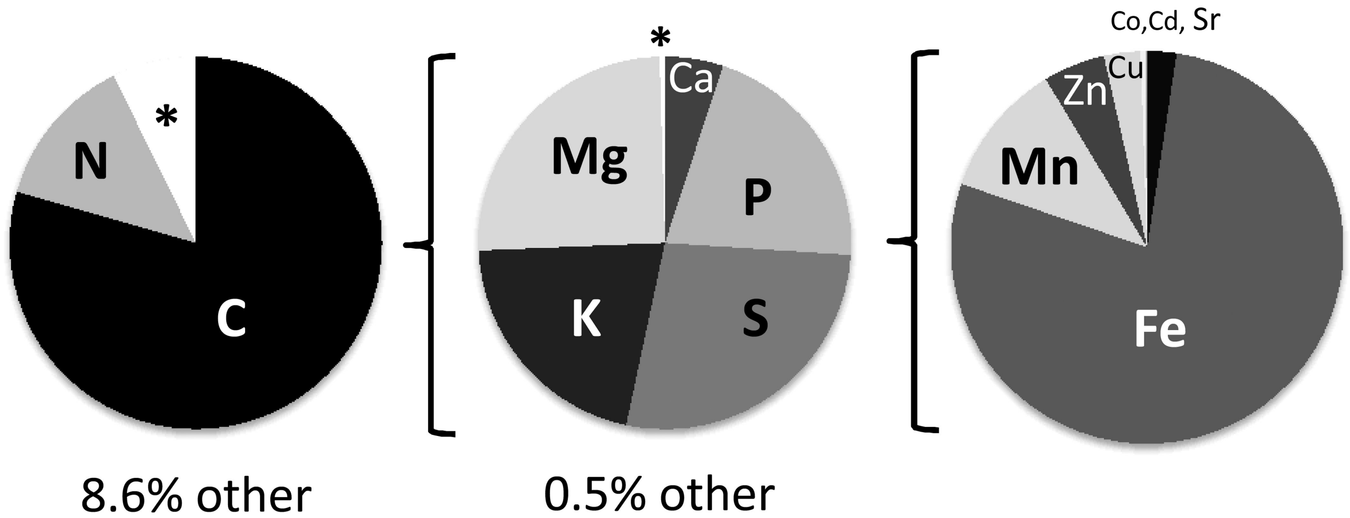

Figure 3.

Atomic composition of *Synechococcus* sp., a representative of the bacterial phytoplankton. For each pie chart, the portion indicated by the asterisk (other) is expanded in the pie chart to the right. On the far right, Sr is indicated by the black slice and the thin white slice between Cu and Sr includes Co and Cd. Plotted with data from (Quigg et al. 2011).

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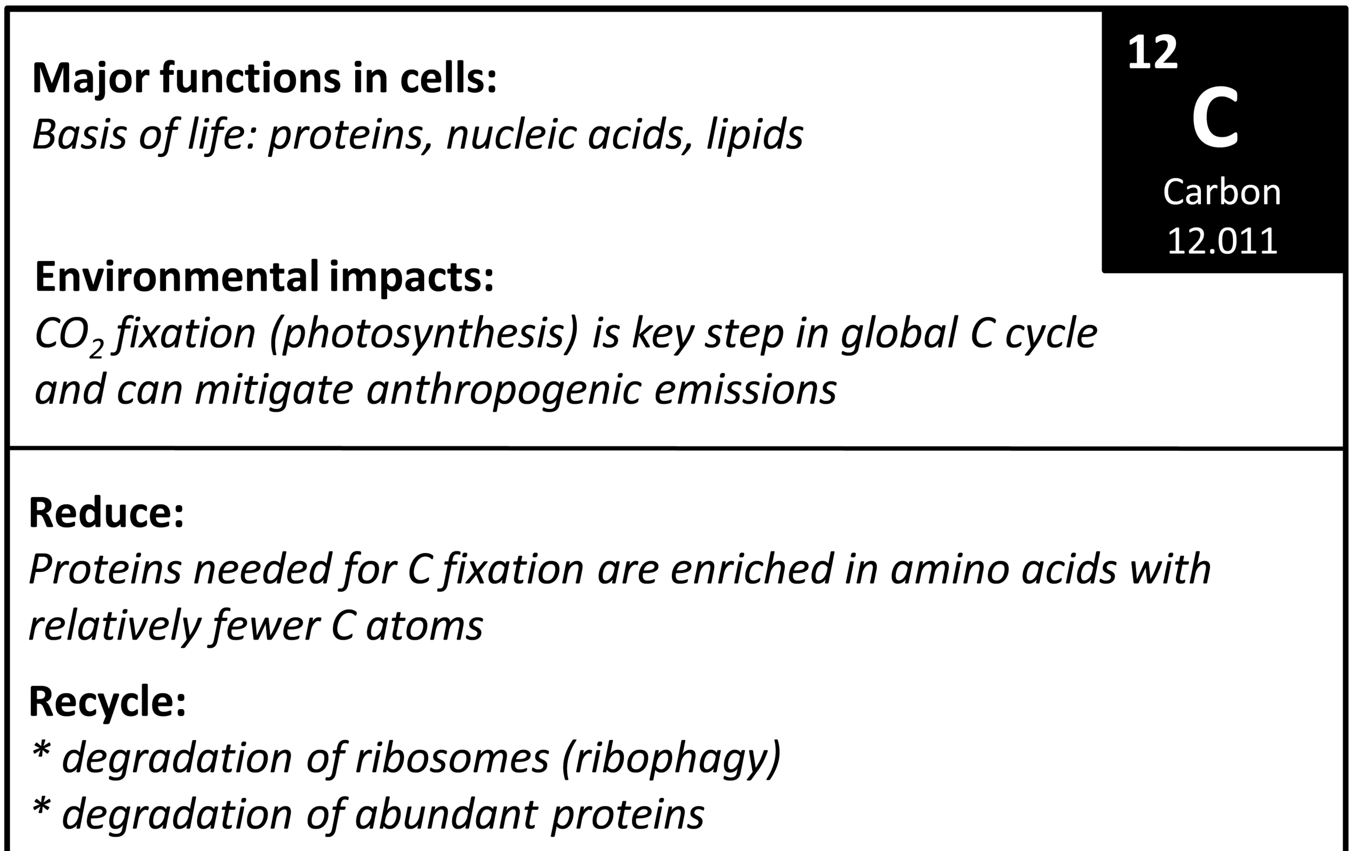

Figure 4.
Overview of the biological roles of C and known sparing and recycling mechanisms (see text for details.)

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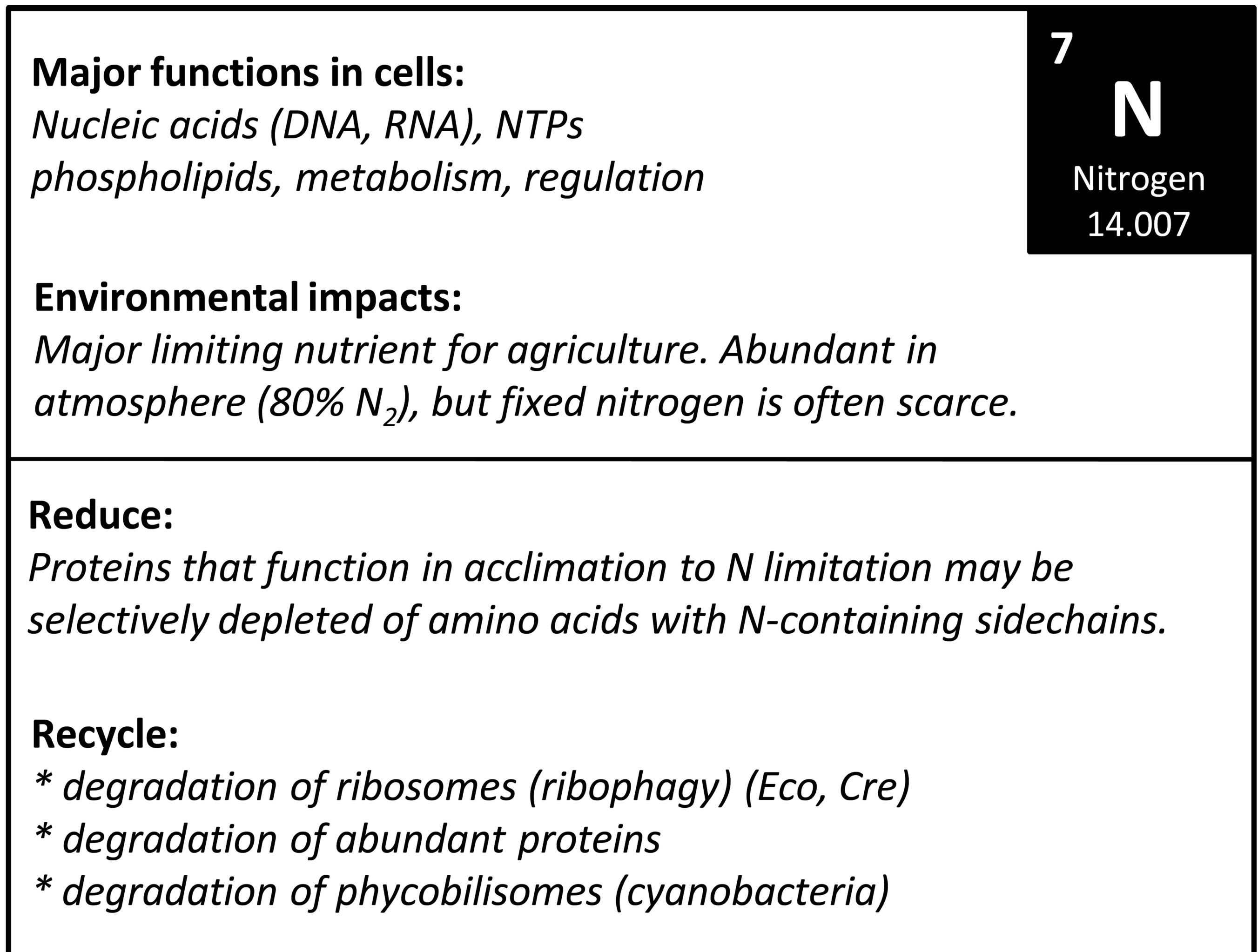

Figure 5.
Overview of the biological roles of N and known sparing and recycling mechanisms.

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16
S
Sulfur
32.065

Major functions in cells:
Proteins (Cys, Met and Fe/S), low molecular weight thiols (glutathione, mycothiol, bacillithiol), biotin, lipoic acid.

Environmental impacts:
Sulfur limitation can affect plant or microbial growth.
Anthropogenic sulfur compounds contribute to acid rain.

Reduce:
Proteins required for S assimilation are depleted of Cys, Met
Substitute: Cells may express S-depleted variants of abundant proteins during acclimation to S limitation (Eco, Sce, Cre)

Recycle:
** degradation of phycobilisomes (Syn)*
** degradation of sulfolipids (SQDG) (Cre)*

Figure 6.
Overview of the biological roles of S and known sparing and recycling mechanisms.

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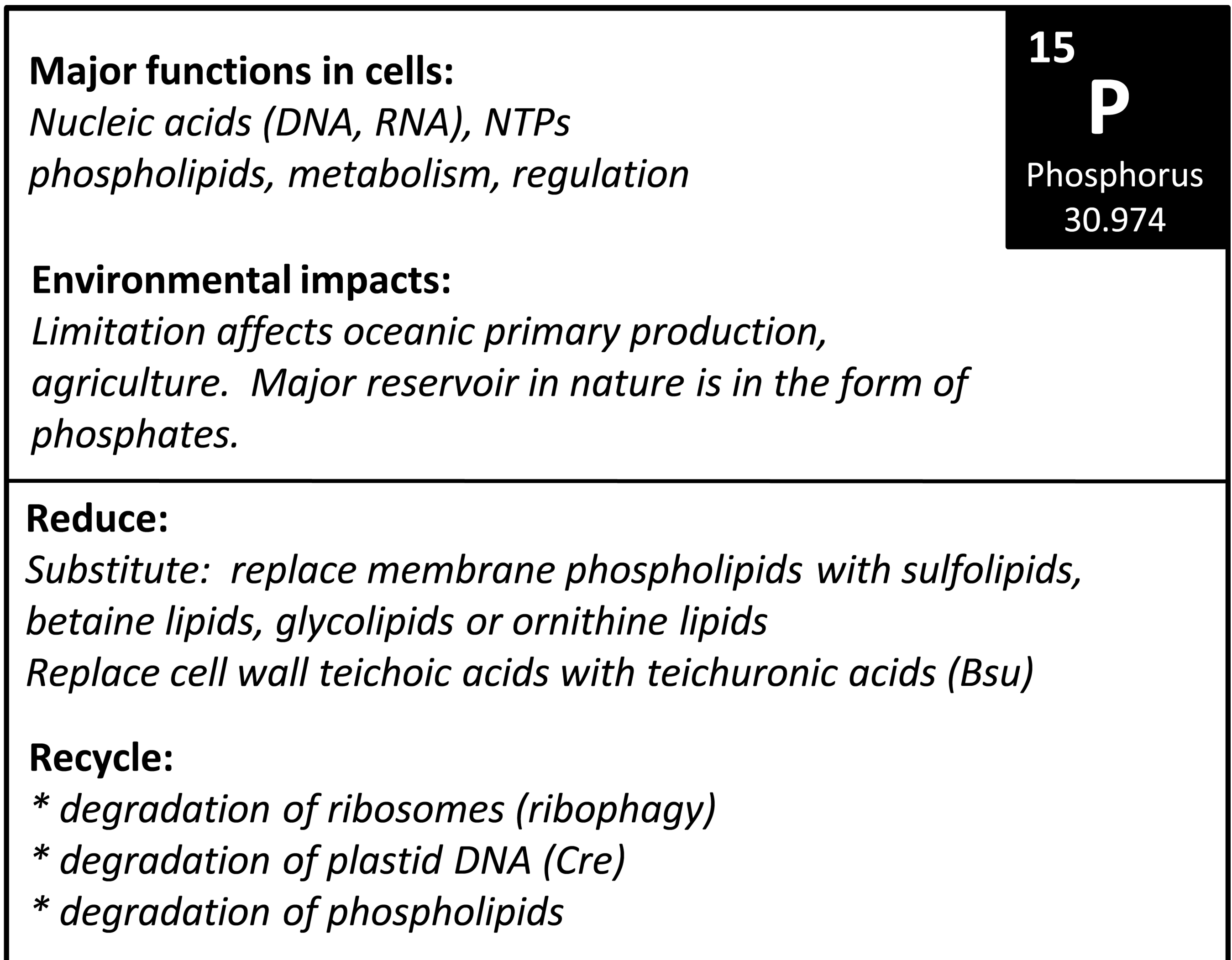

Figure 7.

Overview of the biological roles of P and known sparing and recycling mechanisms.

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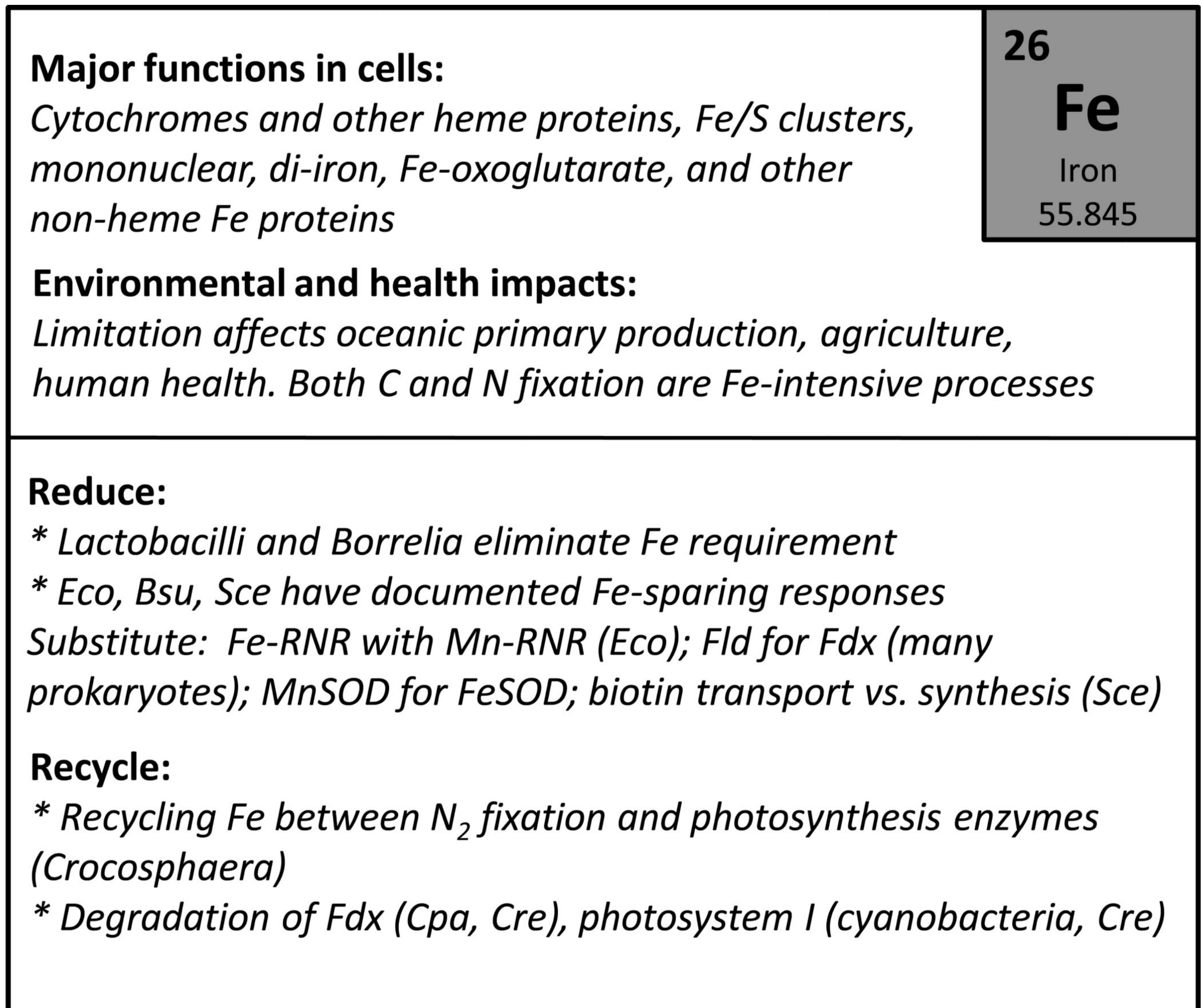

Figure 8.

Overview of the biological roles of Fe and known sparing and recycling mechanisms.

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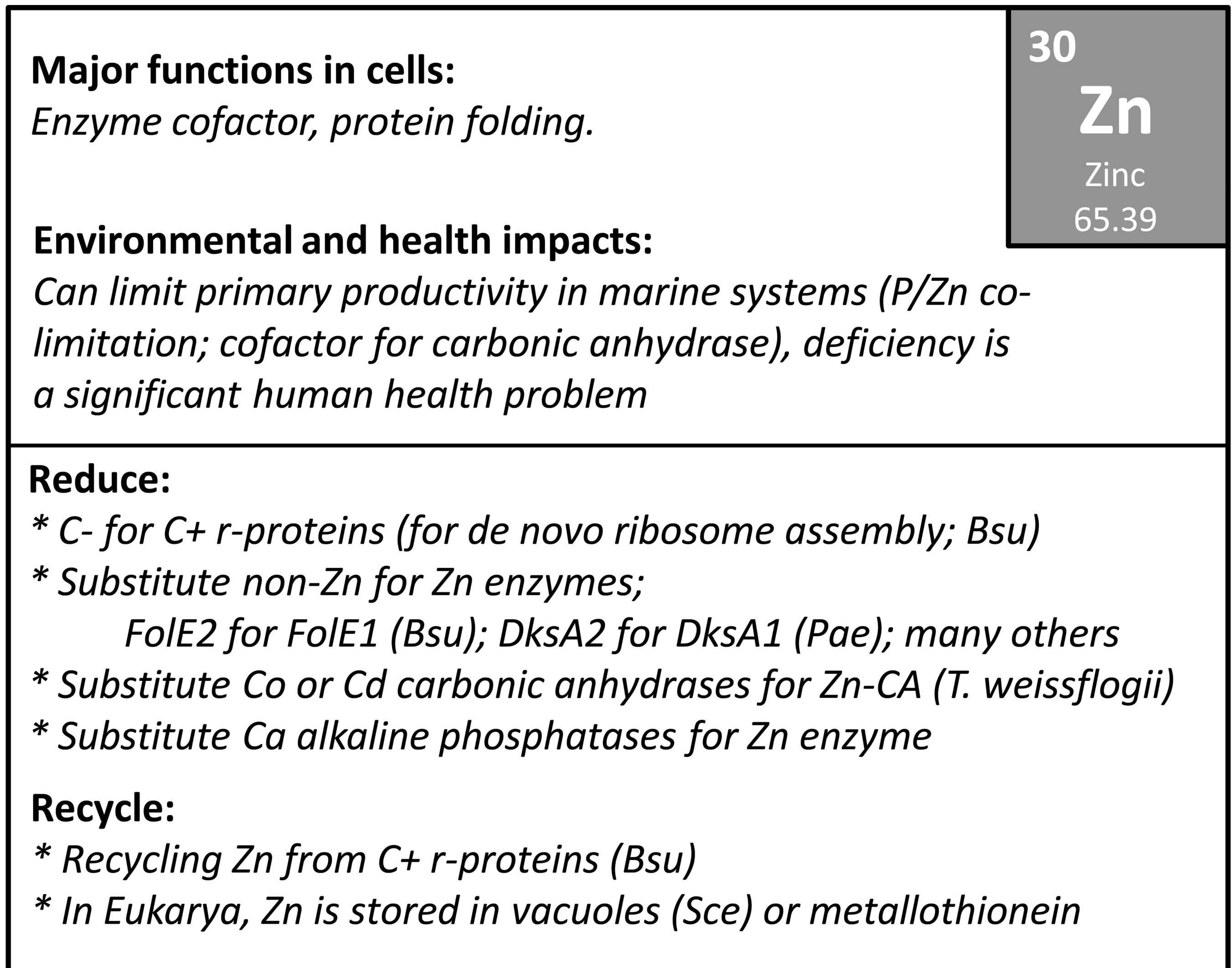

Figure 9.
Overview of the biological roles of Zn and known sparing and recycling mechanisms.

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Figure 10.
Overview of the biological role of Cu and known sparing and recycling mechanisms.

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27 **Co** Cobalt 58.93	28 **Ni** Nickel 58.69
Major functions in cells: *B_{12} cofactor for isomerases, one carbon transfers, and dehalogenases*	**Major functions in cells:** *Enzyme cofactor for SOD, urease, hydrogenases.*
Environmental and health impacts: *some marine systems are B_{12}-limited; cobalamin deficiency is a problem in human nutrition*	**Environmental impacts:** *Required for methanogenesis, substitutes for other metals in phytoplankton*
Reduce: ** Substitute B_{12}-dependent with B_{12}-independent ribonucleotide reductase (Eco, Sco)* ** Substitute B_{12}-dependent with B_{12}-independent methionine synthase (Eco, Cre)*	**Reduce:** ** substitute [NiFe] hydrogenase with [Fe] enzyme (methanogens)* ** substitute Ni-urease with Fe-urease (Helicobacter mustalae)* ** substitute NiSOD with FeSOD (Streptomyces spp.)* **Recycle:** ** Recycle Ni from urease to NiSOD (Syn)*

Figure 11.
Overview of the biological roles of Co (B_{12}) and Ni and known sparing mechanisms.

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Figure 12.
Overview of the biological roles of Mo (W) and Se and known sparing mechanisms.

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Table 1

The elements of microbial cells

Element (symbol)	Major functions and uses in microbial cells
	Required for All Cells
C	basis of all organic molecules
H	H_2O, organic molecules
N	organic molecules, esp. proteins and nucleic acids
O	H_2O, organic molecules
P	nucleic acids, NTPs, metabolites, phospholipids
S	proteins, glutathione and LMW thiols, biotin, lipoic acid, thiamin
Mg	major cation; cofactor for phosphotransferase reactions
Zn	enzyme cofactor, protein folding
	Required for Most Cells
K	major cation, common in cells
Ca	major cation, required by many eukaryotes
Mn	enzyme cofactor, ribonucleotide reductase, SOD, PS II
Fe	heme, iron-sulfur cluster, non-heme enzymes
Co	enzyme cofactor, B_{12}-dependent enzymes
Cu	enzyme cofactor, electron carrier, respiration, SOD
Mo	FeMoCo cofactor (nitrogenase), Mo cofactor enzymes
	Required for Specialized Functions in Some Cells
Se	selenocysteine in proteins
B	plant cell wall, quorum sensing (some AI-2)
Na	used for ion potential in halophiles
Si	some plant cell walls, diatom walls (frustules)
Cl,Br,Fl,I	some bacterial secondary metabolites
V	nitrogenase, haloperoxidases
Ni	urease, SOD (SodN), glyoxalase
Cd	cofactor for carbonic anhydrase (CA) in some marine microbes
W	tungstoenzymes (aldehyde oxidoreductase, formate dehydrogenase, acetyl hydratase)

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Table 3

Microbial Reference Systems for Investigations of Elemental Economy

Domain:Phylum	Organism (abbreviation)	Environment	Nutrition	Notable mechanisms for elemental economy
A. Model systems for molecular genetic studies				
B: Proteobacteria	*Escherichia coli (Eco)*	mammalian intestine	facultative anaerobe	C/N - elemental optimization; recycling by ribophagy S - elemental optimization Fe - Fur regulon; Fe-sparing (RyhB sRNA); isozyme substitution, MnSOD vs. FeSOD; Mn RNR vs. Fe RNR
B: Firmicutes	*Bacillus subtilis (Bsu)*	soil, rhizosphere	facultative anaerobe	C/N - recycling by cannibalism P - P-sparing and recycling by cell wall remodeling Fe - Fur regulon; Fe sparing (FsrA sRNA); flavodoxin substitution for ferredoxin Zn - Zur regulon; ribosomal protein mobilization, FolE2
A: Euryarchaeota	*Halobacterium salinarum (Hsa)*	halophilic archaeon		Fe - Fe acclimation response defined
A: Euryarchaeota	*Methanococcus maripaludis (Mma)*; *Methanosarcina acetivorans (Mac)*		methanogenesis	Ni - Ni sparing response for methanogenesis enzymes Mo/W-isozymes of formylmethanofuran dehydrogenases
E: Ascomycota	*Saccharomyces cerevisiae (Sce)*	yeast, model eukaryote	respiration, fermentation	C/N - elemental optimization; recycling S - S-sparing, substitution with S-depleted isozymes Fe - Fe-sparing (Aft1/Aft2 and Cth1/Cth2 regulons); isozyme substitution Zn - Zn-sparing by Zap1 repression of Adh1 and Adh3
E: Chlorophyta	*Chlamydomonas reinhardtii (Cre)*	soil	photosynthetic and heterotrophic	N - ribophagy during gametogenesis S - S-sparing, substitution with isozymes; recycling of S from sulfolipids P - substitute P-lipids with S-lipids; recycle P from

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Domain:Phylum	Organism (abbreviation)	Environment	Nutrition	Notable mechanisms for elemental economy
				chloroplast DNA by copy number reduction Fe – down-regulation of PS I and Fd, prioritizing respiration over photosynthesis under heterotrophic conditions, chloroplast MnSOD induced Cu – Cyt c_6 for plastocyanin, flavin amine oxidase instead of Cu amine oxidase
B. Reference systems for environmental and genomics-based studies in marine ecosystems				
B: Cyanobacteria	*Prochlorococcus marinus (Pma)*	oligotrophic open ocean	smallest known phototroph,	P - substitute P-lipids with S-lipids Fe - PS I remodeling
B: Cyanobacteria	*Trichodesmium (Tri)*	oligotrophic open ocean	phototroph, diazotroph	Fe- Fe-sparing by down-regulation of nitrogen fixation; Fe mobilization from Dps miniferritin
B: Cyanobacteria	*Synechococcus (Syn)*	mesophilic ocean and freshwater species	phototroph	N - recycling by phycobilisome degradation Fe - Fe sparing; flavodoxin substitution for ferredoxin Ni - recycling of Ni from urease to SodN (proposed)
B. Cyanobacteria	*Synechocystis (Syc)*	freshwater lake	phototroph	Cu - Cyt c_6 for plastocyanin
B: Cyanobacteria	*Crocosphaera watsonii (Cwa)*	oligotrophic open ocean	phototroph, diazotroph	Fe - diurnal cycling of iron between photosynthetic and nitrogen fixation complexes (reduces Fe quota by 40%)