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MINIREVIEW

Physiological and Transcriptional Responses of Different Industrial Microbes at Near-Zero Specific Growth Rates

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The current knowledge of the physiology and gene expression of industrially relevant microorganisms is largely based on laboratory studies under conditions of rapid growth and high metabolic activity. However, in natural ecosystems and industrial processes, microbes frequently encounter severe calorie restriction. As a consequence, microbial growth rates in such settings can be extremely slow and even approach zero. Furthermore, uncoupling microbial growth from product formation, while cellular integrity and activity are maintained, offers perspectives that are economically highly interesting. Retentostat cultures have been employed to investigate microbial physiology at (near-)zero growth rates. This minireview compares information from recent physiological and gene expression studies on retentostat cultures of the industrially relevant microorganisms *Lactobacillus plantarum*, *Lactococcus lactis*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Aspergillus niger*. Shared responses of these organisms to (near-)zero growth rates include increased stress tolerance and a downregulation of genes involved in protein synthesis. Other adaptations, such as changes in morphology and (secondary) metabolite production, were species specific. This comparison underlines the industrial and scientific significance of further research on microbial (near-)zero growth physiology.

Most research in microbial physiology focuses on growing cells, although under natural and industrial conditions, microbes frequently encounter a state in which neither growth nor deterioration of cells occurs. However, the experimental design to study microbes in this clearly relevant physiological state is far from trivial.

In this minireview, we define zero growth as a nongrowing state in which the viability and metabolic activity of a microbial culture are maintained for prolonged periods. As such, zero growth differs from starvation, which is coupled to cellular deterioration, loss of activity, and ultimately, cell death (1, 2). Zero growth also differs from differentiated survival states, such as bacterial or fungal spores, in which metabolism comes to a standstill (3). Conversely, under zero-growth conditions, microbes exclusively use available substrates for processes that contribute to maintenance of cellular integrity and homeostasis (4–7). Such processes include homeostasis of transmembrane gradients of protons and solutes, defense and repair systems, osmoregulation, and protein turnover (8, 9).

In classical food fermentation processes, (near-)zero growth occurs during prolonged periods of extremely restricted availability of energy substrates. Examples include cheese ripening by lactic acid bacteria (LAB) (10–12), wine fermentation by *Saccharomyces cerevisiae* (13, 14), and natto fermentation by *Bacillus subtilis* (15). Despite the severely energy-limiting conditions, microbes manage to survive in these processes for many weeks, while continuing to produce aroma and flavor compounds in the product matrix (10, 13, 15, 16). Another incentive for studying zero-growth physiology is related to the application of microorganisms as cell factories for the production of food ingredients, enzymes, chemicals, and biofuels. In such applications, biomass is essentially an undesirable by-product, especially when costs for inactivation of genetically modified biomass are incurred (17–20). Ideally, product formation should be uncoupled from growth, using zero-growth cultures that retain high productivity and product yields for prolonged periods.

The physiology of nongrowing microbes is mostly studied in batch cultures that proceed from exponential growth to stagnation of growth, or stationary phase (21, 22). This fast transition in batch cultures does not allow researchers to capture the physiology of sustained zero growth. Slow growth of microbes under defined, constant conditions can be studied in chemostat cultures at low dilution rates (23–25). However, chemostat cultures cannot be used to assess extremely low specific growth rates ($<0.01\ h^{-1}$), due to technical limitations related to discontinuous substrate addition, the time required for reaching steady state, and the emergence and selection of adapted mutant variants (26, 27).

The retentostat, or recycling fermenter, is a continuous cultivation setup that was specifically developed to study microbial

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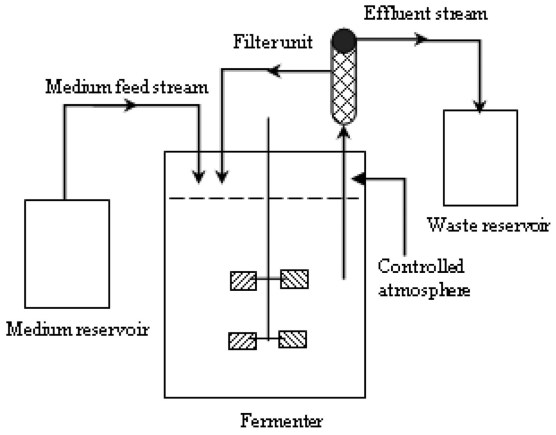

FIG 1 Basic scheme of retentostat cultivation setup. The temperature, pH, and working volume were kept constant during cultivations in all studies.

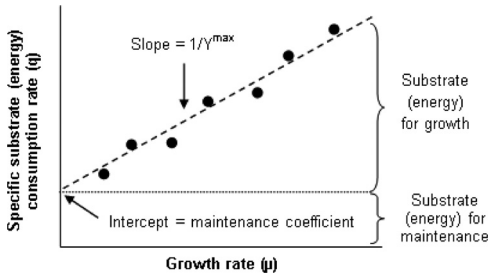

FIG 3 Determination of energy- and substrate-related coefficients with the plot of the specific substrate (energy) consumption rate (q) against growth rate in chemostat and/or retentostat cultivation. The intercept (extrapolation to a zero-growth condition) gives the maintenance coefficient (m), and the slope of the plot (dashed line) gives the inverse of the maximum growth yield (Y^{max}). The dotted line below indicates metabolic energy or substrate for maintenance-related processes.

physiology at extremely low specific growth rates (24, 28). In contrast to chemostats, biomass cannot leave a retentostat via the effluent line but, instead, is retained in the bioreactor by a membrane filter (Fig. 1). Prolonged energy source-limited retentostat cultivation at a constant dilution rate leads to a progressive buildup of biomass (Fig. 2) that coincides with a progressive lowering of the specific rate of substrate consumption. Eventually, this leads to stagnation of growth because all energy substrate provided to the culture is used to meet cellular maintenance requirements (Fig. 3) (5, 6, 28, 29). Retentostat cultivation therefore allows a gradual transition from exponential growth to a (near-)zero growth state in which sufficient energy substrate is provided for maintaining cellular activity and integrity, thereby preventing cell death.

Before the advent of genomics techniques, retentostat cultivation was used to study microorganisms such as the bacteria *Escherichia coli* (30), *Bacillus polymyxa* (31), *Paracoccus denitrificans*, *Bacillus licheniformis* (28), *Tetragenococcus halophila* (32), *Nitrosomonas europaea*, and *Nitrobacter winogradskyi* (33) and the filamentous fungus *Aspergillus niger* (34–37). More recently, the environmentally relevant bacteria *Desulfotomaculum putei* (38) and *Geobacter metallireducens* (39) have been characterized using retentostats. As a multilaboratory collaboration, our groups have combined retentostat cultivation with quantitative physiological analysis and genome-wide expression studies to analyze zero growth of the industrially relevant microbes *Lactococcus lactis* strain KF147, *Lactobacillus plantarum* strain WCFS1, *Bacillus subtilis* (*trpC2 sigF*::Sp^r *amyE*::P*rrnB-gfp*$^+$, derivative of strain 168), *Saccharomyces cerevisiae* strain CEN.PK113-7D (*MAT***a** *MAL* 2-8c *SUC2*), and *Aspergillus niger* strain N402 (*cspA*). The aim of this minireview is to compare the physiological and gene expression responses of different industrially important microbes at (near-)zero growth rates in energy-limited-growth retentostat cultures.

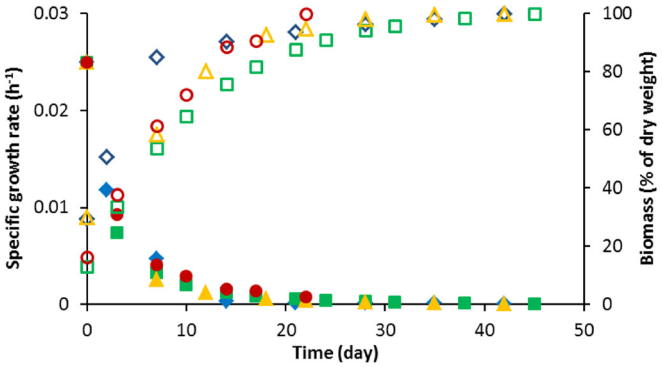

FIG 2 Specific growth rates (closed symbols) and levels of biomass accumulation (open symbols) of *L. lactis* KF147 (diamonds), *L. plantarum* WCFS1 (squares), *B. subtilis* 168 *sigF*::Sp^r *amyE*::P$_{rrnB}$-*gfp*$^+$ (triangles), and *S. cerevisiae* CEN.PK113-7D (circles) during prolonged retentostat cultivation. The biomass concentrations at the end of retentostat cultivations are depicted as 100%. The *L. lactis* KF147, *L. plantarum* WCFS1, *B. subtilis* 168 *sigF*::Sp^r *amyE*::P$_{rrnB}$-*gfp*$^+$, and *S. cerevisiae* CEN.PK113-7D cultures reached their maximal levels of biomass accumulation of 3.7, 6.6, 3.2, and 22.7 g · liter^{-1} after 42, 45, 42, and 22 days of retentostat cultivation, respectively.

PHYSIOLOGY OF MICROBES IN RETENTOSTAT CULTURES

Retentostat cultures are generally started as energy-limited chemostat cultures. After establishing steady-state growth, the effluent line is connected to a biomass retention device and the specific growth rate starts decreasing (28). Depending on the dilution rate of the system and the physiological characteristics of the microbe, reaching extremely low specific growth rates can take several days. Growth rate profiles and quantitative data on specific growth rates for carbon-limited retentostats of different industrial microorganisms (Fig. 2; Table 1) show that growth rates below 0.001 h^{-1} (corresponding to doubling times of several hundred hours) can be reproducibly achieved in retentostats.

Due to the complete cell retention in retentostat cultures,

TABLE 1 Specific growth rates and doubling times of different microorganisms grown in energy-limited retentostat cultures

Microorganism	Reference	Time in retentostat (days)	Specific growth rate (h^{-1})	Doubling time (days)
L. lactis KF147	5	42	0.00011	286
L. plantarum WCFS1	6	45	0.00006	472
B. subtilis 168 *sigF*::Sp^r *amyE*::P$_{rrnB}$-*gfp*$^+$	40	42	0.00006	470
S. cerevisiae CEN.PK113-7D	29	22	0.00063	46
A. niger N402	7	10	0.003	10

even a low death rate is expected to lead to a significant accumulation of dead cells upon prolonged cultivation. Analysis of viability, for example by counting CFUs and/or fluorescence-based live/dead staining (5, 6, 29, 40) is therefore essential. After prolonged retentostat cultivation, the viability of both *L. lactis* and *L. plantarum* remained above 90% (5, 6). Remarkably, the viability of *B. subtilis* remained above 90% as well, while the fraction of cultivable cells decreased to 30% (40). This is most probably due to the observed cell-chain formation under these conditions, which strongly confounds accurate CFU enumeration, rather than to entry of these cells into a viable but nonculturable state (41). Retentostat cultures of the yeast *S. cerevisiae* showed a slow accumulation of nonviable cells, leading to a viability of ca. 80% after 3 weeks of retentostat cultivation (29). Although cryptic growth cannot be fully excluded for all of the microorganisms, the cell debris that may be released from dead cells only provides no or minute amounts of consumable substrates that sustain growth. Cell turnover is therefore most probably minimal in these retentostat cultures. In many microorganisms, severe energy limitation triggers the onset of sporulation. Overkamp et al. (40) and Boender et al. (29) circumvented this problem by using a sporulation-negative strain of *B. subtilis* and a haploid strain of *S. cerevisiae*, respectively. However, in a study on maltose-limited retentostat cultivation of *A. niger*, extensive conidiation occurred already 4 days after the onset of biomass retention (7). Since conidiospores passed the biomass retention device, retentostat cultures of *A. niger* did not reach extremely low specific biomass production rates.

Microscopy of retentostat-grown cultures revealed significant morphological changes at (near-)zero specific growth rates, even in nonsporulating cultures (Fig. 4). The morphology of *L. lactis* cells changed from the typical coccoid form to a rod shape, which caused an approximate 2.4-fold increase in the cell surface/volume ratio after 42 days of retentostat cultivation (estimated from flow cytometry analysis), as the specific growth rate declined during retentostat cultivation (Fig. 4A and B) (5). Similarly, the morphology of *B. subtilis* cells changed from the characteristic short rod shape to substantially elongated cells after 42 days of retentostat cultivation (Fig. 4E and F) (40). Conversely, microscopic examination of chemostat- and retentostat-grown *L. plantarum* cells did not reveal significant changes in morphology (Fig. 4C and D) (6), showing that such a response is not a general phenomenon in firmicutes. Electron microscopy of retentostat-grown *S. cerevisiae* cells showed intracellular accumulation of glycogen and an increased content of lipid droplets in the cytosol (Fig. 4H1) (4), which likely contributed to a concomitant increase in cell size and mass. Notably, cell shape changes have been reported for several microbes when they enter the nongrowing stationary phase of growth (42). The changes reported under those stationary-phase conditions, where cells become smaller and coccoid, are, however, opposite to the ones observed in retentostat cultures (43–45). This might reflect changes in the cell growth and cell division ratio. The low energy availability in retentostat cultures might inhibit the cell division machinery, leading to reduced septum formation and division frequencies. The continuous supply of carbon source may, however, enable individual cells to increase their size and transport capacity by increasing the surface/volume ratio.

ENERGY METABOLISM AT (NEAR-)ZERO GROWTH RATES: IMPACT OF MAINTENANCE ENERGY REQUIREMENTS

In heterotrophs, carbon and energy substrates are often used for both growth and maintenance processes. The distribution of substrate between these two processes can be derived from the relationship between the biomass-specific substrate consumption rate (q_s) and the specific growth rate (μ). When the q_s for maintenance processes (the so-called maintenance coefficient m_s) is growth-rate independent, this relationship is linear and known as the Herbert-Pirt relation ($q_s = m_s + \mu/Y_{sx}^{max}$, where Y_{sx}^{max} is the maximum growth yield, the biomass yield on the growth-limiting substrate at a very high μ, where $m_s \ll q_s$) (Fig. 3) (46, 47). Conventionally, m_s is predicted by extrapolation of measurements obtained from chemostat cultures grown at relatively high specific growth rates (e.g., $\mu \geq 0.05\ h^{-1}$) and the slope of the linear relationship represents $1/Y_{sx}^{max}$ (5, 6, 29, 48).

While in microorganisms, growth rate-independent maintenance energy requirements adequately describe the stoichiometry of biomass and product formation at low to intermediate specific growth rates (49), it is unclear to what extent this concept is generally applicable at (near-)zero growth rates. Moreover, several prokaryotes activate a so-called stringent response at low specific growth rates. This alarmone-mediated response results in a down-regulation of energy-intensive cellular processes (e.g., protein turnover) at low specific growth rates, which reduces the ATP demand for maintenance processes (21, 50–52). Retentostat cultivation enables a much closer approximation of a zero growth rate than chemostat cultivation and, thereby, a better assessment of the growth rate (in)dependence of m_s in different microorganisms.

In recent studies, m_s values were estimated from retentostat cultures of the industrial microorganisms *S. cerevisiae* (29), *L. plantarum* (6), *L. lactis* (5), and *B. subtilis* (Table 2) (40). Contrary to earlier retentostat studies employing other bacteria (28, 30, 31, 33), these experiments did not reveal a stringent response, i.e., the m_s values calculated from (near-)zero growth retentostats were in good agreement with those estimated by extrapolation from chemostat cultivations at high dilution rates (5, 6, 29). For all four microbes, the fraction of the carbon and energy source used for maintenance-associated processes increased from 13 to 31% at the initial μ of 0.025 h^{-1} to above 98% after prolonged retentostat cultivation (Table 2). This virtually exclusive allocation of energy to maintenance is consistent with the (near-)zero growth rates reached at the end of the retentostat experiments. Therefore, the results of the retentostat studies on these four microbes were entirely consistent with the concept of a growth rate-independent maintenance energy requirement.

In *S. cerevisiae*, *B. subtilis*, and *L. plantarum* cultures, the relative concentrations of catabolic products remained essentially constant during the transition from chemostat to retentostat cultivation. The major catabolic products were lactate and low concentrations of acetate, formate, and ethanol for *L. plantarum* (6), CO_2 and H_2O for *B. subtilis* (40), and ethanol and CO_2 for *S. cerevisiae* (29). In contrast, substantial fluctuations in fermentation products were observed in *L. lactis* cultures that were also reflected by modulations in the expression of the corresponding genes. However, these metabolic changes were not predicted to influence the overall energy flux in these cells (5, 53).

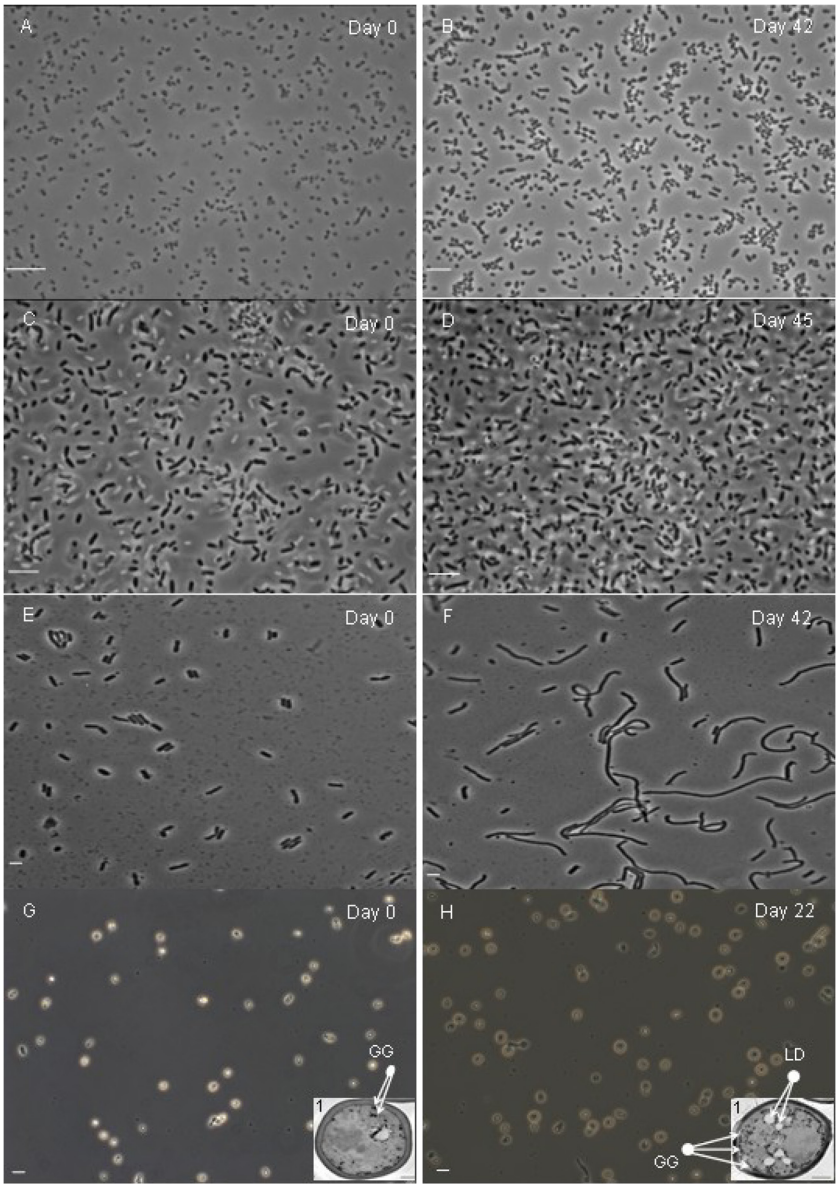

FIG 4 Phase-contrast microscopic images of chemostat cultures (left; day 0) and at the end of retentostat cultivations (right) of *L. lactis* KF147 (A and B), *L. plantarum* WCFS1 (C and D), *B. subtilis* 168 *sigF*::Sp^r *amyE*::P_{rrnB}-*gfp*$^+$ (E and F), and *S. cerevisiae* CEN.PK113-7D (G and H). Scale bars indicate 10 μm. Electron micrographs of *S. cerevisiae* cells from chemostat culture (G1) and after 22 days in retentostat cultivation (H1) were previously published in reference 4. GG, glycogen granules; LD, lipid droplets. Scale bars in insets G1 and H1 represent 1 μm.

Although its growth was limited by carbohydrate substrate availability, *L. lactis* displayed major adaptations of its nitrogen metabolism during extended retentostat cultivation (53). By integration of data from transcriptome analysis and metabolic modeling, *L. plantarum* was predicted to produce plant growth-stimulating metabolites, including indole compounds and phenylacetate, at (near-)zero growth rates (6). Short-term retentostat cultivation elicited significant metabolic changes in *A. niger*, including the production of the secondary metabolites flavasperone, aurasperone B, tensidol B, and fumonisins B_2, B_4, and B_6 (54). These results show that in some but not all (two of the four analyzed here) species, (near-)zero growth elicits the production of secondary metabolites.

SIMILAR TRANSCRIPTIONAL RESPONSES IN PATHWAYS INVOLVED IN NUTRIENT SENSING, ANABOLISM, AND RESPONSE TO STRESS

Besides adaptations in metabolic fluxes, transcriptional adaptations were observed as a function of decreasing growth rate and nutrient availability (Fig. 5). Both in the eukaryotic and prokaryotic kingdoms, dedicated glucose-sensing and signaling cascades (such as the protein kinase A [PKA] and TOR pathways in eukaryotes and catabolite control protein A [CcpA] in Gram-positive bacteria) result in transcriptional reprogramming, including the repression of genes involved in alternative carbon source utilization in glucose-rich environments (55–58). During retentostat

TABLE 2 Substrate-related maintenance coefficient and maximum specific growth yield values and relative distributions of energy and substrate costs of maintenance-associated processes during chemostat and extended retentostat cultivation[a]

Microorganism	Reference	Carbon source	m_s ($mmol_{carbon}$ g_{DW}^{-1} h^{-1})	Y_{sx}^{max} (mg_{DW} $mmol_{carbon}^{-1}$)	$Dist_{chemostat}$ (%)	$Dist_{retentostat}$ (%)
L. lactis KF147	5	Glucose and citrate	1.11	9.80	30.3	99.0
L. plantarum WCFS1	6	Glucose and citrate	0.65	5.54	12.8	98.4
B. subtilis 168 *sigF*::Sp^r *amyE*::P_{rrnB}-*gfp*$^+$	40	Glucose	1.43	7.92	31.4	98.2
S. cerevisiae CEN.PK113-7D	29	Glucose	3.00	2.91	21.3	98.6

[a] Cultivations were performed under anaerobic conditions for all organisms except *B. subtilis*, for which aerobic conditions were used. m_s, maintenance coefficient; DW, dry weight; Y_{sx}^{max}, maximum specific growth yield; $Dist_{chemostat}$ and $Dist_{retentostat}$, relative distribution of energy and substrate costs to maintenance-associated processes during chemostat cultivation, with a dilution rate of 0.025 h^{-1}, and extended retentostat cultivation, respectively.

cultivation, prolonged glucose limitation led to the progressive relief of repression of CcpA targets in prokaryotes and of glucose-repressible genes in *S. cerevisiae*, thereby demonstrating a gradual alleviation of carbon catabolite repression when approaching (near-)zero growth (4, 6, 40, 53). In *L. lactis*, this carbon catabolite repression transcriptional relief was shown to enable retentostat-derived cultures to more rapidly ferment carbon sources other than glucose, illustrating the functional consequence of this transcriptional adaptation (53). Notably, *A. niger* retentostat cultures displayed increased expression of genes involved in nutrient mobilization, including carbohydrate transporters. These evolutionarily conserved adaptive responses enable the microbes to rapidly scavenge alternative carbon and energy sources when they appear in their environment (Fig. 5).

When microbes approach (near-)zero growth rates, the cellular requirement for biosynthetic building blocks strongly decreases. This reduced anabolic demand is reflected in the transcriptome of most organisms by a decreased expression of genes involved in biosynthetic routes. Protein synthesis is the most energy-demanding biosynthetic process (26). Under the severe calorie restriction in retentostat cultures, *B. subtilis*, *S. cerevisiae*, and *A. niger* showed reduced expression of components of the translation machinery, including ribosomal proteins and amino acyl tRNA synthases (Fig. 5) (4, 7, 40, 59). Although chemostat studies of *L. lactis* revealed the existence of a positive correlation between growth rate and the expression of genes involved in translation at growth rates above 0.09 h^{-1} (60), such a correlation was not observed in retentostat cultures of *L. lactis* or *L. plantarum* (6, 53). The absence of such a correlation could not be readily explained by differences in energy levels, as all unicellular organisms showed similar distributions of energy substrate to maintenance-associated processes (Table 2). This suggests an uncoupling between translation and growth rate at a growth rate between 0.025 and 0.09 h^{-1} in LAB. However, the use of different nutrient limitations (glucose and leucine in retentostats and chemostats, respectively) in the two *L. lactis* studies (53, 60) may also have contributed to the observed differences.

The severe energy limitation in retentostats potentially is a stress factor for the culture. Indeed, a third conserved transcriptional response observed in the five microorganisms mentioned above encompassed an increased expression of genes involved in stress responses. Although observed during retentostat cultivation of all five microbes, the responses were diverse. *L. lactis* and *S. cerevisiae* showed increased expression of genes involved in general stress resistance, including heat shock proteins (GroELS and DnaKJ protein complexes in *L. lactis* and Hsp12, Hsp26, Hsp30, Hsp78, and Ssa3 in *S. cerevisiae*) (Fig. 5). These responses were shown to confer increased tolerance of heat stress on yeast and of heat, acid, and oxidative stress conditions on *L. lactis* (4, 61; O. Ercan, H. M. W. den Besten, E. J. Smid, and M. Kleerebezem, unpublished data). General stress response regulons were not significantly induced in retentostat cultures of *B. subtilis* and *L. plantarum* (6, 40). The latter, however, did show induction of the expression of genes involved in the SOS response, including error-prone DNA polymerases (6), which by increasing mutation rates might accelerate evolutionary adaptation and thereby increase the chances of survival. However, the number of generations in the retentostat was too small to directly observe adaptive evolution or

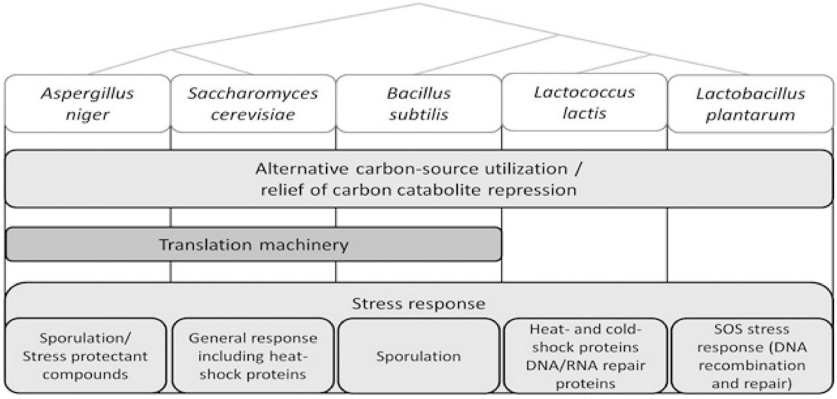

FIG 5 Cross-kingdom-shared transcriptional adaptations to (near-)zero growth. Increased and decreased expression levels of the functional categories during retentostat cultivation are indicated by light- and dark-gray boxes, respectively.

enrichment of fitness-improving mutations (40). *A. niger* showed increased expression of genes involved in the synthesis of stress-protectant compounds during retentostat cultures (7), while the induction of sporulation in this fungus can also be interpreted as an adaptation to the stress imposed by (near-)zero growth (62, 63). True assessment of zero growth in *A. niger* may require the use of a nonsporulating mutant (64), analogous to what has been used for *B. subtilis*.

MICROBE-SPECIFIC TRANSCRIPTIONAL RESPONSES AT (NEAR-)ZERO GROWTH RATES

In addition to similar responses in two or more microorganisms, retentostat cultivation was also found to induce species-specific transcriptional responses. When exposed to nutrient limitation, *B. subtilis* cells are known to increase their expression of genes involved in motility and chemotaxis to enable migration to nutrient-rich environments (65). However, *B. subtilis* retentostat cultures showed downregulated expression of these genes, a response also observed in stationary-phase cultures (40, 66, 67). This could imply that the expression of these genes depends on a minimal carbon or energy source availability, which may not have been met in retentostat cultures. Reduced autolysin expression in growing cultures of *B. subtilis* was associated with cell chain formation and cell wall metabolism (40, 68). Reduced expression of these functions during retentostat cultivation may explain the pronounced morphological changes in *B. subtilis*.

Exopolysaccharide production in LAB has been reported to be strongly growth related, which is in agreement with the repression of these genes during retentostat cultivation of *L. lactis*. Reduced exopolysaccharide expression at (near-)zero growth rates may increase mobility by reducing adhesion and/or biofilm formation and affect susceptibility to polysaccharide-recognizing bacteriophages (69, 70). In addition to the aforementioned production of plant growth-stimulating compounds, the transcriptome of retentostat cultures of *L. plantarum* revealed further responses that could relate to plant environment-associated conditions, including the activation of genes that are shared with other plant-associated bacteria and putatively involved in the degradation of plant-derived material (6).

In anaerobic *S. cerevisiae* retentostat cultures, many genes involved in mitochondrial functions, including respiration, were upregulated at both the transcript and protein level, despite the absence of oxygen (4, 71). This transcriptional reprogramming could not be attributed solely to the alleviation of glucose repression (4, 71) and, therefore, probably reflects a preparation for environmental changes. In general, many yeast genes that were previously shown to be characteristic for quiescent cells (22), i.e., postmitotic cells, were gradually upregulated during retentostat cultivation, suggesting a growth rate-dependent expression of these genes rather than an on-off switch of quiescence upon cessation of growth.

Consistent with the observed sporulation of *A. niger* retentostat cultures, genes involved in conidiation and related processes showed the strongest concerted upregulation in retentostat cultures of this filamentous fungus. This response also included increased expression of genes encoding small cysteine-rich proteins, such as hydrophobins, and of gene clusters involved in secondary metabolism. Conversely, the expression of genes encoding secreted hydrolases was decreased (7), possibly to reduce the energy-intensive production of proteins that do not exclusively benefit the producing cells.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this minireview, we compared the physiological, metabolic, and genome-wide transcriptional adaptations of the industrially important microbes *L. lactis*, *L. plantarum*, *B. subtilis*, *S. cerevisiae*, and *A. niger* to (near-)zero growth rates imposed by energy-limited retentostat cultivation. Some cellular responses observed in retentostats, such as reduced expression of *B. subtilis* genes involved in motility and strongly increased stress resistance of *S. cerevisiae* and *L. lactis*, resembled characteristics of stationary-phase, energy-starved cultures. However, many other responses seen in retentostat cultures differed strikingly from those observed in stationary-phase cultures. For example, the high and stable viability of bacterial and yeast retentostat cultures contrasted strongly with the reported loss of viability in stationary-phase, nutrient-starved cultures. Moreover, in *S. cerevisiae*, glycogen accumulated intracellularly during retentostat cultivation, while carbon and energy starvation leads to mobilization of this storage carbohydrate (4). In *B. subtilis* and *L. lactis* retentostat cultures, the cellular surface-to-volume ratio increased (5, 40), while this ratio decreases in starved bacterial cells (42–45). Furthermore, transcriptional analyses showed even stronger downregulation of biosynthetic processes, such as translation, when *S. cerevisiae* and *L. lactis* retentostat cultures were completely deprived of extracellular energy sources (61, 72). These observations show that retentostat cultivation captures a physiological state that is distinct from starvation in stationary-phase culture, which is still the most intensively used system for studying nongrowing microorganisms.

A significant fraction of the huge amount of nondividing microbial biomass on the planet (73) resides in oligotrophic environments characterized by extreme scarceness but not complete absence of essential nutrients, including energy sources (74). Examples of such environments vary from nutrient-poor environments, such as ocean water (75), to natural biofilms, in which high biomass density, low nutrient availability, and diffusion limitation can together cause extremely low specific growth rates (76, 77). We are convinced that the unique access that retentostat cultures provide to the largely uncharted but ecologically highly important "twilight zone" between growth and starvation amply rewards the efforts involved in setting up and running these rather labor-intensive cultivation systems. Additionally, planktonic growth of microbes in homogeneous retentostat cultures offers unique possibilities to further dissect the effects of attached growth from those of the resulting (near-)zero growth rates in biofilms.

The uncoupling of microbial growth and product formation holds great potential for microbial biotechnology. This is exemplified by near-theoretical yields of the catabolic products ethanol and lactate in anaerobic retentostats of *S. cerevisiae* and LAB, respectively. For example, the yields of yeast biomass and glycerol, which are the major by-products of bioethanol production by *S. cerevisiae* (17), were negligible in retentostat cultures. An important challenge for future research is to combine these near-optimal product yields with a high biomass-specific fermentation rate, for example, by deliberate introduction of futile cycles that cause a net hydrolysis of ATP (78–80). Even greater challenges are associated with anabolic products whose synthesis requires a net input

of ATP, such as enzymes, proteins, and peptides (20, 28, 81), as well as some of the high-value flavor, texture, and health metabolite production processes that employ LAB and *S. cerevisiae* (18, 19, 82, 83). Efficient production of anabolic products by nongrowing cultures will need to overcome the observed downregulation of the protein synthesis machinery during retentostat cultivation and the scarcity of ATP under calorie-restricted conditions. Strategies to genetically engineer strains for efficient conversion of carbon sources into the desirable end product in a growth-uncoupled fashion have been demonstrated for *L. lactis* for the production of its endogenous metabolites (84–87) and the alternative metabolite L-alanine (18). Moreover, retentostat cultures provide an excellent test bed for quantitative analysis of radical synthetic biology strategies aimed at uncoupling native and heterologous protein production by introduction of orthogonal translation systems (88, 89). Also, retentostats are not the only means to uncouple growth and anabolic product formation. Growth limitation by a nonenergy substrate can be explored, provided that overflow metabolism and reduced efficiency of energy source utilization under such energy excess conditions (90, 91) can be prevented. Moreover, cell division can be uncoupled from metabolic activity under glucose excess by continuous cultivation of *S. cerevisiae* in alginate beads (92).

In addition to the potential of retentostats to enable near-theoretical product yields, the strongly increased stress resistance of retentostat-grown *L. lactis* and *S. cerevisiae* provides leads for the design of fermentation protocols that improve survival under subsequent industrial processing and storage conditions. For example, eliciting increased heat and/or oxidative stress tolerance by introducing a (near-)zero growth phase may be explored as a strategy to produce robust starter cultures.

Most of the observed responses under retentostat cultivation, e.g., stringent and SOS responses, etc., are specific to subsets of microbes. However, there are some conserved responses, such as activation of alternative carbon source utilization pathways and the generic chaperonin-type heat shock proteins and reorientation of energy metabolism. These common responses may be universally conserved in most microbes under (near-)zero growth conditions to strictly sustain maintenance-associated processes and not growth.

This study highlights the power of combining controlled cultivation in retentostats with genome-scale analytical techniques. The retentostat cultivation method yields quantitative information on an important domain of microbial physiology that is not experimentally accessible via other methods. We hope that our comparative study underlines both the versatility and additional value of this approach, not only for uncoupling growth and product formation in industrial microbes but also for understanding microbial lifestyles and ecology in their natural habitats and, more tentatively, for the study of cellular ageing (93) of a wide variety of organisms.

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