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MINI-REVIEW

Effects of calorie restriction on life span of microorganisms

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Abstract Calorie restriction (CR) in microorganisms such as budding and fission yeasts has a robust and well-documented impact on longevity. In order to efficiently utilize the limited energy during CR, these organisms shift from primarily fermentative metabolism to mitochondrial respiration. Respiration activates certain conserved longevity factors such as sirtuins and is associated with widespread physiological changes that contribute to increased survival. However, the importance of respiration during CR-mediated longevity has remained controversial. The emergence of several novel metabolically distinct microbial models for longevity has enabled CR to be studied from new perspectives. The majority of CR and life span studies have been conducted in the primarily fermentative Crabtree-positive yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, but studies in primarily respiratory Crabtree-negative yeast and obligate aerobes can offer complementary insight into the more complex mammalian response to CR. Not only are microorganisms helping characterize a conserved cellular mechanism for CR-mediated longevity, but they can also directly impact mammalian metabolism as part of the natural gut flora. Here, we discuss the contributions of microorganisms to our knowledge of CR and longevity at the level of both the cell and the organism.

Keywords Calorie restriction · Microorganisms · Mitochondrial respiration · Metabolism · Life span · Aging

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Introduction

Unlike in many aspects of biology, the calorie restriction–life span connection was first established in a complicated multicellular organism (rat; McCay et al. 1989) and then adapted to a variety of both simpler eukaryotic (Roux et al. 2010) and perhaps prokaryotic microbes (Lele et al. 2008). The advantages of using simpler systems to study the highly conserved phenomenon of calorie restriction include short life span, considerable genetic plasticity, and easier manipulation of diet. While calorie restriction in worms, flies, and mammals usually entails limiting a complex food source such as bacteria or feed pellets, calorie restriction in yeasts and bacteria can be enforced by simple carbon source limitation. Reduction of glucose, which is the preferred carbon source of most microbes, has a wide spectrum of effects in the variety of established microbial life span models, ranging from nearly 100-fold life span extension (van Diepeningen et al. 2010) to threefold life span reduction (Oliveira et al. 2008). This large diversity in CR-mediated life span is a result of the biochemical, genetic, and metabolic identity of each microorganism.

Life span measurements conducted in *Saccharomyces cerevisiae*, the first microbe to gain popularity as a model of aging, have been adapted or modified to address life span in newer microbial aging models, such as *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Candida albicans*, and *Escherichia coli*. One of the two mainstay life span assays in *S. cerevisiae,* chronological life span (CLS), measures how long cells can remain viable in a non-dividing state (such as in media exhausted of nutrients; Fabrizio et al. 2003). CLS can be addressed in most current microbial aging models. Measuring the total division potential of an individual cell, or replicative life span (RLS), is greatly simplified in *S. cerevisiae* due to gross morphological

differences between mother and daughter cell (Mortimer and Johnston 1959). RLS measurement in organisms whose division is morphologically symmetric such as *S. pombe* or *E. coli* is ponderous by comparison, but studies have determined that these organisms have division asymmetries that cumulate after repeated divisions, and therefore these have a replicative life span as well (Barker and Walmsley 1999; Stewart et al. 2005). Certain attributes of new microbial aging models, such as filament senescence in *Podospora anserina* and differentiation in *Caulobacter crescentus*, provide novel methods to determine life span in these unique organisms. Calorie restriction, which extends RLS and CLS in *S. cerevisiae*, provides an example of longevity pathway overlap. However, augmentation or reduction of certain proteins/longevity factors linked to signaling during CR can modulate RLS and CLS differently. The pathways, proteins, and metabolism involved in the initiation or regulation of the CR-mediated longevity in assorted microbial model organisms will be addressed in this review.

Crabtree-positive yeasts as microbial CR models

The diversity of novel yeast and bacterial models for calorie restriction and longevity allows for new metabolic comparisons. Particularly interesting are those among Crabtree-positive yeasts, such as *S. cerevisiae* and *S. pombe*, Crabtree-negative yeasts, such as *K. lactis* and *C. albicans*, and obligate aerobe yeast such as *P. anserina*. The Crabtree effect, coined for Dr. Herbert Grace Crabtree (Crabtree 1929), refers to inhibition of aerobic metabolism when the preferred carbon source, glucose, is available. This inhibition occurs in the presence or absence of oxygen, and the term is not specific to yeasts: many mammalian tumor cells display a Crabtree effect as well (De Deken 1966; Golshani-Hebroni and Bessman 1997). Crabtree-positive yeasts prefer fermentation and actively inhibit aerobic respiration under normal circumstances: they can maintain a pool of stored extracellular carbon and enjoy a selective advantage due to other organisms' sensitivity to ethanol. Crabtree-negative yeasts should not be confused with obligate aerobes: Crabtree-negative yeasts can perform fermentation but generally only choose this form of metabolism when living anaerobically. In Crabtree-negative yeasts, there is no inhibition of aerobic respiration in the presence of glucose, and thus aerobic respiration represents the vast majority of cellular metabolism, regardless of carbon source. Obligate aerobe yeasts cannot ferment and only respire aerobically, providing another category of metabolic diversity. A further metabolic division among microbial model organisms for longevity is prokaryotes and eukaryotes. The mitochondria is the site of the most effective form of energy harvesting (respiration) in both microbial and multicellular eukaryotes, and there is considerable crosstalk between cell and mitochondria to coordinate the demand for ATP, iron–sulfur clusters, and certain amino acids (Liu and Butow 2006; Xu and Moller 2008; Zelenaya-Troitskaya et al. 1995; Veatch et al. 2009). Bacteria, however, are their own energy powerhouses, so energy coordination is less complicated. Prokaryotic model organisms such as *E. coli* and *C. crescentus* therefore provide mitochondria-independent insight into the mechanisms of CR (or at least information independent of mitochondria-to-cell signals). Similar to microbial eukaryote models, prokaryote models come in a variety of flavors, including facultative anaerobes (*E. coli*) and obligate aerobes (*C. crescentus*). A brief summary of aforementioned microbes and their diverse metabolic responses to CR is shown in Fig. 1.

Saccharomyces cerevisiae

The quintessential microbial aging model, *S. cerevisiae*, is a commonly used organism for life span and calorie restriction studies for a multitude of different reasons. For one, mother–daughter cell asymmetry in *S. cerevisiae* is easily observed under the microscope, allowing development of the RLS assay, the first method to measure life

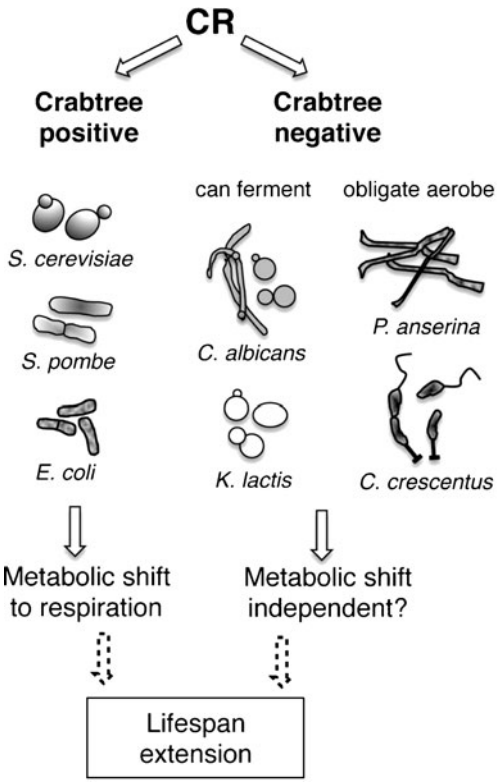

Fig. 1 A brief summary of various microbes and their diverse metabolic responses to CR

span in microbes (Mortimer and Johnston 1959). Well-established molecular genetic techniques (recombination), genome sequence availability, and a prokaryotic-like lack of introns made *S. cerevisiae* a popular model organism for a multitude of different processes and assisted in giving budding yeast a head start in the nascent field of longevity studies. Following soon after, *S. cerevisiae* was also the vanguard microbe for the study of CLS (Fabrizio et al. 2003). Although RLS and CLS are addressing two very different forms of longevity, CR is a uniting factor: both RLS and CLS are extended dramatically by moderate (0.5% glucose) or severe (0.05% glucose) CR. How CR mediates life span extension, however, differs between life span measurements.

As a Crabtree-positive yeast, *S. cerevisiae* is able to repress mitochondrial oxidative respiration machinery when its preferred carbon source, glucose, is plentiful. In lieu of respiring, when glucose is abundant in its growth media, *S. cerevisiae* ferments glucose to ethanol, promoting the buildup of a metabolic by-product which it is highly tolerant of, while eliminating harmful acidic intermediates like acetic acid or lactic acid, which is the end product of mammalian fermentation. The ethanolic fermentation pathway employed by Crabtree-positive yeasts like *S. cerevisiae* and *S. pombe* has the ancillary benefits of both rapidly conferring a selective advantage against bacteria and providing a form of extracellular energy storage that not many other organisms can utilize. When glucose is scarce, such as during CR, *S. cerevisiae* focuses on a more efficient form of energy metabolism, mitochondrial respiration (Lin et al. 2002). This switch from fermentation to respiration is the driving force behind many physiological changes associated with longevity, although some aspects of CR appear to be independent of respiration, as illustrated by mutants in *S. cerevisiae* and Crabtree-negative and obligate aerobic organisms (Kaeberlein et al. 2005a; van Diepeningen et al. 2010). Development of many genetic mimics of CR, such as strains where pro-growth glucose-regulated kinases have been deleted, may provide further insight into the molecular mechanism of CR. Among those reported CR genetic mimics, strains with reduced target of rapamycin (TOR; Kaeberlein et al. 2005b), protein kinase A (PKA; Lin et al. 2000; Longo 2003), or Sch9 (an S6 kinase and AKT homolog; Longo 2003; Kaeberlein et al. 2005b; Easlon et al. 2007) activity have both increased RLS and CLS and thus closely resemble CR life span phenotypes. TOR kinase is pro-growth in the presence of certain amino acids and activates ribosomal production and proliferation while inhibiting stress resistance, mitochondrial respiration, and stationary-phase entry (Wullschleger et al. 2006; Schieke and Finkel 2007; Powers et al. 2006). PKA is activated in response to glucose through the G-protein Ras1/2 and adenylate-cyclase-mediated production of cyclic AMP (Lin et al. 2000; Longo 2003). Like TOR kinase, PKA is pro-growth and upregulates cell proliferation while preventing stationary-phase entry and aerobic respiration (Portela and Moreno 2006). However, unlike moderate CR (0.5% glucose), reduction of PKA, deletion of Sch9, or inhibition of TOR via rapamycin result in a mild to severe growth defect, and this growth defect may be contributing to longevity in either or both life span measurements, perhaps in a CR-independent fashion. There is a mammalian precedent for this: dwarf mice tend to live longer than their larger littermates (Bartke et al. 2001).

The delay in replicative aging caused by CR in *S. cerevisiae* is strongly linked to a number of associated factors. An increase in respiration causes a concomitant decrease in nicotinamide adenine dinucleotide, reduced form (NADH), which is a competitive inhibitor of enzymes that require oxidized nicotinamide adenine dinucleotide, oxidized form (NAD^+) as a cofactor (Lin et al. 2004). Many enzymes, particularly metabolic ones, require NAD^+ as a cofactor, but the ones most closely associated with CR and life span are the sirtuins, characterized by the founding member in *S. cerevisiae*, silent information regulator 2 (Sir2; Lu and Lin 2010). Sirtuins are NAD^+-dependent protein deacetylases: the activity Sir2 is best known for the deacetylation of histones H3 and H4. During CR, a decrease in cellular and nuclear NADH, enforced by a metabolic shift to mitochondrial respiration, results in increased or stabilized Sir2 activity. A primary target of Sir2 in *S. cerevisiae* replicative aging is the tandem ribosomal DNA (rDNA) repeats in the nucleolus. In a Sir2 deletion strain, acetylated histones are relaxed from nucleolar rDNA, allowing recombination machinery to produce extrachromosomal rDNA plasmid-like DNA circles (ERCs). These rDNA circles, or ERCs, are semi-autonomous: they can replicate on their own, like plasmids, and gradually accumulate during aging (Sinclair and Guarente 1997; Lu and Lin 2010). Sir2 does not only prevent ERC formation, it also regulates asymmetrical inheritance of ERCs and other aggregates during cell division (Erjavec and Nystrom 2007). Deletion of the replication fork block protein Fob1 results in much lower rDNA recombination and ERC formation and extends life span considerably (Defossez et al. 1999). However, rDNA repeats are not the only cellular target for Sir2: Sir2 also silences chromatin in the mating loci and telomeric and subtelomeric regions (Moazed et al. 1997; Xu et al. 2007). Since ERCs are not found in many other model organisms for aging but Sir2 orthologs are, the main effect of Sir2 in these other aging models may be due to telomeric or subtelomeric silencing (Dang et al. 2009). Asymmetrical inheritance is also a critical aspect of CR-mediated replicative longevity: if *S. cerevisiae* cells cannot restrict damaged or aggregate proteins and ERCs exclusively to the mother cell, daughter cells will have decreased RLS. In *sir2*Δ mutant

strains, RLS is dramatically reduced with or without CR treatment, but there is some controversy as to whether Sir2 can function independently of CR in replicative aging (Kaeberlein and Powers 2007). It is possible that CR decreases the abundance of damaged or aggregated proteins in part by Sir2-independent means but reduces rDNA recombination through Sir2. This explanation may help resolve the controversial role of Sir2 in CR and RLS and is supported by studies in Crabtree-negative yeasts and bacteria.

A metabolic shift from fermentation to respiration appears to be responsible for long CLS in calorie-restricted cells as well. Similarly, respiration is required for CLS extension by reduced-growth pathway activity (Lavoie and Whiteway 2008). In contrast to replicative aging, cellular stresses during chronological aging include cell intrinsic factors, such as hydrogen peroxide and superoxide, and cell extrinsic factors, such as acetic acid and ethanol (Fabrizio et al. 2005; Magherini et al. 2009; Burtner et al. 2009; Takeda et al. 2010). Reduction of PKA, TOR, and Sch9 activity confers considerable CLS extension and, like CR, increases both respiration and cellular resistance to a variety of stresses (Bonawitz et al. 2007; Lavoie and Whiteway 2008; Pan and Shadel 2009). Oxidative scavenging contributes to chronological longevity: overexpressions of both cytosolic and mitochondrial superoxide dismutases together results in extended CLS (Fabrizio et al. 2004). The role of Sir2 in CLS is even more enigmatic than in RLS. Although sirtuin activity is required for 0.5% glucose CR in RLS, Sir2 actually has an inhibitory effect upon the CLS of several long-lived CR-mimicking mutants (*sch9*Δ and a transposon-disrupted adenylate cyclase mutant, which has low PKA activity), and deletion of Sir2 confers a minimal CLS extension (Fabrizio et al. 2005). Since during CR *S. cerevisiae* cells primarily respire, ultimately producing the harmless by-products CO_2 and H_2O, ethanol does not accumulate at high concentrations in the medium. Ethanol, while not being particularly toxic on its own, can contribute to chronological aging either by later conversion to acetic acid (Burtner et al. 2009) or on its own accord (Fabrizio et al. 2005). Additionally, mitochondrial stability is a major contributor to chronological longevity in *S. cerevisiae*. Mitochondrial stability and CLS can be increased by oxidative scavenger activity, especially those that reside in the mitochondria such as mitochondrial superoxide dismutase (Sod2; Harris et al. 2003). Not surprisingly, CR is a potent activator of both the oxidative and general stress response (Wang et al. 2009). Although the majority of cellular reactive oxygen species (ROS) are produced by mitochondrial respiration, calorie-restricted cells possess a lower concentration of ROS than untreated cells (Barros et al. 2004; Wang et al. 2009): this is likely the result of upregulation of the oxidative stress response. Programmed cell death (PCD) is also linked to mitochondrial function, both in mammals and *S. cerevisiae*, and contributes to chronological aging (Herker et al. 2004). Since single-celled budding yeast seems to live in an environment of pure everyone-for-itself competition, there would seem to be no reason for a programmed cell death routine in *S. cerevisiae*. There is speculation that PCD, like aging, contributes to the rejuvenation of a yeast culture by eliminating damaged cells, cells with dysfunction mitochondria, and cell which have undertaken a process which they are incapable of completing, such as mating (Hamann et al. 2008). In the case of extremely short-lived mutants, such as respiration-defective strains lacking cytochrome c1, mitochondria become punctuate and destabilized as early as the diauxic shift (Easlon et al. 2007; Skinner and Lin, data not shown). Interestingly, *Rho*^{0} *S. cerevisiae* strains (cells which have lost their mitochondrial DNA (mtDNA)) actually exhibit slower kinetics of chronological aging than WT cells, despite their inability to respire (Mazzoni et al. 2005). Surprisingly, *S. cerevisiae* shares apoptotic pathway components with mammals. Budding yeast possess a pro-apoptotic caspase (Yca1), which, when deleted, extends CLS (Herker et al. 2004). Additionally, PCD can be attenuated in *S. cerevisiae* by overexpressing the anti-apoptotic mammalian Bcl2 (Longo et al. 1997). Calorie restriction may contribute both to mitochondrial efficiency, through upregulation of mitochondrial electron transport chain components, and mitochondrial stability, through optimization of oxidative scavengers like superoxide dismutases and catalases. With stable and necessary mitochondria, PCD can be prevented, resulting in extended chronological longevity during CR.

Overall, the contributions of *S. cerevisiae* to the studies of CR and aging have been considerable and have helped pave the way for further research in metazoans and other microbial model organisms. Not only did *S. cerevisiae* help define aging measurements in other microbes, but the characterization of Sir2, mitochondrial respiration, and TOR, PKA, and Sch9 growth kinases in CR-mediated life span all originated from the budding yeast. However, certain aspects of *S. cerevisiae* make parallels with metazoans difficult, but collaboration with the many other microbial models for aging that have been developed recently may greatly aid in resolving the microbial mechanisms behind CR.

Schizosaccharomyces pombe

Another Crabtree-positive and well-studied, both genetically and metabolically, yeast, *S. pombe*, probably has the distinction of being the second most popular microbial aging model. Like *S. cerevisiae*, aging factors such as pro-

growth kinases and sirtuins are highly conserved between *S. pombe* and mammals. Certain characteristics including characterized RNAi machinery and non-essential protein kinase A make *S. pombe* longevity pathways distinct. Differences like these make *S. pombe* a valuable complement to *S. cerevisiae* for the study of life span and cellular aging.

One overwhelming benefit of studying *S. cerevisiae* in preference to *S. pombe* is gross morphological asymmetry between mother and daughter cells during cell division. This makes RLS measurements pedestrian in *S. cerevisiae* and exhausting in *S. pombe*. However, fission yeast does have a finite RLS and demonstrates division asymmetry analogous to *S. cerevisiae* (Erjavec et al. 2008). The effects of CR upon the RLS of *S. pombe* are currently unknown, but studies are likely already underway.

Chronological life span, on the other hand, is more thoroughly characterized in fission yeast. CR in *S. pombe*, as a threefold or 40-fold reduction in glucose, extends CLS dramatically and enhances cellular resistance to heat and peroxide (Chen and Runge 2009). Similarly, deletion of PKA (Pka1; which is not lethal in *S. pombe*) or the AKT homolog Sck2 resulted in an increased CLS, and deletion of both Pka1 and Sck2 extended CLS longer than either deletion alone (Roux et al. 2006), suggesting that the two kinases function in complementary or partially overlapping pathways to regulate CLS. Calorie restriction during CLS has been suggested to function in *S. pombe* by both inhibition of PKA signaling and activation of mitogen-activated protein (MAP) kinase, Sty1. Indeed, CR in rich medium (yeast extract) cannot extend CLS in fission yeast without Sty1 (Zuin et al. 2010). In contrast to parallel studies in *S. cerevisiae*, although oxygen consumption was also increased in calorie-restricted cultures (indicating higher mitochondrial respiration and a metabolic shift), CR increased intracellular ROS concentration, which may play a role in activating Sty1. However, CLS measurements may not be directly comparable between budding and fission yeasts. It should be noted that yeast-extract-based rich media contains autolyzed *S. cerevisiae* extract and therefore may not be as optimal a growth medium for fission yeast as it is for budding yeast. In addition, *S. pombe* has a shorter CLS on yeast-extract-based rich media than on minimal media, again dissimilar to *S. cerevisiae*.

Although Sir2 may negatively regulate CLS in certain mutant strains in *S. cerevisiae*, there is no evidence yet that Sir2 functions similarly in *S. pombe*. In fact, in the absence of stress attenuation proteins glutathione (Gsh1) and copper/zinc cytosolic superoxide dismutase (Sod1), deletion of Sir2 reduces CLS even further, although the double-deletion *gsh1*Δ*sod1*Δ strain is short-lived to begin with (Mutoh and Kitajima 2007). This seems to suggest that Sir2 is fulfilling an additional function in *S. pombe*, regulating certain aspects of the stress response. However, characterization of Sir2 in fission yeast remains incomplete: although it is required for certain aspects of asymmetrical protein segregation, its impact upon CR in *S. pombe* RLS is yet unknown and its effects upon AKT homologs are untested.

Life span studies in *S. pombe* have already uncovered several important aspects of the CR response in Crabtree-positive organisms, including the function of MAP and AKT kinases in CLS. We eagerly await further revelations from fission yeast, perhaps including the characterization of Sir2 in the other well-known Crabtree-positive model of aging.

Crabtree-negative yeasts, facultative anaerobes, and obligate aerobes as microbial CR models

What distinguishes Crabtree-negative yeasts and bacteria such as *E. coli* and *C. crescentus* is their inability to inhibit respiration in the presence of glucose in favor of fermentation. Since these organisms primarily respire under normal life span assay conditions, CR does not activate a fermentation-to-respiration switch as in Crabtree-positive yeasts. Crabtree-negative (and obligate aerobe) yeasts therefore provide a unique platform to study the respiration switch-independent mechanisms of CR. Perhaps these respiration switch-independent mechanisms can be even further explored using obligate aerobes like *P. anserina* and *C. crescentus*.

Kluyveromyces lactis

An ascomycetus budding yeast found in milk, *K. lactis*, has well-characterized genetics and is heavily studied for biological and industrial reasons. Unlike the Crabtree-positive yeasts, CR (by fourfold glucose limitation, 0.5% glucose) induces a remarkably dramatic reduction of CLS (up to threefold) and does not alter NADH–cytochrome c reductase activity or respiration (Oliveira et al. 2008). Response to CR by a metabolic switch, which is critical to chronological longevity in Crabtree-positive yeasts, is absent in *K. lactis*, as is the prolonged CLS. *K. lactis* therefore complements studies on Crabtree-positive yeast, highlighting the importance of the CR-induced fermentation-to-respiration switch. However, despite its nature as an asymmetrically dividing budding yeast, RLS on *K. lactis* has not been reported. It would be interesting to note if CR can extend RLS but not CLS and if CR can induce a metabolic shift in this organism during replicative aging.

Candida albicans

More commonly known for its role in nosocomial, vaginal, and oral infections, *C. albicans* has recently also been

established as a Crabtree-negative microbial model for aging. Although *C. albicans* prefers respiration to fermentation in the presence of oxygen, this organism has an atypical response to glucose concentration: higher concentrations of glucose increases resistance to certain stresses (Rodaki et al. 2009), whereas restriction of glucose increases stress resistance in *S. cerevisiae* (Wang et al. 2009) and *S. pombe* (Zuin et al. 2010). *C. albicans*, which is normally benign intestinal microflora, exists in one of two diverse states: as single cells or as invasive filaments. This organism is amenable to RLS analysis, and both the single-celled and filamentous forms have similar RLSs (Fu et al. 2008). Seemingly, in contrast to *K. lactis*, CR administered to *C. albicans* extends RLS by up to 33%, and increased dosage of Sir2 extends RLS as well. To date, only RLS has been studied in *C. albicans*. A thorough analysis of both life span measurements could be more enlightening than one on its own, and *C. albicans*, like *K. lactis*, seems amenable to both RLS and CLS.

Podospora anserina—an obligate aerobe aging model

Despite its complex filamentous nature, *P. anserina* holds many firsts in microbial aging studies. Its characteristic hyphal senescence phenotype, described nearly 60 years ago (Rizet 1953), combined with ease of culture and genetics, laid the groundwork for its continued development as a fungal aging model. Plate-cultured *P. anserina* hyphae grow radially to a certain length; then the expanding hyphal tips begin to swell, branch abnormally, and finally rupture, causing the culture to cease growth. Eventually, the entire mycelium becomes unable to produce new filaments (infertile) as well. Although conventional life span measurements (RLS and CLS) are complicated in filamentous fungi, the unusual senescent phenotype allows for other life span measurements. For example, the length of mycelium may correspond loosely to RLS, and the time the culture can remain fertile (reproductive life span) may be comparable to CLS. CR, manifested as moderate (0.2% glucose, 10-fold reduction) or severe (0.02% glucose, 100-fold reduction), results in a dramatic increase in both mycelia size before senescence and period of fertility (van Diepeningen et al. 2010).

The mitochondrial toolkit of *P. anserina* includes an electron transport chain similar to that in *S. cerevisiae*, but with two key differences. Complex I in *P. anserina* is a proton pump, like complex I in metazoans, but different from aforementioned single-protein mitochondrial NADH dehydrogenase in *S. cerevisiae*. Secondly, *P. anserina* possesses an alternative oxidase (AOX), which receives electrons from ubiquinone and bypasses both complexes III and IV to deliver electrons directly to molecular oxygen. Utilizing the proton-pumping complex I and AOX, an electron can flow down the electron transport chain with a minimum amount of energy harvested (one proton), but much less potential ROS production, since complex III is a major site of ROS generation. Deletion of Cox5 or Cyc1, required subunit of complex IV and cytochrome C, respectively, results in an enormous life span extension (both reproductive and mycelia size), suggesting that either activation of AOX can promote life span or cytochrome-mediated respiration itself is the cause of aging and senescence in *P. anserina* (Scheckhuber and Osiewacz 2008). Recent studies indicate that constitutive activating mutations in AOX transcription factors Rse2 and Rse3 can reduce or eliminate the long life span conferred by *cox5*Δ or *cyc1*Δ deletions (Sellem et al. 2009). Additionally, it has been shown that overexpression of AOX protein not only suppresses the long life span of Cox5 deletion mutants but also restores senescence (Lorin et al. 2006). In light of this new information, it would appear that the halt of respiration, not the activation of AOX, is responsible for the dramatic lengthening of life span by *cox5*Δ or *cyc1*Δ deletion. The link between CR and mitochondrial respiration, which is well-documented in *S. cerevisiae*, has not been thoroughly addressed in *P. anserina* at present, but preliminary studies indicate that CR and severe CR can extend life span in AOX deletion mutants (mutant strains defective in AOX activity; van Diepeningen et al. 2010). It would be interesting to note if CR can extend or further extend life span in a *cox5*Δ or *cyc1*Δ mutant, given the much clearer relationship between life span and respiration in *P. anserina*'s distant cousin, *S. cerevisiae*. CR in *P. anserina* does lower the production of ROS, similar to *S. cerevisiae*, and optimized ROS scavenging may be a contributing factor to the life span increase during CR.

Suggested "senescence factors" for *P. anserina* myceliums include mtDNA instability, unchecked mitochondrial production of ROS, and changes in mitochondrial morphology. Accumulation of damaged or rearranged mtDNA, particularly of the well-characterized intron α, is evident at senescent hyphal bud tips and greatly reduced in severely calorie-restricted *P. anserina* hyphae (van Diepeningen et al. 2010). Mitochondrial production of ROS is reduced by CR or severe CR as well, as mentioned earlier, and mitochondria in CR- or severe-CR-treated mycelia arrange in healthy tubular structures, in contrast to the punctuate form of senescent non-CR bud tips. CR and severe CR therefore appear to contribute to the alleviation of all three of these "senescence factors" and, unlike Cox5 or Cyc1 deletions, do not result in sterility or require AOX for viability. CR in *P. anserina*, as an obligate aerobe, cannot initiate a fermentation-to-respiration metabolic switch, so the dramatic life span extension conveyed by CR and severe CR must originate from elsewhere. A full mechanistic explanation of CR in *P. anserina*, outside of an

incomplete connection to mitochondrial ROS production and increased mtDNA stability and mitochondrial morphology, is still unavailable. However, this organism, as one of the very few obligate aerobe eukaryotic fungal models of aging, may contribute significantly to our knowledge of the fermentation-to-respiration metabolic-shift-independent events initiated by calorie restriction.

Escherichia coli—a bacterial facultative anaerobe aging model

Although *S. pombe* cell division is morphologically symmetrical, segregation of proteins, lipids, and organelles into mother and daughter cells is dissimilar at the molecular level. *S. pombe* therefore provides a eukaryotic example of aging in symmetrical organisms. Studies on *E. coli* also demonstrate that replicative aging occurs in symmetrically dividing bacteria as well and additionally proves that aging is not wholly dependent upon mitochondria. During division of the rod-shaped bacterium, the old and new copies of bacterial chromosome migrate and bind to opposite poles of the dividing cell before cytokinesis. By following the old "mother cell" pole, replicatively aged cells (more than six divisions) show a reduced growth rate and an increased mortality. Asymmetrical distribution of protein aggregates correlates with aging in *E. coli*: aggregates tend to migrate toward the old "mother cell" pole and are thereby excluded from the daughter cell (Lindner et al. 2008). In yeast models, aging has been associated with symmetrical cell division, and CR enhances the mother cell's ability to retain damaged proteins, lipids, and organelles, enhancing the fitness of her daughter. Contrastingly, CR in *E. coli* has recently been shown to enforce symmetrical cell division while prolonging longevity (Lele et al. 2008), although it is possible that CR simply reduces oxidative damage and protein aggregation below a detectable limit. This would mean that mother cell retention of aggregates and damage is preserved during CR, but the amount of aggregates and damaged proteins is significantly reduced in both mother and daughter cells, similar to what occurs during replicative aging in *S. cerevisiae* (Reverter-Branchat et al. 2004).

Caulobacter crescentus—a bacterial obligate aerobe aging model

Yeast and bacteria with symmetrical division were thought to be immune to the effects of aging until recently. The *Caulobacter* life cycle begins as a motile flagellate, or "swarmer," and culminates as a reproducing sessile cell anchored to a surface with a holdfast or "stalked" cell. This unique asymmetrical division not only enables mother–daughter discrimination but also facilitates mother cell retention. Through the use of a microscopy flow chamber, the motile daughter cells can be continuously washed out while tallying the mother cell's RLS (Ackermann et al. 2003, 2007). Unlike with *E. coli*, the effects of CR upon *Caulobacter* are untested currently, but a comprehensive study would be illuminating and is likely currently underway. Information from *C. crescentus* would be complementary to RLS measurements in *K. lactis* and *C. albicans*: if the RLS of *C. crescentus* could be extended by CR, this would indicate either a respiration-independent component to replicative longevity or an unforeseen metabolic shift by CR (in an obligate aerobe).

Metabolic preference of CR in microbes and its relevance to mammalian aging

While mitochondrial respiration plays a role in microbial aging, there is still no clear consensus as to what exactly that role is. In the obligate aerobe *P. anserina*, inhibition of respiration can extend life span even more dramatically than CR. In addition, CR does not enforce a metabolic switch to respiration since this organism cannot ferment and has no options other than respiration. The mechanism of CR-mediated longevity is clearer for the Crabtree-positive yeasts: CR does enact a metabolic shift to respiration, so respiration could be responsible for the multitude of life-span-extending changes during CR (increased resistance to stress, Sir2 activity, etc.). Since CR does not induce a metabolic shift in the Crabtree-negative yeast *K. lactis*, CR does not extend CLS, as expected. However, RLS is extended by CR in the Crabtree-negative yeast *C. albicans*. In aggregate, these studies suggest that perhaps RLS and CLS are controlled by different CR-mediated factors: respiration and metabolite production play a central role in chronological longevity, while sirtuin activation and division asymmetry have a considerable impact upon replicative longevity. Controlling the concentration of ROS and the stability of mitochondria are critical for both life span measurements, since dysfunctional mitochondria, in many of these microbial species, eventually result in cellular death or senescence.

Though metazoans are much more complicated than single-celled or filamentous microbes, microbes, especially the eukaryotic variety, are very similar to metazoans at the cellular level. Many CR and longevity-associated factors/pathways are highly conserved from yeast to mammals, including mitochondrial respiration, Sir2 family proteins (sirtuins), pro-growth TOR and PKA signaling pathways, and metabolic pathways such as NAD^+ biosynthesis. *E. coli*, though lacking mitochondria and chromatin, nevertheless possess a homolog to Sir2 (CobB) which is important in chemotaxis and may offer insight into early

connections between sirtuins and metabolism (Landry et al. 2000). The significance of some of these pathways in mediating beneficial effects of CR was first recognized in simple model organisms, which was later found parallel in complex higher eukaryotes. To date, the detailed mechanisms for how these longevity factors/pathways respond to CR and elicit diverse beneficial effects in different cell types/organisms have remained unclear. Contradictions also persist as to whether these factors indeed affect longevity by similar mechanisms in mammals. For example, whether increased or decreased mitochondrial activity is beneficial to life span and whether mitochondrial respiration is the major mediator of CR (Guarente 2008) are still highly debatable. It has been suggested that lowering the electron transport activity is beneficial for longevity (Kim 2007), which is in line with studies of cytochrome c deletion in *P. anserina*. However, inhibition of the electron transport activity is detrimental to life span in *S. cerevisiae*, which also blocks certain CR-induced effects in yeast and mammalian cells (Lin et al. 2002, 2004; Guarente 2008). In metazoans, several aging-related phenotypes have also been associated with a decline in electron transport activity (Lin and Beal 2006; Someya et al. 2007). Much clarification has yet to be done on the effects of mitochondria and other longevity factors on CR and aging. Microbial models not only provide a powerful genetic tool for the identification of critical components in CR and aging but also serve as a platform for studying these longevity factors at the biochemical/molecular level.

Which of these models more closely resemble human aging, Crabtree-positive or Crabtree-negative yeasts? Since most human cells primarily respire when oxygen is available and ferment to lactate when it is not, at first glance, it would appear that Crabtree-negative yeasts have the edge in relevance. However, calorie restriction does induce a metabolic response in mouse white adipose tissue (Nisoli et al. 2005): mitochondrial biogenesis is increased and fatty acids are more readily consumed. Perhaps this represents a change from energy storage to utilization. The CR-induced metabolic shift from ethanol production, which could be considered a form of energy storage, to aerobic respiration, which is obviously a form of energy utilization, may actually be more similar to what happens in certain human cells, like adipose tissue. Similarly, one might wonder which of these life span measurements (RLS, CLS, *P. anserina* senescence) might be the most relevant to mammalian aging. CLS measures the survival of cell in stationary phase, and the vast majority of human cells at any given time post-development are in G_0. On the other hand, RLS measures division potential and would be of more use for the study of dividing cells. Cells actively dividing in mammals include germ cells and a plethora of different types of stem cells. However, while microbial mother cells sacrifice themselves for their progeny by retaining damaged and aggregated proteins or DNA, stem cells thrust accumulated damage upon their progeny, thereby keeping themselves pristine. Nevertheless, RLS may currently be the most accurate microbial parallel to stem cell aging. Therefore, a combination of the aforementioned life span measurements would probably be the best way to uncover new conserved determinants of aging. Conserved longevity factors such as the TOR, PKA kinases, and the sirtuins are excellent targets for therapeutic interventions. Rapamycin, a TOR inhibitor, has been shown to extend life span in yeast (Powers et al. 2006) and mice (Harrison et al. 2009). Resveratrol, a potent sirtuin activator, can prolong longevity in yeast and metazoans (Wood et al. 2004; Valenzano et al. 2006). As more conserved longevity factors are revealed, the potential for life-span-extending therapeutics increases, and microorganisms, due to rapid screening and life span procedures, remain at the forefront of this field.

Calorie restriction and intestinal microflora

Although microbial model organisms can assist in elucidating highly conserved mechanisms, mediating the metabolic response to CR in mammalian cells, microbes also play a direct role in mammalian metabolism. Outnumbering our own cells nearly 10 to 1 and our genes 100 to 1, the human intestinal microbiome is extremely diverse, and some consider it to be the only organ “outside the body.” Microbial metabolism is infinitely more complex and diverse than that of their mammal hosts, and much of what we eat cannot be digested without the aid of gut microflora. Obese mice with mutated leptin genes (ob/ob) have dramatic changes to gut microbe composition relative to lean littermates (Turnbaugh et al. 2006), but whether gut microflora changes are the cause or a symptom of obesity is still unclear (Backhed 2009). The supremacy of Bacteroides or Firmicutes bacterial phyla, which account for 90% of the species found in the gut microflora, is linked to obesity: obese mice have a significantly higher percentage of Firmicutes inhabiting their gut, whereas lean mice have fewer Firmicutes and more Bacteroides (Turnbaugh et al. 2006). It is thought that ob/ob mice and possibly other forms of obesity both encourage the proliferation of Firmicutes and have increased energy harvesting and storage potential, possibly through utilization of Firmicutes metabolic by-products. Remarkably, if the gut microbiome of an ob/ob mouse is transplanted into a lean littermate, body fat begins accumulating at a significantly higher rate. Thus, gut microbiota composition can play a significant part in energy harvesting, energy storage, and development of obesity.

Since gut microflora composition could potentially be both an indicator of obesity or a direct therapeutic target, the field of gut microbial genomics and metabolomics has been expanding rapidly. After the aforementioned landmark studies in mice, human microflora is being studied extensively. In humans (by studying the fecum of pairs of obese or lean twins and their mothers), obese individuals not only harbored less Bacteriodes but also had less overall gut bacterial diversity (Turnbaugh et al. 2009). It is unclear whether the lower bacterial diversity in obese subjects is due to a more homogenous (high in fats, protein, and carbohydrates) diet, although a lower variety of dietary intake could result in a less diverse microbiome. Other than Firmicutes, *Achaea* methanogens were enriched in obese fecum (Zhang et al. 2009). Methanogens could interact metabolically with H_2-producting bacteria (such as Prevotellaceae, phylum Bacteriodes), resulting in more efficient conversion of hard-to-digest fiber polysaccharides to short-chain fatty acids. Short-chain fatty acids like acetate and succinate are readily absorbed by the gut lining, unlike their complex fiber precursors. Increased absorption of short-chain fatty acids can then be stored as fat. The obesity-associated microbiome may be even more insidious and send signals for fat deposition to their host.

But what about the roles of intestinal microbiota in CR? A recent study in Labrador retrievers determined not only that CR can extend the life span of dogs, but also, in aged dogs (9 years), the quantity of certain microbial aromatic metabolites (hippurate, PAG, and 4-HPPA) in urine significantly increased relative to those fed a rich diet (Wang et al. 2007). The microbial mammalian co-metabolite hippurate has an inverse correlation with obesity; low levels of hippurate are also related to high blood pressure and type II diabetes (Calvani et al. 2010). Additionally, the levels of metabolites such as creatine, lactate, acetate, and succinate were decreased in the urine of dietary-restricted dogs. Lactate and acetate are fermentation by-products and can be absorbed through the gut lining, indicating increased bioavailability of energy. If more energy is available, possibly due to a preponderance of intestinal Firmicutes or methanogens, more energy can be stored. Aromatic metabolite production, primarily generated by bacteria, in urine by CR may indicate a microfloral remodeling. Reduced consumable energy provided by gut microflora leads to more efficient energy harvesting (respiration) as well as less energy storage, promoting stable mitochondrial function, and ultimately lean and long-lived hosts (Fig. 2). Naturally, it would be exciting to investigate whether treatment with any of these CR-linked microbial chemicals, like hippurate, can help induce a CR response. Failing manipulation of mammalian cells by microbial by-products, could CR microflora be transmissible, like obesity microflora in rats? If CR-related microbiota, transplanted into another animal, can induce a CR-like effect, even in the short term, this could open the door to future probiotics.

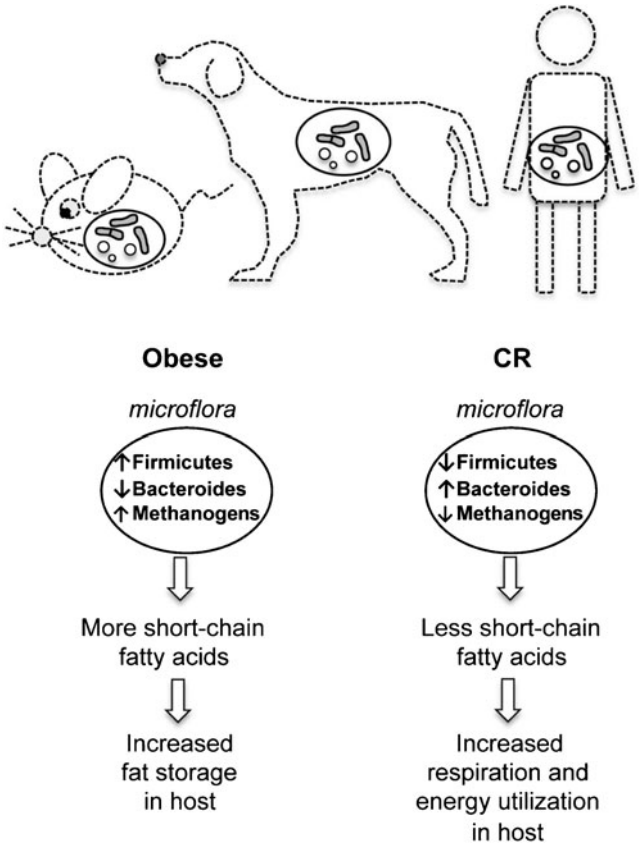

Fig. 2 Intestinal microflora may contribute to CR- and obesity-induced metabolic changes in host organisms

Conclusion

Insight into the mechanisms behind CR has been greatly facilitated by studying a variety of microbial model organisms. Although the importance of mitochondria and respiration in longevity is not fully understood in any organism, the combined power of metabolically distinct microbial model organisms is expanding our understanding of the respiration–calorie restriction connection. Certain life span measurements (CLS) appear to be wholly dependent upon a fermentation-to-respiration metabolic shift, while others (RLS) seem to be affected by additional CR-mediated factors as well. Future studies in prokaryotes may reveal novel non-mitochondrial components of the CR response. Microbes are more similar to metazoans than we would expect: they share pathways for programmed cell death and all exhibit a form of aging. Study of CR and longevity in microbial models has already revealed much about aging in human cells and, due to considerable relative simplicity, remains a powerful strategy for further characterization of the CR response. But microbes can help us

understand CR both within and without: not only can microbial model organisms enlighten us on how our cells age, but intestinal microbes can control our exposure to nutrients and enforce CR exogenously. In the long term, we should strive to understand how we age on a cellular level and how calorie restriction could potentially promote longevity. But in the short term, perhaps we should also look to the microbes within us.

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