Isolated Microbial Single Cells and Resulting Micropopulations Grow Faster in Controlled Environments

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Singularized cells of *Pichia pastoris*, *Hansenula polymorpha*, and *Corynebacterium glutamicum* displayed specific growth rates under chemically and physically constant conditions that were consistently higher than those obtained in populations. This highlights the importance of single-cell analyses by uncoupling physiology and the extracellular environment, which is now possible using the Envirostat 2.0 concept.

The output of any process involving microbial cells is composed of the physiological activity of each individual cell in the population. Although the average readout of bulk measurements suggests a homogeneous distribution of cellular activity among population members, this is certainly not the case (13, 27). Studies at the single-cell level revealed substantial cell-to-cell differences within clonal populations, occurring on all hierarchical levels from genome to phenome (3, 10, 18, 38, 45). This heterogeneity has its origin in random events, such as variations in molecule location and quantity, or can arise from the active generation of diversity. These processes result in the continuous cycling of the cell through a multitude of physiological states (36). Changes in concentrations of low abundant molecules at the top of the regulatory hierarchy govern global gene expression and protein synthesis as well as metabolite formation. They play a particularly important role, since small deviations result in high relative changes (1, 40, 42). The resulting transitions of individual cells among a variety of phenotypes are assumed to statistically increase the capability of a population to cope with fluctuating environments and therefore represent an important competitive advantage (5). The frequency and extent of these transitions were shown to correlate with frequency and extent of the environmental changes (1, 5). Thus, a constant and totally defined extracellular environment, diminishing physiological oscillations, is a key requirement for the mechanistic description of any cellular biochemical network operation in its native state of equilibrium. Furthermore, environmental control is mandatory to accurately analyze the impact of environmental changes on cellular physiology (2, 16). Detailed knowledge about such responses would allow the improvement of efficiency and performance of any bioprocess.

From the strong interrelation of physiology and environment, it follows that conventional bulk cultivation technologies, where changes in the cells’ immediate environment are many and ubiquitous, do not allow the analysis of specific and stable phenotypes (14, 25). Cells, whether in a stirred system or in a static environment, are subject to perpetual changes in terms of mechanical force, ion equilibrium, nutrient supply, concentrations of toxic or inhibiting metabolites, quorum sensing, and oxygen availability (35). Even in strongly agitated small-scale fermentors, cells spend considerable periods of time in stagnant zones, depleted of substrate and oxygen, leading to impaired growth and macromolecule synthesis rates (11).

Thus, it can be concluded that a comprehensive analytical approach for the understanding of cellular phenomena must tackle the challenges of total environmental control and time-resolved analysis of single cells. Only such an approach will reveal how extracellular perturbations affect the functional state of the cell and hence the functional structure of a microbial population.

The Envirostat 2.0 meets this challenge with an integrated microfluidic Lab-on-a-Chip system, allowing isolation, contactless cultivation, and time-resolved analysis of single cells in a microflow (F. S. O. Fritzsch, K. Rosenthal, A. Kampert, S. Howitz, C. Dusny, and A. Schmid, submitted for publication). The contactless isolation and retention of the cell were realized by negative dielectrophoresis (nDEP) with electrodes in a symmetrical octupole arrangement and an electrode tip distance of 20 μm (43). Optimal supply of nutrients and oxygen as well as immediate removal of metabolites was ensured through a continuous medium flow for cell perfusion. Since the mass transfer in this system relies not only on passive diffusion but also on enforced convection, the extracellular environment of the target cell always has a steady, environmentally static composition. A previous study substantiated this assumption by computational fluid dynamics (24). It was demonstrated that even for extracellular substances with high conversion rates like glucose and ethanol, only minimal changes in medium composition occur in the direct proximity of the cell surface at flow rates similar to those applied during this study. Environmental control via a moving liquid distinguishes the Envirostat 2.0 system from most other microfluidic devices for single-cell analysis that operate mainly in batch mode. The Envirostat periphery also enables exact control of the temperature in the cultivation section of the chip (24).

Using this single-cell analysis system, the influence of stable environments on the growth of single cells was systematically investigated and compared with the corresponding properties of populations.

However, the individuality of a single cell is difficult to qua...
tify, as access is limited by sample processing and assay sensitivity (7, 15). Therefore, we considered the growth rate in place of the physiologically entire cell, which can be ambiguously determined on both the population and single-cell levels by optical means (29, 31). Owing to its dynamic nature and strong connection to the overall biochemical network, growth is widely used as a proxy for global cellular physiology, providing information about fitness and functionality of the cell (41).

Here, we present a data set containing specific growth rates of single cells and micropopulations cultivated under precisely defined (micro)environmental conditions employing the Envirostat 2.0 single-cell analysis system (Fig. 1). Three microbial and industrially relevant strains, *Pichia pastoris* Mut³, *Hansenula polymorpha* RB11 Conphys, and *Corynebacterium glutamicum* ATCC 13032, were investigated. Growth experiments at the single-cell level were performed at 30°C with a perfusion flow rate of 7.2 nl min⁻¹ standard yeast peptone dextrose (YPD) medium for the yeast strains and 9.0 nl min⁻¹ brain heart infusion (BHI) medium for *Corynebacterium glutamicum* ATCC 13032. Cells were cultivated in shaken submerged cultures prior to analysis in the Envirostat 2.0 chip and taken from the early exponential growth phase. Cell growth was monitored by time-lapse microscopy (Fig. 1). Additional details on materials, experimental setup and procedures, data acquisition and octupole cage operation are provided in the supplemental material.

The volume growth of all three surveyed microbial strains followed a strictly exponential increase from single-cell stage to micropopulations of up to 8 cells (Fig. 2).

Notably, single cells and micropopulations exhibited a consistent and robust increase in cell volume when the respective cultivation experiments were compared. The determined specific growth rates were highly similar, ranging from 0.34 h⁻¹ to 0.36 h⁻¹ for *Pichia pastoris* Mut³, 0.45 h⁻¹ to 0.49 h⁻¹ for *Hansenula polymorpha* RB11 Conphys, and 0.55 h⁻¹ to 0.61 h⁻¹ for *Corynebacterium glutamicum* ATCC 13032. These unusually high and stable growth rates are most probably attributable to the controlled extracellular environment and hence diverge from the findings on single-cell growth made earlier by other research groups (4, 22). The earliest studies on single-cell growth were based on cinematographic analysis of baker’s and fission yeast carried out on tempered object slides with thickened medium or agar plates (20, 28, 33). Many investigations focused on yeasts because of its comparably large size and well-defined shape, allowing reliable size- and volume-based measurements of single cells (20, 28, 30, 34, 47). More recent studies also include the investigation of bacterial strains and the analysis of cells trapped in micrometer structures (6, 12, 41, 44). The results and conclusions regarding growth heterogeneity and the general character of cell proliferation diverged and were partially contradictory. A closer look at the cultivation techniques applied during these studies reveals one common feature that might have been responsible for the observed inconsistencies. These studies gave valuable insight into the nature of growth and proliferation of single cells, yet the cultivation techniques utilized lack adequate environmental control and possibly influenced the observations made. Nutrient and oxygen depletion as well as changes in pH may have affected physiology, thereby causing significant alterations in the metabolic network function and stress responses, which certainly leads to variations in growth behavior (23). Mechanical confinement of bacterial cells was also shown to entail deformation and morphological diversification (32). Furthermore, functional changes induced by cell surface interactions and a possible lack of hydrodynamic stimulation cannot be excluded (8, 41). One particularly important example for such a change in phenotype triggered by cell surface interaction is biofilm formation, because the vast majority of microbes associate in biofilms (26, 39).

However, studies on the robustness of growth of single bacterial cells cultivated in microfluidic channels revealed an exceedingly stable rate of length increase over many generations, which coincide with our observations (46). A recent study reported increased growth rates of bacterial microcolonies with up to 250 cells that were confined in a monolayer bioreactor (17).

All mentioned experimental drawbacks and the resulting limitations in description could be avoided during this study by the use of the Envirostat 2.0 analysis platform. Nevertheless, detrimental effects of electric fields on microbial growth have been described (37). It was also shown that the impact of the electric field on growth is strongly dependent on the applied voltage and frequency and diminishes at low voltages and high frequencies (19). Since lowest possible voltage and highest applicable frequencies for the dielectrophoretic trapping of the single cells were applied during this study, it can be assumed that the electric field at these parameters does not influence, or only negligibly influences, microbial physiology.

Accordingly, our investigations suggest that the observed increase and robustness in growth rate are connected to the steady extracellular environment.

In order to assess the impact of inhomogeneous environments on cellular growth, the kinetic data from single-cell cultivation experiments were compared to growth kinetics of shake flask cul-

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**FIG 1** Cultivation of *Pichia pastoris* Mut³ (A), *Hansenula polymorpha* RB11 Conphys (B), and *Corynebacterium glutamicum* ATCC 13032 (C) in the Envirostat 2.0 system. Cells were trapped in the center of the octupole cage by nDEP. The volume growth of cells was monitored by video surveillance.
tivations and diluted populations (Table 1). During the course of a batch shake flask cultivation, cells are subjected to continuous and drastic concentration changes (e.g., glucose depletion), caused by the steady consumption of nutrients and production of extracellular metabolites. For example, the pH of a strongly buffer-

ered cultivation medium (100 mM potassium phthalate) with 20 g liter\(^{-1}\) glucose dropped from 7 to 3.2 at the end of the exponential growth phase during batch cultivation of Hansenula polymorpha RB11 Conphys. The environmental stress caused by the changes in medium composition was assumed to be reflected in reduced growth rates. Furthermore, the extent of changes in the extracellular environment should correlate with the number of active cells and diminish along with a decreasing cell number.

Indeed, in comparison to shake flask experiments, single cells in the Envirostat 2.0 showed strong increases in volume growth: 48% for Pichia pastoris Mut\(^a\), 120% for Hansenula polymorpha RB11 Conphys, and 32% for Corynebacterium glutamicum ATCC 13032. Volume growth rates of isolated cells were also compared to growth rates of diluted populations. Diluted populations were assumed to propagate under unlimited growth conditions at very low cell concentrations, since the environmental composition is hardly changed by the metabolic activity of the few cells (21). This assumption could be confirmed by measurement of the medium pH during the cultivation of diluted populations, which was not changed by cellular activity. Cultures with low cell titers were grown from singularized cells by the dilution-to-extinction method to reveal the existence of stable growth phenotypes that exhibit growth behavior deviating from the average population growth rates (Table 1) (9). Growth experiments with diluted populations were performed in 100-well microtiter plates at 30°C (Bioscreen C MBR; Growth Curves AB Ltd., Finland) in the same media that were used for the single-cell experiments. All tested microbial cells showed higher specific growth rates during growth analysis at extremely low biomass concentrations in comparison to standard shaking flask cultivations. Average specific growth rates of diluted populations were determined as 0.27 h\(^{-1}\) for Pichia pastoris Mut\(^a\), 0.34 h\(^{-1}\) for Hansenula polymorpha RB11 Conphys, and 0.48 h\(^{-1}\) for Corynebacterium glutamicum ATCC 13032, corresponding to respective increases in growth rate of 17%, 61%, and 9%. Enhanced growth is most likely attributed to the better availability of nutrients and oxygen in comparison to shake flask cultivations and the minor changes of the medium composition in low-cell-titer cultivations. The frequency distribution of growth rates of the diluted populations grown from single cells was normally distributed, with a significantly broader range of observed growth rates than in single-cell cultivations (Fig. 3). The broad distribution of growth rates is possibly attributable to the inheritance of growth characteristics of the individual single-inoculum cell, which determined the capacity of the populations to grow in inhomogeneous environments over many generations.

When the results of the single-cell experiments with cultivations with low and high biomass concentrations are compared, the consistently higher specific growth rates of single cells are striking. Isolated cells of Pichia pastoris Mut\(^a\) and Hansenula polymorpha

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Shake flask</th>
<th>Microtiter plate</th>
<th>Envirostat 2.0</th>
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</thead>
<tbody>
<tr>
<td>P. pastoris Mut(^a)</td>
<td>0.23 ± 0.01</td>
<td>0.27 ± 0.03</td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td>H. polymorpha RB11 Conphys</td>
<td>0.21 ± 0.01</td>
<td>0.34 ± 0.03</td>
<td>0.46 ± 0.02</td>
</tr>
<tr>
<td>C. glutamicum ATCC 13032</td>
<td>0.44 ± 0.01</td>
<td>0.48 ± 0.04</td>
<td>0.58 ± 0.02</td>
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**TABLE 1** Specific growth rates of the analyzed microbial strains in different cultivation systems
RB11 Conphys showed 1.26- and 1.35-fold higher average volume growth rates than diluted populations when cultivated in the En-
vironstat 2.0 system (Table 1). The growth rates of single
bacterium glutamicum
and population effects.

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This is, to the best of our knowledge, the first systematic inves-
tigation of microbial growth at the single-cell level in controlled
environments and with contactless cell retention.

In summary, our results indicate that despite the existence of
cell-to-cell differences in clonal populations, these inherent dif-
ferences do not manifest themselves in variable growth rates under
controlled cultivation conditions. It is rather the extracellular en-
vironment which can dictate the effective maximal growth rate.
This principle was shown to be valid for two unicellular microbial
eukaryotes and one prokaryote.

This study demonstrates the benefit of elucidating cellular
physiology and its response to perturbations at a single-cell level,
unbiased by uncontrollable changes in the extracellular environ-
ment and population effects.

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by M. Piontek (ARTES Biotechnology, Langenfeld, Germany).

FIG 3 Frequency distribution of specific growth rates of diluted populations.
The growth rates of Pichia pastoris Mut+ (A), Hansenula polymorpha RB11
Conphys (B), and Corynebacterium glutamicum ATCC 13032 (C) were deter-
mined during the exponential growth phase at 30°C and low cell densities
within a range of 1 × 10^7 cells ml^-1 to 2 × 10^7 cells ml^-1 (optical density at 600
nm ~ 0.01 to 0.1). The frequency of a specific growth rate as a fraction of all
conducting growth experiments (Pichia pastoris Mut+, n = 56; Hansenula poly-
morpha RB11 Conphys, n = 63; Corynebacterium glutamicum ATCC 13032,
with 1.26- and 1.35-fold higher average volume growth rates than diluted populations when cultivated in the En-
virostat 2.0 system (Table 1). The volume growth of single Coryne-
bacterium glutamicum ATCC 13032 cells exceeded specific growth
rates of diluted populations by a factor of 1.21.

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