Emergence of Phenotypically Distinct Subpopulations Is a Factor in Adaptation of Recombinant \textit{Saccharomyces cerevisiae} under Glucose-Limited Conditions

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\textbf{ABSTRACT} Cells cultured in a nutrient-limited environment can undergo adaptation, which confers improved fitness under long-term energy limitation. We have shown previously how a recombinant \textit{Saccharomyces cerevisiae} strain, producing a heterologous insulin product, under glucose-limited conditions adapts over time at the average population level. Here, we investigated this adaptation at the single-cell level by application of fluorescence-activated cell sorting (FACS) and showed that the following three apparent phenotypes underlie the adaptive response observed at the bulk level: (i) cells that drastically reduced insulin production (23%), (ii) cells with reduced enzymatic capacity in central carbon metabolism (46%), and (iii) cells that exhibited pseudohyphal growth (31%). We speculate that the phenotypic heterogeneity is a result of different mechanisms to increase fitness. Cells with reduced insulin productivity have increased fitness by reducing the burden of the heterologous insulin production, and the populations with reduced enzymatic capacity of the central carbon metabolism and pseudohyphal growth have increased fitness toward the glucose-limited conditions. The results highlight the importance of considering population heterogeneity when studying adaptation and evolution.

\textbf{IMPORTANCE} The yeast \textit{Saccharomyces cerevisiae} is an attractive microbial host for industrial production and is used widely for manufacturing, e.g., pharmaceuticals. Chemostat cultivation mode is an efficient cultivation strategy for industrial production processes as it ensures a constant, well-controlled cultivation environment. Nevertheless, both the production of a heterologous product and the constant cultivation environment in the chemostat impose a selective pressure on the production organism, which may result in adaptation and loss of productivity. The exact mechanisms behind the observed adaptation and loss of performance are often unidentified. We used a recombinant \textit{S. cerevisiae} strain producing heterologous insulin and investigated the adaptation occurring during chemostat growth at the single-cell level. We showed that three apparent phenotypes underlie the adaptive response observed at the bulk level in the chemostat. These findings highlight the importance of considering population heterogeneity when studying adaptation in industrial bioprocesses.

\textbf{KEYWORDS} population heterogeneity, heterologous protein production, \textit{Saccharomyces cerevisiae}, chemostat cultivation, proteomics, flow cytometry

A growing number of pharmaceuticals, food ingredients, and other valuable chemicals are produced commercially by recombinant microbial cell factories. Expression of heterologous proteins confers a burden to the host strain which can result in an adverse selection toward low-producing cells during production (1).

The chemostat mode of cultivation is an efficient strategy for industrial production processes, especially in continuous manufacturing, as the cells in the bioreactor can be...
kept in a constant growth environment for long periods with a constant output (2). In this steady-state growth environment, the cells are exposed to nutrient limitation, e.g., glucose, either as a kinetic limitation of the cellular transport of nutrients or as a limitation of the metabolic enzymes converting the nutrients (3). The nutrient-limited conditions in the chemostat impose a constant selective pressure on the production organism. Isogenic cells cultured in glucose-limited environments often undergo adaptation increasing fitness benefits under long-term energy limitation.

We have shown previously how a recombinant *Saccharomyces cerevisiae* strain producing a heterologous protein adapts over time with respect to the population average protein levels (4). Over approximately 30 generations of glucose-limited growth, we observed a drastic decrease in recombinant protein production to almost half of the maximum value together with significant changes in the intracellular proteome. The underlying mechanisms of this adaptation are currently unknown but could not be associated with genetic mutations (unpublished results).

In nature, microorganisms live in complex communities consisting of many different species, subspecies, and genetic variants. Phenotypic heterogeneity can occur in populations of genetically identical cells with respect to different traits, including metabolism and morphology (5). Phenotypic population heterogeneity with respect to metabolic activity has been shown in chemostat cultivations of microbes (6, 7).

In this study, we have investigated the adaptation of a recombinant *S. cerevisiae* strain in glucose-limited cultures at the single-cell level. The results show that the bulk adaptive outcomes observed at the culture level, i.e., protein levels, physiology changes, and loss of productivity, are a mixed response composed of at least three apparent phenotypes or subpopulations. The results highlight the importance of considering population heterogeneity when studying adaptation.

RESULTS AND DISCUSSION

**Fluorescence-activated cell sorting (FACS) analysis of chemostat cultivations with *S. cerevisiae* C.U17.** We cultivated the recombinant *S. cerevisiae* strain C.U17 in duplicated chemostats and performed time-resolved analysis of the population with respect to particle size measured by forward scatter light area (FSC-A; used as a proxy for morphology) (Fig. 1A). We observed the differentiation of three main subpopulations over time with respect to FSC-A (Fig. 2; see Fig. S2 and S7 in the supplemental materials for replicates). We named the three subpopulations population 1 (small particles), population 2 (medium particles), and population 3 (large particles). Population 1 corresponded to 23% of the total population after 270 h of glucose-limited growth, population 2 corresponded to 46%, and population 3 corresponded to 31% (Fig. 3A).

**Morphology of FACS-sorted populations.** We FACS sorted each of the three subpopulations based on FSC-A and inspected the subpopulations by microscopy (Fig. 1A and B). We observed that population 1 contained primarily single cells where about a fourth presented small round buds, whereas population 2 consisted of multibudded cells with a more ellipsoidal cell shape (Fig. 3B; see Fig. S21 to Fig. S26 in the supplemental material). Population 3 contained branches of elongated cells with multiple buds (pseudohyphae).

Morphological changes toward a more filamentous and pseudohyphal growth are known effects of chemostat growth and also a known adaptive response of cells in a nutrient poor environment as a strategy to forage for nutrients (8–10). This information suggests that population 3 has emerged as a result of the glucose-limited conditions.

Flocculation and wall growth have been observed in prolonged chemostats where cells stick to the surface of the culture vessels (11). However, wall growth has not been observed in this study.

Morphological changes exhibited by cell subpopulations comprising large single-budded and multibudded cells have been observed previously in industrial scale chemostat cultivations with *S. cerevisiae* and were related to hypoxia (12). In the current study, no sign of oxygen limitation was found during the chemostat cultures (see Fig. S12 in the supplemental material). Thus, we link the formation of the subpopulations to the selective pressure of the constant glucose-limited environment. We confirm this
FIG 1 Overview of the experimental setup. (A) The monoclonal initial cell clone was used to inoculate a chemostat culture. After 271 h of continuous cultivation, a sample of adapted cells was taken (end sample cells) and stored as a (Continued on next page)
link by glucose pulse experiments with the initial cell clone (C.U17 strain) in a scale-down reactor system conducted as a control experiment in parallel with the glucose-limited chemostats. A uniform particle size and morphology with no significant changes over time could be detected in cultures exposed to continuous glucose pulsing (Fig. 4; see Fig. S13 and S14 in the supplemental material for replicates).

**Maximum growth rate of FACS-sorted subpopulations.** Each of the FACS-sorted individual populations had maximum growth rates equal to or higher than the initial cell clone (Fig. 5A). Population 1 had a significantly higher maximum growth rate on glucose than the other populations, which can be explained by a lower metabolic burden of the heterologous insulin production (two-sided t test, \( P < 0.05 \)). This result is further discussed in section “Heterologous insulin production.”

**Reinitiated chemostat cultures of FACS subpopulations.** Each of the FACS-sorted subpopulations were propagated in batch cultures and stored as glycerol stocks (Fig. 1A and B).

We cultured each of the propagated populations in new chemostat cultures in order to characterize the populations individually, with respect to particle size (FSC-A), heterologous insulin production, and intracellular proteome (Fig. 1C). We also compared the subpopulations to the end sample cells from which they were sorted with respect to heterologous insulin production. Biomass concentrations measured as cell dry weight for each cultivation can be found in Fig. S16 in the supplemental material.

(i) **Particle size (FSC-A).** We observed that the particle size of some of the cells in population 2 and population 3 after propagation in batch cultures had changed toward particle sizes typical of population 1 (see Fig. S3, S4, S5, S8, S9, and S10 in the supplemental material). This finding indicates that the morphology of the populations is reversible. However, the particle size increased again over time in the reinitiated chemostats.

(ii) **Heterologous insulin production.** The production of the recombinant insulin decreased over time when the initial cell clone was cultivated under prolonged glucose-limited conditions, whereas a stable insulin production was found in the scale-down reactor system with continuous glucose pulsing (Fig. 5B; see Fig. S15 in the supplemental material). This finding is in line with our previous observations where we coupled the decline in insulin yield to changes in the intracellular proteome of the strain observed in measurements at the culture level (4). The end sample cells continued to produce insulin at the same low level when cultured de novo in chemostats, while the subpopulations showed divergent phenotypes with respect to heterologous insulin production (Fig. 5B). In the beginning of the reinitiated cultures, the sum of the insulin yields of population 1 to 3 was \( \sim 1.8 \) mg/gDW (adjusting for the ratios between the three subpopulations at the end of the cultivations with the initial cell clone). This yield approximates the insulin yield observed by bulk measurements in the end of the cultivations with the initial cell clone. This information indicates that the insulin yields of the three populations were conserved during the batch propagation of the FACS-sorted cells (estimated to more than 40 generations).

Only small amounts of insulin could be detected in cultures with population 1, and the population had a significantly lower plasmid copy number than population 2 (Fig. 5C). The production of a heterologous protein is a burden for the host (13), and cells with reduced productivity will have a growth advantage compared with cells, which are not able to adapt (1). We observe this advantage as a higher maximum growth rate for population 1 under glucose-rich conditions (Fig. 5A). Therefore, we suggest that population 1 has arisen mainly due to the burden of the heterologous protein production and due to a lesser extent to the glucose-limited conditions. The determined maximum growth rate for population 1 (0.33 h\(^{-1} \pm 0.01\)) is slightly lower than the growth
FIG 2 Distribution of cells with FSC-A corresponding to population 1 [log₂(FSC-A) of <16.6], population 2 [16.6 < log₂(FSC-A) of <17.7], or population 3 [log₂(FSC-A) of >17.7] at different time points during a representative culture of the initial cell clone. (A) Density plot of log₂(FSC-A) at different cultivation time points. A total 100,000 cells were analyzed at each time point. (B) Fraction of cells with an FSC-A corresponding to each of the three populations as function of cultivation time.

rate of the wild-type CEN.PK113-7D strain (0.37 h⁻¹ ± 0.01), which has been used previously as a reference strain for comparison with the C.U17 strain (14).

The bulk measurements of insulin in the initial cell clone reached a new steady state after around 150 h of glucose-limited growth (Fig. 5B). Moreover, a steady state
between the three morphological phenotypes seemed to occur after around 200 h of chemostat cultivation (Fig. 2B). Cultivations with the end sample cells for another 250 h under glucose-limited conditions confirm the steady state with respect to insulin production and morphology (Fig. 5B, Fig. 6). This result indicates that none of the three populations had a significantly higher fitness under chemostat conditions than the other. Based on the maximum growth rate experiments, we would have expected that population 1 should take over the entire population in the chemostat and that population 2 and population 3 gradually would be washed out. However, the increase in fitness of chemostat-adapted cells is often specific to the low nutrient-limited environment and many organisms adapted to chemostat conditions show reduced growth capabilities in nutrient-rich environments (15–17). Thus, a higher maximum growth rate under glucose-rich conditions will not be an adequate measure of fitness under glucose-limited conditions.

Yeast cells release a variety of different metabolites and prefer the uptake of extracellular metabolites over self-synthesis (18). Population heterogeneity can emerge as a consequence of metabolic cooperation between cells, and a population as a whole can benefit from the division of labor between individuals (19, 20). We speculate that the three populations in the chemostat cooperate in metabolism and exchange metabolites and that this may explain the observed steady state between the subpopulations. However, this suggestion needs to be further investigated.

(iii) Intracellular proteome. The FACS-sorted cells were further characterized with respect to their intracellular proteome during chemostat growth. We compared the intracellular proteome in the beginning of chemostat cultures of the three populations with our previously reported proteomics data set from the beginning of chemostat cultures with the initial cell clone (4). Moreover, we compared the proteome of the three individual populations in the beginning of the chemostat cultures and again in the end of the cultivations.

In the beginning of the chemostat cultures, population 2 differed from population 1 and the initial cell clone with respect to proteins involved in the central carbon...
FIG 4 Distribution of cells with FSC-A corresponding to population 1 \([\log_2(\text{FSC-A}) < 16.6]\), population 2 \([16.6 < \log_2(\text{FSC-A}) < 17.7]\), or population 3 \([\log_2(\text{FSC-A}) > 17.7]\) at different time points during a (Continued on next page)
metabolism (Table 1; see Table S6 and S7 in the supplemental material). The levels of these proteins were significantly lower in population 2 (see Fig. 7 for example of glycolytic proteins). Moreover, simulations of chemostat growth of population 1 and population 2 using the enzyme-constrained genome scale metabolic model ecYeast8 (21) indicated that the two populations might differ with respect to intracellular fluxes in central metabolism (see Table S9 and S10 in the supplemental material), a finding that will have to be confirmed experimentally in future work. Protein synthesis is an energetically expensive process. Cells, which can economize the protein synthesis, e.g., by reducing the production of overexpressed proteins in a nutrient-limited environment, will have an advantage over cells, which cannot adapt. The reduced capacity of the central carbon metabolism, including the glycolysis and tricarboxylic acid, is a well-known adaptive response to chemostat conditions (22–24). This information suggests that the establishment of population 2 is a response to the glucose-limited conditions and confers a fitness advantage in the chemostat.

A total of 6% of the measured proteome differed between population 1 and the initial cell clone (log2 fold change of >0.5 or log2 fold change of < −0.5; q-value of <0.05) (Table S6). No differentially expressed GO terms could be found related to these proteins (false discovery rate [q value] <0.05). However, levels of the proteins expressed from the selective markers URA3 and HIS3 were significantly lower in cultivations with population 1 than those of cultivations with the initial cell clone and population 2 (see Fig. S17 and S18 in the supplemental material) and can be coupled to a significant lower plasmid copy number in population 1 than that in population 2 (two-sided t test, P < 0.05) (Fig. 5C). A decline in plasmid copy number over cultivation time has been measured previously in the bulk of chemostat cultivations with the initial cell clone in the time frame investigated in this study (14) and may be related to the proportion of population 1 increasing over time. In general, several proteins involved in the biosynthesis of uridine are significantly decreased in the beginning of cultivations with population 1 compared with those with population 2 (q value of <0.05) (see Fig. S17 for levels of the specific proteins). This finding is not the case for the biosynthesis pathway of histidine (see Fig. S18 for levels of the specific proteins).

After 271 h of glucose-limited growth, only 16 proteins differed with more than 0.5 log2 fold change between population 1 and population 2 (q value of < 0.05), (see Table S6 and S8 in the supplemental material). This finding indicates that two adaptive mechanisms occurred over time in population 1. In the original chemostat cultures, the fitness of the cells was increased toward the burden of the heterologous insulin production by a decrease of the insulin productivity. In the reinitiated chemostat cultures, the fitness was increased toward the glucose-limited conditions by a decrease in the overcapacity of especially enzymes linked to the central carbon metabolism. We have shown previously that the adaptation to glucose-limited conditions is enhanced by the production of a heterologous product (4). This information may explain the delayed adaptation in population 1 with respect to the reduction of enzymatic overcapacity compared with population 2. During the first 100 h of the reinitiated chemostats with population 2, there seems to be a further reduction in the glycolytic capacity of the cells (Fig. 7).

No significantly expressed proteins could be found between population 2 and population 3 in the beginning of chemostat cultivations, whereas 50 proteins differed between population 1 and population 3 (log2 fold change of >0.5 or log2 fold change of < −0.5, q value of <0.05) (Table S6). Population 3 consisted of cells with multiple simultaneously attached buds (Fig. 3B) that span a larger variety of cells with respect to particle size. Therefore, a larger variation was observed between replicates with this population (see Fig. 4).
FIG 5 (A) Maximum growth rate in batch cultures. Error bars indicate differences between three biological replicates. Population 1 had a significantly higher maximum growth rate than the other (Continued on next page)
S19 in the supplemental material). This variation explains the lower level of significantly changing proteins between population 3 and the other subpopulations.

As described in section “Particle size (FSC-A),” the particle size of some of the cells in population 2 and population 3 had changed toward the particle sizes typical of population 1 in the reinitiated chemostats where the proteomics samples were taken. Therefore, the proteomics data cannot be used to support the differences in morphology between the three populations.

(iv) Cell synchrony. We have shown previously that the initial cell clone synchronized growth during the first 100 h of chemostat growth (4). The time point where the synchronized growth stopped correlated with the time point where the heterologous insulin production started to decrease (Fig. 5B, Fig. S12). This result also correlated well with the time point where the population heterogeneity with respect to particle size (FSC-A) arose in the culture (Fig. 2B). Neither the end sample cells nor population 3 showed synchronized growth in the reinitiated cultures. We explain this finding by the larger fraction of population heterogeneity in the reinitiated chemostats already from the beginning of the cultures (Fig. 6; see Fig. S5, S6, S10, and S11 in the supplemental material). This heterogeneity was conserved during the initial batch propagation. Population 1 showed synchronized growth for 150 h, whereas population 2 showed synchronized growth for 50 h. The stop of synchronized growth of population 2 correlated with an increase in population heterogeneity in the reinitiated cultures with respect to particle size (FSC-A) (Fig. S9 and S12).

Three apparent phenotypes underlie the adaptive response observed at the bulk level. We have shown previously that the recombinant S. cerevisiae strain (C.U17) adapts in a reproducible manner at the average population level for five replicated cultures with respect to changes in heterologous insulin production and intracellular proteome under prolonged glucose-limited conditions (4). The diversity of adaptation varies as a function of the distribution of fitness effects between beneficial outcomes (25). Thus, if an adaptive path confers a much greater selective advantage than other selective outcomes, that path will be highly reproducible. Due to the reproducibility of the phenotypic outcome of chemostat cultivations with the initial cell clone, we expected the adaptation to be driven by the selection of a single clone with a large relative selective advantage. In the present study, however, we demonstrate that the adaptation is not caused by the selection toward a single beneficial phenotype. Instead, the isogenic strain differentiated into subpopulations and reproducibly established three main subpopulations after 271 h of glucose-limited growth (Fig. 3A). Our results indicate that three apparent phenotypes underlie the adaptive response observed at the bulk level (Fig. 8). We speculate that this phenotypic heterogeneity is a result of different mechanisms to increase fitness. Population 1 has increased fitness by downregulating heterologous insulin production by decreasing the plasmid copy number, whereas population 2 has adapted toward the glucose-limited conditions. Population 3 seems to be a response to both the burden of the heterologous insulin production and the glucose-limited conditions having a phenotype with reduced insulin productivity and pseudohyphal growth.

Adaptation and evolution in chemostats have been highly investigated at the average population level. However, only a few studies have investigated adaptation in terms of isogenic cells differentiating into phenotypic subpopulations (6). Our results highlight the importance of considering population heterogeneity when studying adaptation, as bulk adaptive outcomes observed at the culture level can be a mixed response composed of different phenotypes or subpopulations. A better understanding of the mechanisms...
FIG 6 Distribution of cells with FSC-A corresponding to population 1 \([\log_2(FSC-A) < 16.6]\), population 2 \([16.6 < \log_2(FSC-A) < 17.7]\), or population 3 \([\log_2(FSC-A) > 17.7]\) at different time points during a
(Continued on next page)
behind the adaptive phenomenon in chemostats may reveal new targets for metabolic engineering of production strains or process optimization that can prevent adaptation and product loss in industrial chemostats.

Conclusions. In this study, we investigated adaptation at the single-cell level and showed that an isogenic, recombinant *S. cerevisiae* strain differentiated into three subpopulations, as follows: (i) cells that drastically reduced insulin production (23%), (ii) cells with reduced enzymatic capacity of the central carbon metabolism (46%), and (iii) cells that exhibited pseudohyphal growth (31%). This finding indicates that the bulk adaptive outcome is a mixed response composed of at least three apparent subpopulations emerged as a response to the selective pressure of the chemostat and the burden of the heterologous insulin production.

**MATERIALS AND METHODS**

**Strains and experimental overview.** An isogenic culture of the recombinant *S. cerevisiae* strain C.U17 was cultured in prolonged chemostat cultivations (14). The strain contains a 2-μm vector with an *TPI1* promoter and a gene encoding a single-chain insulin precursor. Both *HIS3* and *URA3* were used as auxotrophic selection markers. The C.U17 strain is referred to as the initial cell clone throughout the manuscript. After 271 h of glucose-limited growth, end sample cells from one of the cultivations were collected and stored as glycerol stocks at −80°C. These cells are referred to as end sample cells. One of the glycerol stocks were thawed, washed three times in phosphate-buffered saline (PBS), and used for FACS sorting of three individual populations separated based on particle size measured as forward scatter light area (FSC-A) (Fig. 1A). A total of 10,000 sorted cells from each population were propagated in individual shake flasks with minimal medium at 30°C, harvested at an optical density at 600 nm (OD600) of 20, and stored as glycerol stocks at −80°C (Fig. 1B). The glycerol stocks were used to reinitiate new chemostat cultures with each of the FACS-sorted populations (Fig. 1C).

**Chemostat cultivations.** Aerobic chemostat cultivations were performed in 0.5-L fully instrumented and automatically controlled BioSTAT reactors (Sartorius Stedim Biotech S.A., Germany). The strains were cultured in duplicates as previously described by Wright et al. (4) at a temperature of 28°C, pH of 5.9, aeration rate of 2vvm, and dilution rate of 0.1 h⁻¹. A minimal medium with a glucose concentration of 75 gL⁻¹ was used, and cultures were propagated in individual shake flasks with minimal medium at 30°C, harvested at an optical density at 600 nm (OD600) of 20, and stored as glycerol stocks at −80°C (Fig. 1B). The glycerol stocks were used to reinitiate new chemostat cultures with each of the FACS-sorted populations (Fig. 1C).

**FIG 6** Legend (Continued)

representative culture of the end sample cells. (A) Density plot of log2(FSC-A) at different cultivation time points. A total of 100,000 cells were analyzed at each time point. (B) Fraction of cells with an FSC-A corresponding to each of the three populations as function of cultivation time.

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| GO description | Proteins |
|----------------|----------|
| Oxidation-reduction process | Fas2p, Fas1p, Ald4p, Pgk1p, Tdh2p, Fdh1p, Fox2p, Tdh1p, Kgd1p, Gut2p, Cyb2p, Idp2p, Lys9p, Cta1p, Arg15.6p, His9p, Yhb1p, Adh2pAdh1p, Mdh1p, Mcl1p, Pox1p, Idh1p, Ndi1p, Ydi124wp, Hom2p, Mdh3p, Sod1p, Gdh2p, Gcy1p, Mae1p, Sdh1p, Dld1p, Pox1p, Idp3p, Gnd2p, Rnr4p, Idh2p, Adh3p, Bna1p, Sdh2p, Erg11p, Gcv1p, Ahp1p, Cir2p, Gpd1pCy1p, Zta1p, Rip1p, Yml113wp, Rnr1p, Mdh2p, Gre2p, Nde2p, Adh5p, Bdh1p, Bna4p, Sdh4p, Sdh3p, Ifa38p, Met12p |
| Tricarboxylic acid cycle | Ac01p, Kgd1p, Ldp2p, Cit1p, Mdh1p, Lsc2p, Mdh3p, Sdh1p, Ldp3p, Idp2p, Mls1p, Cit2p, Sdh2p, Icl1p, Cit3p, Mdh2p, Sdh4p, Sdh3p |
| Fatty acid metabolic process | Fas2p, Fas1p, Fox2p, Pox1p, Pot1p, Faa2p, Cat2p, Faa4p, Yat2p, Yat1p, Faas3p, Dci1p, Ifa38p |
| Fatty acid beta-oxidation | Fox2p, Pox1p, Mdh3p, Pot1p, Ldp3p, Tes1p, Eci1p |
| Gluconeogenesis | Pgk1p, Eno2p, Tdh2p, Fba1p, Enol1p, Tdh1p, Gpm1p, Tpi1p, Pyc2p, Mdh2p |
| Glyoxylate cycle | Idp2p, Mdh3p, Ldp3p, Icl2p, Mls1p, Cit2p, Icl1p |
| Glycolytic process | Pgk1p, Enol2p, Cdc19p, Tdh2p, Fba1p, Enol1p, Glk1p, Tdh1p, Gpm1p, Tpi1p |
| NADH oxidation | Gut2p, Adh2p, Adh1p, Ndi1p, Adh3p, Gpd1p, Nde2p, Adh5p |
| Lipid metabolic process | Fas2p, Fas1p, Fox2p, Ino1p, Pox1p, Pot1p, Faa2p, Gtp2p, Cat2p, Faas4p, Erg11p, Yat2p, Yat1p, Faas3p, Dci1p, Plb2p, Ifa38p, Tgl1p |
| Carboxylic acid metabolic process | Mdh1p, Mdh3p, Icl2p, Leu9p, Icl1p, Mdh2p |
| Amino acid catabolic process to alcohol via Ehrlich pathway | Adh2p, Adh1p, Adh3p, Adh5p |
| Malate metabolic process | Mdh1p, Mdh3p, Mae1p, Mdh2p |
| Isocitrate metabolic process | Ldp2p, Idh1p, Ldp3p, Idh2p |
| Cellular respiration | Sdh1p, Sdh2p, Sdh4p, Sdh3p |

*α=48 h of chemostat growth; log2 fold change of <-0.5; q value of <0.05.*
Levels of glycolytic enzymes measured during replicated chemostat cultivations with the initial cell clone, population 1, population 2, and population 3. Only proteins, which differ significantly between population 1 and population 2 in the beginning of the cultivations (≤48 h of chemostat growth), are pictured (q value of <0.05, log₂ fold change of >0.5). The proteomics data for the initial cell clone were obtained from Wright et al. (4).
g/L without uracil and histidine supplements was used. Each cultivation was initiated with 8-h batch phase and 52-h fed-batch phase. Cell dry weight and extracellular heterologous insulin production were measured each day as previously described by Wright et al. (4).

Duplicated cultivations with the initial cell clone were performed in a scale-down reactor system with glucose pulses. The setup of the cultivations was the same as described previously but instead of a continuous media supply, the medium was dosed in pulses. Thus, the media, which should be added to the reactor in a time interval of 84 s in order to obtain a dilution rate of $0.1 \text{ h}^{-1}$, were instead added in for 22 s followed by a 62-s pause with no medium addition.

**Fluorescent activated cell sorting (FACS).** Samples for flow cytometry analysis were collected every day and stored in glycerol at $-80^\circ\text{C}$. Prior to analysis, the samples were thawed and washed three times in PBS. Flow cytometry analysis and cell sorting with respect to particle size (FSC-A) were performed using a Sony cell sorter (SH800S). A total of 100,000 cells were analyzed in each sample using a 100-μm microfluidic sorting chip. The samples were diluted to obtain an event rate below 1,000 eps. The raw flow cytometry data (fcs files) were analyzed in the software environment R version 3.6.1 using the flowCore package (26).

Stacked density plots of the log2(FSC-A) distribution at different time points were constructed by application of the ggplot2 package in R (27). Histogram bar charts of log2(FSC-A) were constructed by sorting the cell count data into 833 uniformly sized bins using the build-in hist function in R.

**Analysis of intracellular proteins.** A minimum of four samples were withdrawn for analysis of intracellular proteins at different time points of the reinitiated chemostat cultivations of the three FACS sorted populations (see Table S4 in the supplemental materials for an overview of the different samples). The samples were stored at $-80^\circ\text{C}$ before further processing. Intracellular proteins were quantified by label-free quantification as described previously by Wright et al. (4). For an analysis of the samples, liquid chromatography was performed on a CapLC system (Thermo Fisher Scientific) coupled to an Exploris 480 mass spectrometer (Thermo Fisher Scientific). The peptides were separated with a flow rate of 1.2 μL/min on a 75-μm by 15-cm 2-μm C₁₈ easy spray column. A stepped gradient, going from 4% to 40% acetonitrile in water over 50 min, was applied. Mass spectrometry (MS)-level scans were performed with the following settings: Orbitrap resolution, 60,000; AGC target, 1.0e6; maximum injection time, 50 ms; intensity threshold, 5.0e3; and dynamic exclusion, 25 s. Data-dependent MS2 selection was performed in Top 12 mode with high-energy collisional dissociation (HCD) collision energy set to 30% (AGC target, 1.0e4; maximum injection time, 22 ms).

**Data processing of proteome data.** For analysis of the thermos rawfiles, Proteome Discover 2.3 (Thermo Fisher Scientific) was applied. The following settings were used for the analysis: fixed modifications, carboxymethyl (C); and variable modifications, oxidation of methionine residues. We used a first search mass tolerance of 20 ppm and an MS/MS tolerance of 20 ppm. Trypsin was selected as an enzyme and allowed one missed cleavage. False discovery rate was set at 0.1%. The data were searched against the S. cerevisiae database retrieved from Uniprot with proteome identifier (ID) AUP000002311 and the

**FIG 8** The adaptive outcome observed at the culture level is a mixed response of at least three apparent phenotypes. We speculate that population 1 is mainly an outcome of the selective pressure of the glucose-limited conditions, whereas population 2 is a reaction toward the protein burden of the heterologous insulin production. Population 3 seems to be a product of both the selective pressure of the heterologous insulin burden and the glucose-limited conditions. A higher resolution of the images of the three populations can be found in Fig. S21 to S26.
Batch variations between different proteome data sets were reduced by scaling each protein such that the mean log2(abundance) was the same between data sets. A differential expression analysis was performed between population 1, population 2, and population 3 for samples taken in the beginning of the cultures (≤48 h of chemostat growth) and again in the end of the cultures (after 254 h of chemostat growth). Only proteins which were measured in all samples between the compared populations were included in the analysis, meaning that 2,635 proteins were compared between population 1 and population 3, 2,811 proteins were compared between population 1 and population 2, and 2,679 proteins were compared between population 2 and population 3. The analysis was performed using the EdgeR package (28) in R version 3.6.1. The proteomes from the beginning of chemostat cultures with the three populations were furthermore compared with a previously published proteome of the initial cell clone (4). For an overview of the samples used for the comparison, see Table S5 in the supplemental material. A total of 2,716 proteins between population 1 and the initial cell clone were compared, 2,770 proteins between population 2 and the initial cell clone were compared, and 2,692 proteins were included in the comparison of population 3 and the initial cell clone.

For each comparison between two strains, proteins were grouped in clusters depending on whether the level of the proteins were higher (log2 fold change of >0.5, q value of <0.05) or lower (log2 fold change of <−0.5, q value of <0.05) in strain A than that in strain B. Gene Ontology (GO) process terms were obtained online from http://current.geneontology.org/annotations/sgd.gaf.gz on 16 November 2020. A one-sided Fisher’s exact test was used to investigate whether the protein clusters, were enriched with proteins annotated with certain GO process terms (q value of <0.05). The test was performed using the R package bc3net (29).

Microscopy. The morphologies of FACS-sorted populations were inspected visually using an LMI-005 Leica microscope and an SP8 confocal microscope.

**Determination of maximum growth rate in batch cultures.** Maximum growth rates were determined based on OD600 measurements from exponentially growing cells in 100-ml shake flasks with minimal medium without uracil and histidine supplements and with 3% (vol/vol) glucose (14). Three biological replicates were performed for each strain. Moreover, the maximum growth rates of population 1 and population 2 were determined based on cell dry weight measurements for exponentially growing cells in 2L bioreactors with minimal medium without uracil and histidine supplements (see Fig. S1 in the supplemental material). To determine whether the growth rates differed between the populations, a two-sided t test was performed using the scipy.stats package in Python (30).

Genomic extraction was performed using a QIAamp DNA blood minikit. Plasmid copy numbers relative to the housekeeping gene ACT1 were determined by quantitative PCR (qPCR) using an Agilent Technologies Stratagene Mx3005P instrument. Each sample of template DNA was diluted to a concentration of 20 ng/µL resulting in a final PCR concentration of 1 ng/µL. The PCR mixture consisted of 1 µL template DNA, 1 µL forward primer, 1 µL reverse primer, 10 µL SYBR master mix, and 7 µL Milli-Q H2O (see Table S2 and S3 in the supplemental materials for the applied primers and thermal profile for the qPCR). The plasmid copy numbers relative to the housekeeping gene are reported as the log fold change to a calibrator sample taken from one of the reinitiated chemostat cultures of population 1 after 24 h of cultivation. To determine whether the relative plasmid copy number differs between the two populations, a two-sided t test was applied on all the analyzed samples, using the scipy.stats package in Python (30).

**Data availability.** The proteomics data generated in this work has been deposited online to https://doi.org/10.11583/DTU.17080931.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**

PDF file, 3.1 MB.

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N.R.W. and N.P.R. were employed by the company Novo Nordisk A/S. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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