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Adaptive responses of genome-reduced *Bacillus subtilis* during enzyme secretion in a breathing vessel culture

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Abstract

Background The utilisation of microbes for the production of enzymes and pharmaceutical proteins is an important step towards a sustainable future. However, the energy requirement for agitation, aeration and cooling during industrial fermentation processes is substantial. To address this challenge, we have previously developed a 'breathing' polytetrafluoroethylene fermenter vessel that allows effective gas exchange with ambient air and, thus, does not require sparger aeration. The present study aimed to explore the potential application of the breathing vessel for enzyme production by the Gram-positive bacterial cell factory *Bacillus subtilis*. Here, we compared production of the secreted α-amylase AmyQ by the genome-reduced multiple protease-deficient *B. subtilis* strain IIG-Bs-27-31 during parallel culturing in breathing vessels and shake flasks. Enzyme yields and the cellular and extracellular proteome compositions were assessed.

Results We observed comparable growth characteristics and AmyQ yields per liter in both culture systems. However, proteome analyses indicated statistically significant differences ($p < 0.05$, $\log_2$fold change>|0.5|) in the utilization of carbon sources and stress responses between cells grown in the breathing vessels versus shake flasks. In particular, bacteria in the breathing vessel presented activation of the Sigma B-dependent general stress response, presumably to sustain bacterial growth and viability. In contrast, bacteria grown in shake flask presented activated cell envelope stress pathways and typical sheer stress symptoms.

Conclusions While the overall AmyQ yields per liter were similar in both fermentation systems, the total enzyme yields in the breathing fermenter were significantly higher due to the 15-fold increase in culture volume. Our findings imply that breathing fermentation vessels are suitable for *B. subtilis* enzyme production and, upon further scale-up, they may represent sustainable and cost-effective alternatives to traditional fermentation systems for microbial cell factories.

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Keywords *Bacillus subtilis*, 'breathing' fermenter, Industrial enzyme production, Proteomics, Genome reduction, Stress response

Background

The aerobic soil bacterium *Bacillus subtilis* has become a model organism for studies on Gram-positive bacteria, ever since it was identified over 7 decades ago [1]. At the same time, it was developed into a popular workhorse for industrial protein production thanks to its high secretory capacity, the 'generally recognised as safe' (GRAS) status given to many of its products by the United States Food and Drug Administration (FDA), and the wide range of possibilities to genetically engineer this organism. The study of *B. subtilis* is nowadays supported by effective tools, such as the SubtiWiki database, which provides easy access to the well-annotated genome of the *B. subtilis* type strain 168, as well as this organism's gene regulatory circuits and metabolic pathways [2, 3]. Considerable effort has been put into improving *B. subtilis* to obtain higher production yields, as exemplified by the removal of multiple protease-encoding genes and, more recently, systematic genome-reduction [4–9]. In recent years, a 'cloud' of genome-reduced strains has thus been generated and, subsequently, several of these strains have been investigated in terms of recombinant protein production, stress responses and metabolic changes [10–17]. Particularly, intermediate strains of the genome-reduced 'mini- and midi*Bacillus*' phylogeny have shown promising results when it comes to lowered stress responses during recombinant protein production and reduced product degradation [11]. Interestingly, these strains lack not only multiple extracellular proteases, but also the intracellular protease *aprX*, which sets major limits to the degradation of difficult-to-produce proteins [10].

Utilizing microbes for enzyme and pharmaceutical protein production has been an important step towards a more sustainable future [18]. However, industrial fermentation processes require energy for agitation, aeration and cooling. Especially while the cell mass increases, oxygen transfer to the growth medium can become an increasingly limiting factor for microbial growth and product formation [19]. To address this limitation, fermentation strategies such as enhanced aeration, higher agitation speeds and increased pressure can be applied [20]. However, this also comes at the expense of increased energy consumption. To tackle this challenge, we have recently developed a so-called 'breathing' fermentation system that supports effective growth of *B. subtilis* [21]. This novel system features a fermentation vessel made from expanded polytetrafluoroethylene (ePTFE) that facilitates effective gas exchange [22]. Based on our previous observations, we hypothesized that the breathing fermentation vessel is potentially attractive for enzyme production. Accordingly, our present study was aimed at: (i) obtaining proof-of-principle for this hypothesis through comparative analysis of the production of an industrially relevant enzyme in the breathing vessel and shake flasks, and (ii) defining the stress responses and metabolic adaptations in bacteria grown in the breathing vessel.

The α-amylase AmyQ from *Bacillus amyloliquefaciens* was selected as a representative secreted protein of industrial relevance, because it was used as a model protein in various previous studies involving both conventional batch fermentation systems and shake flasks [23–29]. Furthermore, we selected the genome-reduced *B. subtilis* strain IIG-Bs-27-31 as expression host for AmyQ, because it shows substantially reduced product degradation [10, 16], and because it grows well both in shake flaks and the breathing fermenter. Lastly, a proteomics analysis was applied to detect any differential stress responses, as well as gene regulatory and metabolic adaptations. This is the first study that explores the combined use of a genome-reduced *B. subtilis* strain and a breathing fermenter with a passive aeration system for industrial enzyme production.

Methods

Bacterial strains

In this study, the genome-reduced *B. subtilis* strain IIG-Bs-27-31 was used, which is a derivative of *B. subtilis* strain 168. The genome size of IIG-Bs-27-31 is 3.41 Mb and, compared to 168, it lacks 19.13% of the genome [10]. For production of the α-amylase AmyQ of *B. amyloliquefaciens*, plasmid pKTH10 was used [30]. This plasmid is a derivative of pUB110 and carries a kanamycin resistance marker.

Growth conditions

B. subtilis was routinely grown at 30 °C in Lysogeny Broth (LB; BD Difco, USA) supplemented with 10 µg/ml kanamycin using baffled shake flasks and shaking at 180 revolutions per min (rpm). Fermentation medium (FM), consisting of 2% yeast extract, 2.5% peptone, 1% $NaH_2PO_4.H_2O$, 1% $Na_2HPO_4.H_2O$ and 0.5% potato dextrose (Carl Roth GmbH, Karlsruhe, Germany), was used for comparative studies in shake flasks and the breathing fermenter vessel. The respective pre-cultures were started by inoculation of 9 mL of FM with 1 mL of an overnight culture in LB. The pre-cultures were then incubated overnight at 37 °C with shaking at 180 rpm as previously described [24].

Shake flask cultures

The main shake flask cultures were started by inoculation of 100 mL FM in 1 L baffled shake flasks with pre-cultures in FM to a final optical density at 600 nm (OD_{600}) of 0.05. Incubation was carried out at 37 °C with shaking at 180 rpm.

Fermentation

Fermentations using the breathing vessel set-up were performed as previously described [21]. A schematic representation of the setup is shown in Fig. 1. Briefly, the fermenter was loaded with 1.5 L FM and, prior to inoculation, it was run overnight to verify the absence of contaminating microorganisms. On the next day, the fermenter was inoculated with the pre-culture in FM to a final OD_{600} of 0.05. Fermentations were performed at 37 °C with stirring at 800 rpm for up to 48 h.

Sampling overview

The present study included analyses of the culture OD_{600} and pH, analyses of AmyQ production levels by Western blotting and α-amylase activity measurements, and genome-wide protein expression analyses by mass spectrometry (MS). To this end, samples were collected at different time points during the breathing fermenter and shake flask cultivations. An overview of the sampling time points and the subsequently performed analyses is presented in Table 1.

Western blotting

Samples were collected from shake flasks or fermenter runs and standardized to an OD_{600} of 2. Subsequently, the samples were centrifuged to separate cells from the culture medium. Cell pellets were resuspended in 100 µL of lysis solution consisting of 100 mM Tris-HCl (pH 8.0), 1 M KCl, 200 mM EDTA (pH 8.0), 1 mg/mL lysozyme

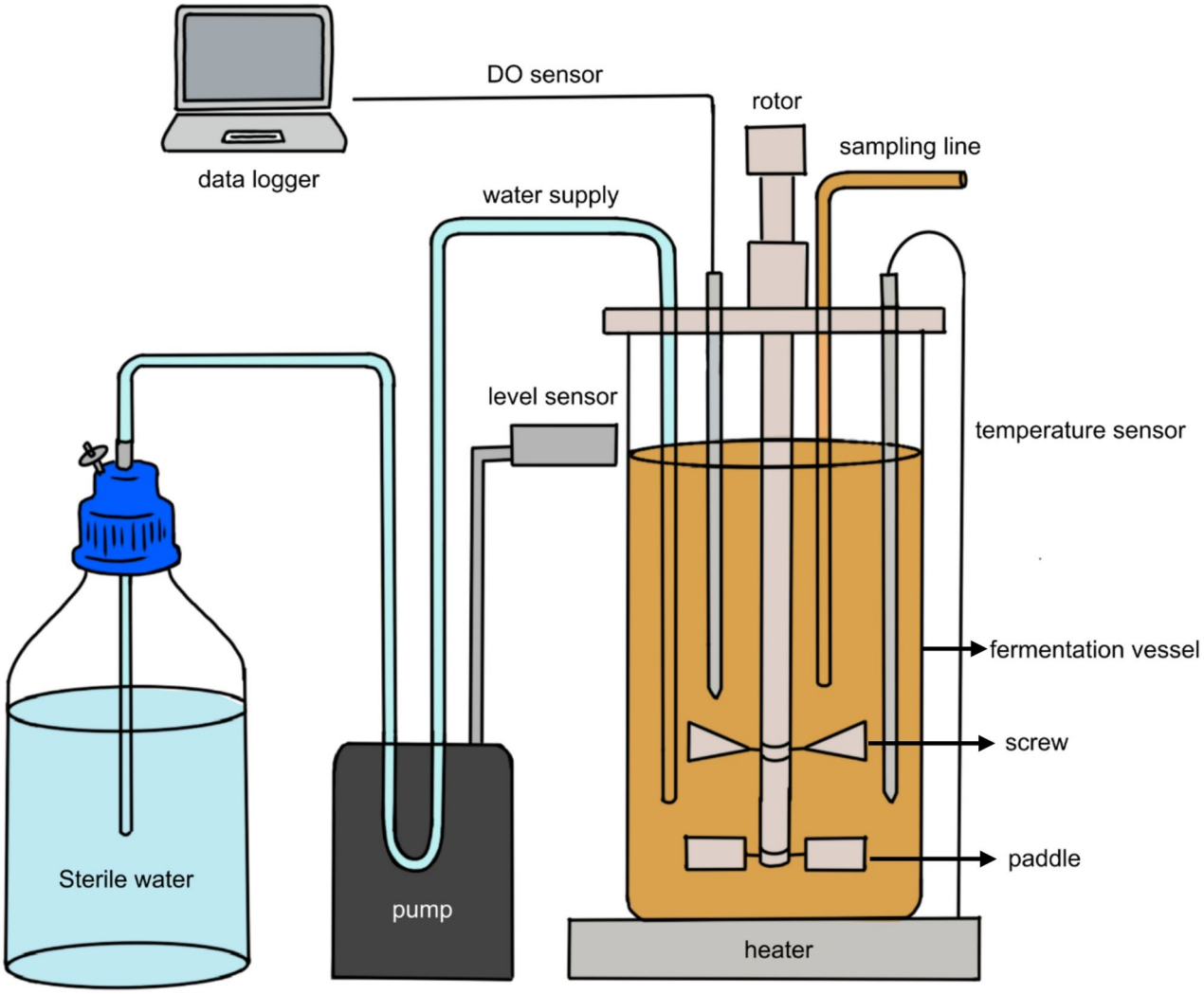

Fig. 1 Schematic representation of the 'breathing' fermenter setup

Table 1 Sampling overview

Time Point (h)	OD600	pH	Western blot	Amylase activity	Proteomics
0	☑	☑	☐	☐	☐
1	☑	☑	☐	☐	☐
2	☑	☑	☐	☐	☐
3	☑	☑	☐	☐	☐
4	☑	☑	☑	☐	☐
5	☑	☑	☐	☐	☐
6	☑	☑	☐	☐	☐
7	☑	☑	☐	☐	☐
8	☑	☑	☑	☑	☑
9	☑	☑	☐	☐	☐
10	☑	☑	☐	☐	☐
11	☑	☑	☐	☐	☐
12	☑	☑	☑	☐	☐
24	☑	☑	☑	☑	☑
28	☑	☑	☑	☐	☐
32	☑	☑	☑	☑	☑
36	☑	☑	☑	☐	☐
48	☑	☑	☑	☐	☐

Time points at which particular samples were collected and analysed are indicated with a check mark

and 1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated at 37 °C for 10 min. After the incubation, the cells were sonicated for 5 min with cycles of 2 s sonication and 8 s intervals. 25 µL of 5x sodium dodecyl sulphate (SDS) buffer consisting of 250 mM Tris-HCl (pH 6.8), 8% SDS, 0.1% bromophenol blue, 40% glycerol and 100 mM DTT were added to each tube and the tubes were incubated at 95 °C for 10 min. Proteins in the growth medium fractions (corrected for an OD_{600} of 2 as indicated above) were concentrated using an Amicon Ultra Centrifugal Filter Unit with 3 kDa molecular weight cut-off (MWCO; Merck KGaA, Darmstadt, Germany). After filtration, 5x SDS buffer was added to each tube and the tubes were incubated at 95 °C for 10 min.

SDS-PAGE gels were prepared in two steps. First a separating gel was cast by combining 5 mL deionized H_2O, 3.8 mL 1.5 M Tris-HCl (pH 8.8), 0.15 mL 10% SDS, 6 mL 30% Acrylamide/Bis Mixed Solution (29:1) (Nacalai Tesque Inc., Kyoto, Japan), 0.15 mL 10% ammoniumpersulfphate (APS) and 0.006 mL tetramethylethylenediamine (TEMED). Once the separating gel had solidified, a stacking gel was cast on top of it by combining 4.1 mL deionized H_2O, 0.75 mL 1 M Tris-HCl (pH 6.8), 0.06 mL 10% SDS, 1 mL 30% Acrylamide/Bis Mixed Solution (29:1) (Nacalai Tesque Inc., Kyoto, Japan), 0.06 mL 10% APS and 0.006 mL TEMED. For SDS-PAGE, equal sample volumes were loaded on the gel and electrophoresis was carried out at 40 mA, 300 V for 2 h.

After the protein separation by SDS-PAGE, Western blotting was conducted. First, polyvinylidene fluoride (PVDF) membranes were soaked in methanol. Then blotting buffer was prepared, which consisted of 100 mL 10x Tris-glycine buffer, 700 mL deionized H_2O and 200 mL methanol. Filter papers and the activated PVDF membranes were immersed in the blotting buffer for 30 min. Proteins were transferred to the PVDF membranes by semi-dry blotting at 15 V, 297 mA for 1 h. After the transfer, the membranes were blocked with TBS-T with 5% skim milk. TBS-T consisted of 675 mL deionized H_2O, 75 mL 10x TBS (pH 7.6) and 750 µL Tween20. 10x TBS consisted of 24 g Tris and 88 g NaCl in 1 L of deionized H_2O. To visualize the presence of AmyQ or TrxA, the membranes were incubated with specific polyclonal rabbit antibodies (1:5,000) and incubated for 1 h at room temperature. Subsequently, the membranes were washed with TBS-T and incubated with a secondary mouse-anti rabbit IgG horseradish peroxidase conjugate (1:1,000) (Cosmo Bio Co., LTD, Tokyo, Japan) for 1 h. The membranes were then washed three times with TBS-T. For visualization of bound antibodies, Chemi-Lumi One L (Nacalai Tesque Inc., Kyoto, Japan) reagents were mixed at a 1:1 ratio and incubated with the membrane. The chemiluminescence of the membrane was recorded

immediately using a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

α-Amylase activity assay

α-Amylase activity was measured using Ethylidene-4-Nitrophenyl-aD-Maltoheptaoside (ethylidene–[pNP]-G7, EPS) as the substrate. First, tubes containing 500 µL of EPS solution and 500 µL of enzyme solution consisting of glucoamylase and α-glucosidase were incubated at 37 °C for 5 min. 100 µL of growth medium fraction was added to the mix and the tubes were incubated for exactly 10 min at 37 °C. 2 mL of the stop solution containing 1.0 M sodium carbonate was added to each tube to stop the reaction. The absorbance of the samples was measured at 400 nm (Es). For the blank measurement, the reaction solution was preheated at 37 °C for 15 min and 2 mL stop solution containing 1.0 M sodium carbonate was added. Lastly, 100 µL growth medium fraction was added and the absorbance was measured at 400 nm (Eb). The amylase activity (U/mL) was calculated as follows:

$$\frac{(\mathrm{Es}-\mathrm{Eb})\times 3.1\ \mathrm{mL\ (Total\ Volume)}\times \mathrm{Dilution\ factor}}{18.1\ \mathrm{(Molar\ Absorptivity\ Coefficient\ of\ PNP)}\times 0.1\ \mathrm{mL\ (Sample\ Volume)}\times 10\ \mathrm{(Measurement\ Time\ (min))}}$$

Here, 1 U of α-amylase activity is defined as the amount of enzyme that releases 1 µmol of PNP per min from the EPS.

To calculate the specific α-amylase activity, the total protein concentration in each sample was measured using the bicinchoninic acid (BCA) assay (Sigma-Aldrich, Burlington, MA). Briefly, BCA Reagent A containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide was mixed with BCA Reagent B containing 4% cupric sulfate. A 100 µL aliquot of the growth medium fraction was added to 2 mL of the mixture and incubated at 37 °C for 37 min. After cooling the absorbance was measured at 562 nm. The protein concentration in the sample was determined according to a calibration curve that was prepared with bovine serum albumin (BSA) standards. The specific secreted α-amylase activity (U/mg protein) was calculated by dividing the α-amylase activity by the total extracellular protein concentration.

Sample preparation for mass spectrometry

For MS analyses, cell pellets and growth medium samples collected from 4 independent shake flask or fermenter cultures were prepared as described for the Western blotting experiments except that the addition of 5x SDS buffer was omitted. For the collection of proteins from growth medium fractions, StrataClean beads (Agilent, Santa Clara, USA) were used as described previously [31]. To this end, the concentrated samples were adjusted to equal volumes with 50 mM Tris-HCl (pH 8.0). StrataClean beads were prepared by incubating 20 µL of beads suspension with 37% HCl at 100 °C for 6 h. Subsequently, the tubes with the beads were centrifuged at 3,500 x g for 5 min at room temperature, the supernatant was discarded, and the beads were washed twice with 200 µL of 50 mM Tris-HCl (pH 8.0). The washed beads were then mixed with the samples and incubated overnight at 4 °C on an overhead shaker. Next day, the tubes were centrifuged at 10,000 x g for 45 min at 4 °C and the supernatant fraction was discarded. Lastly, the beads with bound proteins were washed with 1 mL of distilled water and centrifuged at 20,000 x g for 5 min. The supernatant was discarded, and the beads were dried in a vacuum centrifuge for 20 min.

Beads were incubated for 10 min at 95 °C with 20 µL of Laemmli sample buffer, consisting of 125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 3.75% β-mercaptoethanol, 20 mM DTT and 0.04% bromophenol blue. The resulting samples were loaded onto 4–20% Mini-PROTEAN TGX precast protein gels (Bio-Rad, Hercules, CA, USA) for electroelution of the bound proteins at 180 V in 15 min. Once the proteins had run for ± 1 cm per lane, the electrophoresis was stopped, and the gels were stained with Coomassie brilliant blue dye. The individual protein-containing gel lanes were excised from the gels, cut into smaller pieces (2 × 2 mm), destained and treated with trypsin overnight as previously described [31]. The resulting peptides were eluted with ultrapure water by sonication and desalted using U-C18 Zip Tips (Merck Millipore, MA, Burlington, USA) according to the manufacturer´s instructions.

Mass spectrometry

Peptides were separated on an Easy nLC 1200 coupled online to an Orbitrap Elite mass spectrometer (Thermo Scientific, CA, USA). In-house self-packed columns (i.d. 100 µm, o.d. 360 µm, length 20 cm) containing 3 µm Reprosil C18 reversed-phase material (ReproSil-Pur 120 C18-AQ, Dr. Maisch HPLC GmbH, Ammerbuch-Entingen, Germany) were loaded with peptides in solvent A (0.1% [v/v] acetic acid) at a maximum pressure of 400 bar. To identify proteins from the growth medium samples, the elution of peptides was performed with a nonlinear 100 min gradient from 1 to 99% solvent B (0.1% v/v acetic acid in 95% [v/v] acetonitrile) at a constant flow rate of 300 nL/min at 45 °C. To identify cellular proteins, a 180 min gradient was used for peptide elution.

MS-spectra of the eluting peptides were recorded at a resolution of $R = 60{,}000$ with lockmass correction activated. Up to 20 dependent scans (MS/MS) according to precursor intensity were performed in the linear ion trap after collision-induced dissociation fragmentation (CID). Dynamic exclusion was enabled.

Data analysis

The raw MS data was searched in MaxQuant (version 2.4.13.0) using the *B. subtilis* SubtiWiki proteome annotation for the genome reduced strain IlG-Bs27-47-31 (downloaded on 27/02/2024) with manually added sequences for AmyQ and the pKTH10-encoded kanamycin resistance protein (3,464 entries in total). Common laboratory contaminants and reverse entries were added by MaxQuant. The following parameters were used for database searches: peptide tolerance, 4.5 ppm; primary digest reagent, trypsin; oxidation M (+ 15.9949) and acetylation N, K (+ 42.0106) as variable modifications. Match between runs was enabled with default settings. Results were filtered using a 1% false discovery rate on spectrum, peptide and protein levels. A minimum of two unique peptides per protein were a prerequisite for identification (Supplementary Table S1). LFQ values were used as proxy for protein abundance [32].

Data analysis was performed using Perseus 2.0.11 and Python 3.11. Data cleaning and manipulation was performed using the Pandas (https://pandas.pydata.org/) and Numpy (https://numpy.org/) libraries. Data visualization was performed using the Matplotlib (https://matplotlib.org/) and Seaborn (https://seaborn.pydata.org/) packages. For enrichment analysis, the Fisher exact test from the Scipy library (https://scipy.org/) was used. Functional categories and regulon data were obtained from SubtiWiki [2].

Results and discussion

Growth comparison of the genome-reduced *B. subtilis* strain IIG-Bs-27-31 during shake flask versus breathing vessel cultivation

To investigate the growth behaviour of *B. subtilis* strain IIG-Bs27-31 carrying the AmyQ-encoding plasmid pKTH10 in the breathing vessel, it was cultured for 48 h using 'fermentation medium' (FM) and the OD_{600} was monitored at hourly intervals. We decided to continue the culturing for 48 h to assess whether the cells might be prone to lysis at late time points of culturing, which is sometimes observed with *B. subtilis*. For comparison, the same strain was cultured in 1 L baffled shake flasks using the same FM medium. This revealed minor differences in the growth of the bacteria in the two different culture systems (Fig. 2). While the shake flask cultures reached higher OD_{600} values during the first ± 8 h post inoculation, the breathing vessel cultures showed

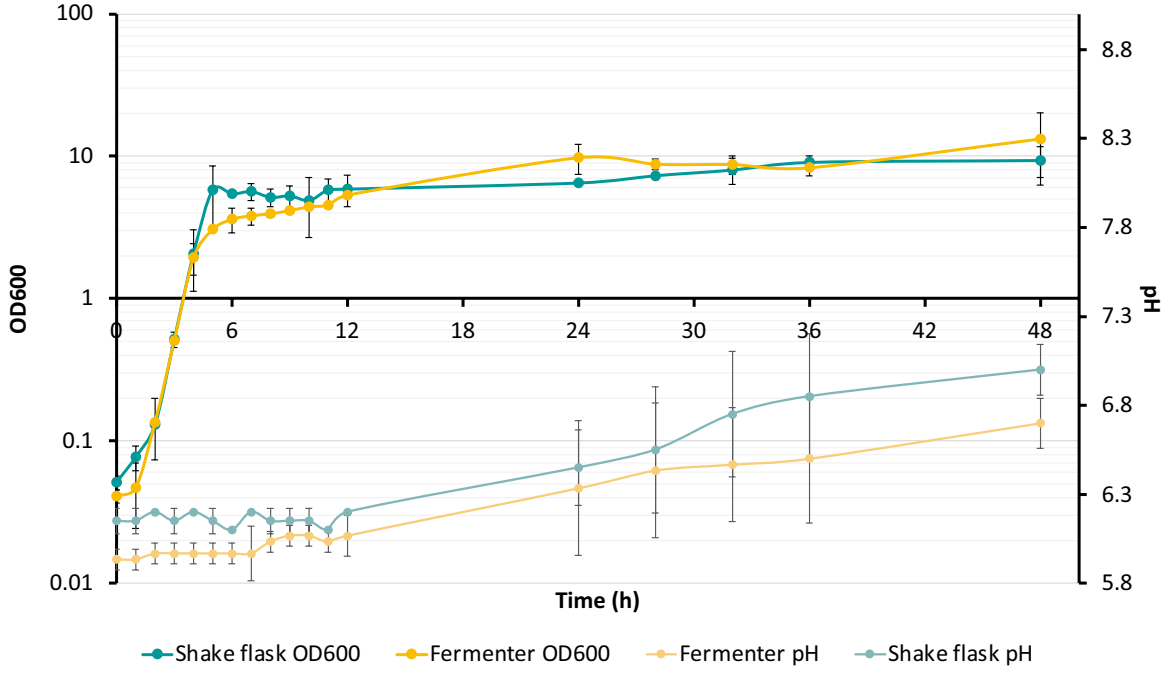

Fig. 2 Comparison of the growth of *B. subtilis* IIG-Bs27-31 pKTH10 in shake flasks and the breathing fermenter. Cultures of *B. subtilis* strain IIG-Bs27-31 pKTH10 in shake flasks (100 mL FM medium) and breathing vessels (1.5 L FM medium) were carried out at 37 °C with, respectively, 180 rpm shaking or 800 rpm mechanical stirring. Both types of cultures were inoculated with pre-cultures in FM medium to a starting OD_{600} of 0.05. OD_{600} values are represented in the primary y-axis and pH values are represented in the secondary y-axis. Data points and error bars indicate the mean values and standard deviations (SD) from 3 independent experiments

continued growth up until ± 14 h post inoculation and reached a higher maximum OD_{600}. After 36 h of culturing the OD_{600} values of the shake flask and breathing fermenter cultures were about the same, and none of the cultures showed signs of bacterial lysis up until 48 h of culturing. Nonetheless, we observed a trend towards differences in the pH of the cultures in the breathing vessel and the shake flask cultures once the 'stationary' growth stage was reached, where the pH in the shake flasks was slightly higher (Fig. 2). Since this trend was consistently observed, we concluded that the physiological processes in the respective cells differed, possibly due to slight differences in the carbon source that was consumed by the bacteria. For instance, if amino acids or proteins are used as the preferred carbon source, more ammonia will be produced which will lead to an increase of the pH. Thus, the slightly higher pH values measured in the shake flasks may be regarded as an indication that proteins and amino acids were preferentially utilized as carbon source by the bacteria growing in shake flasks and/or that sugars could be the preferred carbon source for bacteria growing in the breathing fermenter setup. Here it should be noted that for comparability of the breathing fermentation system and the shake flasks, we did not implement pH control systems, which had the advantage that we could directly measure pH fluctuations in both culture systems.

AmyQ production in different cultivation systems

Previous batch fermentations with an engineered *B. subtilis* strain 168 showed that AmyQ production was evident as early as 3 h post inoculation in FM medium [24]. Accordingly, in our current study, effective secretion of AmyQ was observed from ± 4 h post-inoculation both in the breathing fermenter and shake flasks (Fig. 3), which corresponds to the late-exponential growth phase (Fig. 2). Furthermore, as shown by Western blotting, in both cultivation systems the secreted AmyQ levels showed a substantial increase after 24 h of culturing. Yet, in the breathing fermenter, the extracellular AmyQ levels were slightly reduced after 48 h of culturing (Fig. 3, bottom panel). The latter may be a result of proteolytic degradation caused by residual protease activities of the IIG-Bs27-31 strain [10, 33]. While the data in Fig. 3 seem to suggest that higher AmyQ secretion levels were reached in the breathing fermenter compared to the shake flasks, this turned out to be a trend rather than a systematic feature of the breathing fermenter setup. Nonetheless, while the secreted AmyQ levels obtained in the breathing fermenter were in some experiments comparable to those obtained in the shake flasks, in none of our experiments the AmyQ levels achieved in the breathing fermenter were lower than in the shake flasks. Overall, the extracellular AmyQ levels reached a maximum after ± 32 h of culturing.

Based on our Western blotting results, as shown in Fig. 3, for all further investigations samples were collected at 8, 24 and 32 h post inoculation, here respectively defined as the early-, mid- and late-stationary growth phases. To independently verify the results obtained by Western blotting, we also measured the α-amylase enzymatic activity in samples that were collected from the breathing fermenter and shake flask cultivations. As shown in Fig. 4, comparable levels of AmyQ activity were observed at the 8, 24 and 32 h time points post-inoculation, although they trended to be slightly lower at 24 h in the breathing fermenter samples. Altogether, we

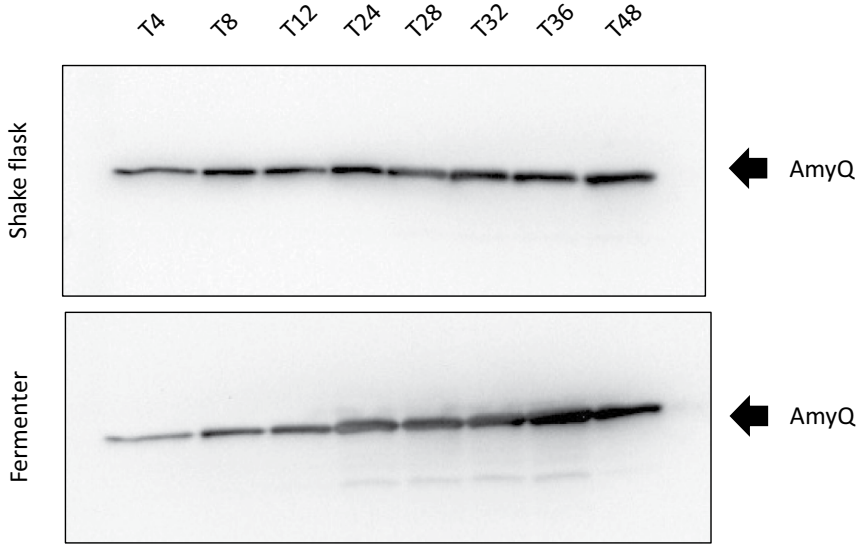

Fig. 3 Extracellular AmyQ levels during culturing in shake flask or the breathing fermenter. *B. subtilis* strain IIG-Bs27-31 carrying pKTH10 was grown on FM medium in shake flask or the breathing fermenter for 48 h. Samples were collected at the indicated time points and standardized at OD_{600} of 1. AmyQ levels were assessed by Western blotting with AmyQ-specific polyclonal antibodies. The experiment was performed in triplicate

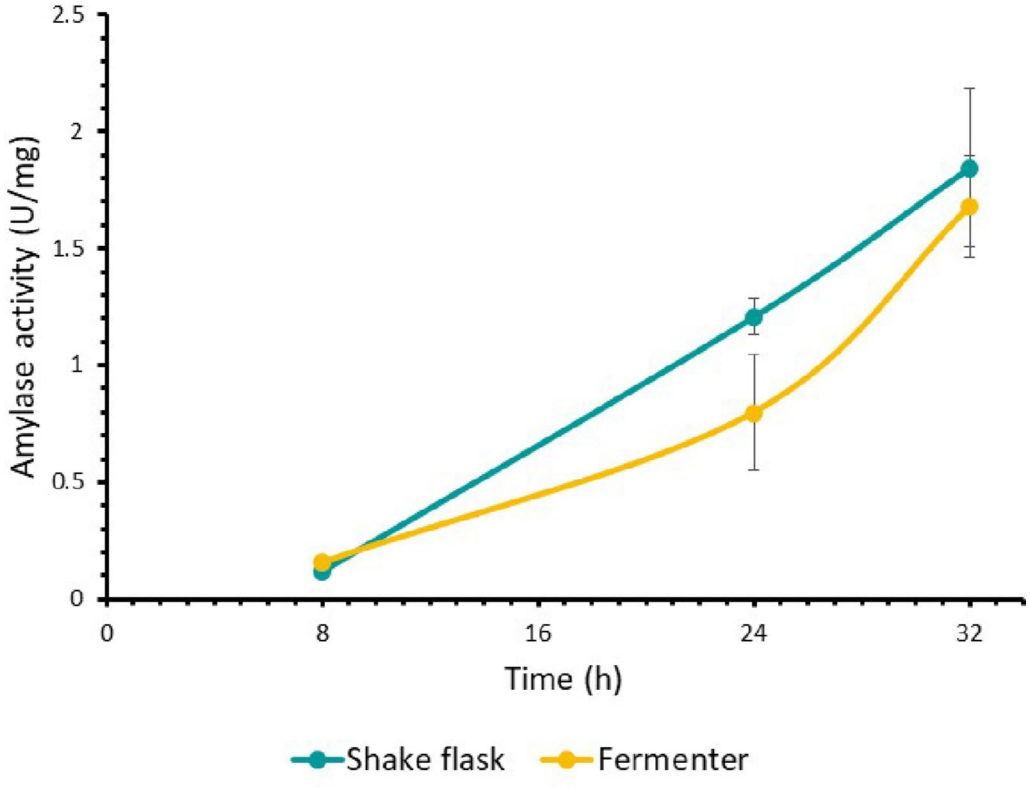

Fig. 4 Specific AmyQ activity levels during shake flask or breathing fermenter cultivation. *B. subtilis* strain IIG-Bs27-31 carrying pKTH10 was grown on FM medium in shake flasks or the breathing fermenter for 32 h and samples were collected at 8, 24 and 32 h post inoculation for α-for amylase activity assays. α-Amylase activity was measured using EPS as the substrate. 1 U of α-amylase is defined as the amount of enzyme that releases 1 µmol of PNP per min from EPS. The specific amylase activity (U/mg protein) was calculated by dividing the amylase activity by the protein concentration. Data points and error bars indicate the mean values and standard deviations (SD) from 3 independent experiments

conclude from the Western blotting and AmyQ activity measurements that the production of secreted AmyQ did not differ significantly in both systems. However, the overall yields in the breathing fermenter were significantly higher in view of the higher culture volume of 1.5 L compared to the 100 mL culture volume of the shake flasks.

Proteome analyses

To gain insights into potentially different cellular stress responses and metabolic adaptations when the bacteria were grown in the breathing vessel or in shake flasks, a detailed proteome analysis by MS was performed on cells and culture supernatant samples collected over time from 4 independent cultures. Specifically, samples were collected during the early- (T8), mid- (T24) and late-stationary (T32) phases to assess stress responses and metabolic adaptations during stages where AmyQ was actively produced (Fig. 4), while growth (rate-)dependent proteomic signatures that can potentially overshadow any differences arising from AmyQ production stress would presumably be minimal (Fig. 2). As a first approach, we inspected the presence of AmyQ in the bacterial cells and in the culture supernatants. Consistent with the results of our Western blotting and AmyQ activity analyses, per time point of sampling post inoculation, we identified comparable levels of AmyQ in the culture supernatants of the breathing fermenter and shake flasks (Supplementary Table S1; Fig. 5b, d, f). Intriguingly, however, especially at T8 and T24 the cell-associated AmyQ levels were significantly higher in the bacterial cells grown in the fermenter than in the bacterial cells harvested from shake flasks (Fig. 5a, c and e; indicated in bold lettering). This higher abundance of AmyQ in the bacterial cells indicates that the breathing fermenter system supports higher production levels of AmyQ than the shake flasks. However, since similar levels of AmyQ were detected in the culture supernatants, this suggests that the rate of AmyQ synthesis outcompeted the rate of AmyQ secretion in bacteria grown in the breathing fermenter. To investigate the possible cause(s) of this finding, we first inspected the levels of identified proteins that are required for protein secretion. These included the signal recognition particle (SRP) and SRP receptor proteins Ffh and FtsY, the translocation motor proteins SecA and SecDF, the translocation channel protein SecY, the signal peptidases SipS, SipT and

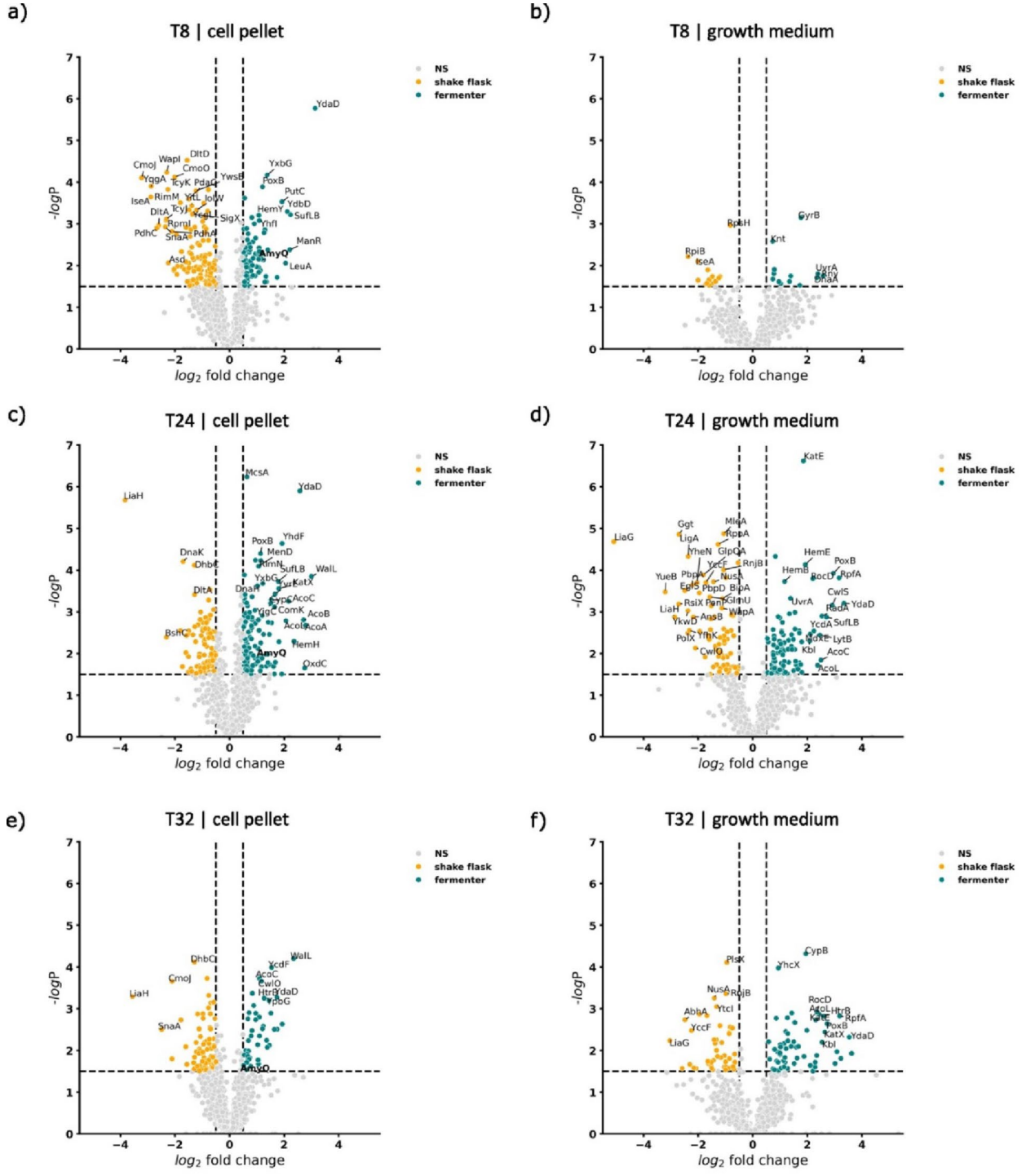

Fig. 5 Volcano plots comparing the cellular and extracellular protein abundances in shake flasks versus the breathing fermenter. Proteins that were identified at significantly higher abundance in bacterial and growth medium samples from shake flask cultivation are indicated in orange, and proteins that were significantly more abundant in the respective samples from breathing fermenter cultivation are indicated in green ($p < 0.05$ and $\log_2$fold change>|0.5|). Data from 4 independent experiments are presented

SipV, and the extracytoplasmic protein folding catalyst PrsA [34]. Intriguingly, at all time points of sampling, the Ffh, SecA and PrsA proteins were slightly more abundantly present in cells grown in the breathing fermenter (Supplementary Table S1). This suggests that the targeting of secretory proteins to the membrane, translocation across the membrane and post-translocational protein folding were probably not impaired in bacteria growing

in the breathing fermenter, despite the fact that they accumulated AmyQ. On the other hand, in cells from the breathing fermenter, the levels of the signal peptidase SipS were significantly reduced, suggesting that the processing of translocated precursor proteins was a limiting step in the secretion of AmyQ. This is in line with previous observations showing that SipS can be a limiting factor in protein secretion in *B. subtilis* [35–37].

We also examined whether some of the residual cell-associated and/or extracellular proteases of *B. subtilis* IIG-Bs-27-31 were more abundant in samples from the breathing fermenters compared to samples from the shake flasks, in order to assess whether any translocated AmyQ might have been lost due to proteolysis by bacteria cultured in the breathing fermenters. This revealed that several cytoplasmic proteases were upregulated in cells from the breathing fermenter, including the ClpP protease and the associated protein unfoldase ClpC, the ClpQ protease and the associated unfoldase ClpY, as well as the LonA protease (Supplementary Table S1) [38]. Here it was noteworthy that ClpQ and ClpY were more strongly upregulated than ClpP, suggesting an enhanced requirement for protein unfolding activity rather than protein degradation. Intriguingly, a previous study showed that inactivation of LonA enhanced the secretion of the xylanase XynA and the α-amylase AmyM [39]. Thus, the enhanced level of LonA might also be counterproductive for the secretion of AmyQ, but this would impact the extracellular levels of AmyQ indirectly rather than directly. Another protease that was seemingly present at elevated levels in bacteria grown in the breathing fermenter compared to bacteria grown in shake flasks was the membrane-associated quality control protease FtsH. This was inferred from the levels of this protein in the respective culture supernatant samples, especially at T8 (Supplementary Table S1). However, while FtsH has been implicated in the turnover of SecY, the SecY levels in cells from the breathing fermenter were unaffected, and FtsH has not been implicated directly in protein secretion [40]. Lastly, the quality control proteases HtrA and HtrB, which both have dual extracytoplasmic protease and chaperone functions [24, 41], were detected at significantly elevated levels in culture supernatant samples from the breathing fermenter at T24 and T32 compared to culture supernatant samples from shake flasks collected at these time points. In addition, HtrB was detected at elevated levels in the respective cells especially at T32 (Supplementary Table S1; Fig. 5f). This is indicative of a so-called secretion stress response triggered by unfolded or misfolded AmyQ at the membrane-cell wall interface [23, 24, 27–29], and it suggests that in the breathing fermenter some of the translocated AmyQ may have been degraded by these quality control proteases. Thus, it seems that relatively low levels of the signal peptidase SipS and relatively high levels of the proteases LonA, FtsH, HtrA and HtrB may have set limits to AmyQ secretion by bacteria grown in the breathing fermenter and to AmyQ accumulation in the respective culture supernatant.

Stress responses and stress management

To obtain a clear overview of pathways or regulons that are represented at elevated or lowered levels in one or more of the tested conditions, we performed enrichment analyses on the identified cellular (Fig. 6) and extracellular proteins (Fig. 7) based on functional categories. In addition, we performed a regulon-based enrichment analysis on the identified cellular proteins (Fig. 8). Only proteins with statistically significant different abundances between conditions were included in these analyses ($p < 0.05$ and $\log_2$fold change>|0.5|).

The regulon-based enrichment analysis highlighted the afore-mentioned secretion stress response involving induction of the quality control proteases HtrA and HtrB. This is, in fact, consistent with the observation that cells grown in the breathing fermenter displayed a significantly higher abundance of cell-associated AmyQ, which may have been sensed by the CssRS two-component regulatory system that controls *htrA* and *htrB* gene expression in order to manage cell envelope integrity [27, 42]. If so, this would mean that at least a part of the cell-associated AmyQ accumulates at the membrane-cell wall interface where it can generate secretion stress stimuli that are sensed by the CssS sensor protein, and which are then transmitted to the CssR response regulator leading to enhanced *htrA* and *htrB* expression [23, 24, 27–29]. In addition, the cells grown in the fermenter displayed enrichment of protein quality control pathways, but only at the late stationary phase (T32) time point (Fig. 6f). Likewise, in culture supernatant fractions collected from the breathing fermenters at the late stationary phase (T32), we noticed significant enrichment of proteins associated with the sigma factor B (SigB)-dependent general stress response of *B. subtilis* and functional categories of heat shock and protein quality control (Fig. 7d). Notably, analysis of cells grown in the fermenter and sampled at earlier time points already revealed enrichment in SigB-regulated general stress proteins, such as YdaD (Fig. 5). These observations are suggestive of SigB-mediated silencing of alternative developmental programs in order to manage exogenous stress and starvation stimuli and to enable the growth and survival of vegetative *B. subtilis* cells [43].

Cell envelope biogenesis

The proteomics data revealed that cells grown in shake flasks were enriched for proteins encoded by several regulons related to membrane and cell wall biogenesis.

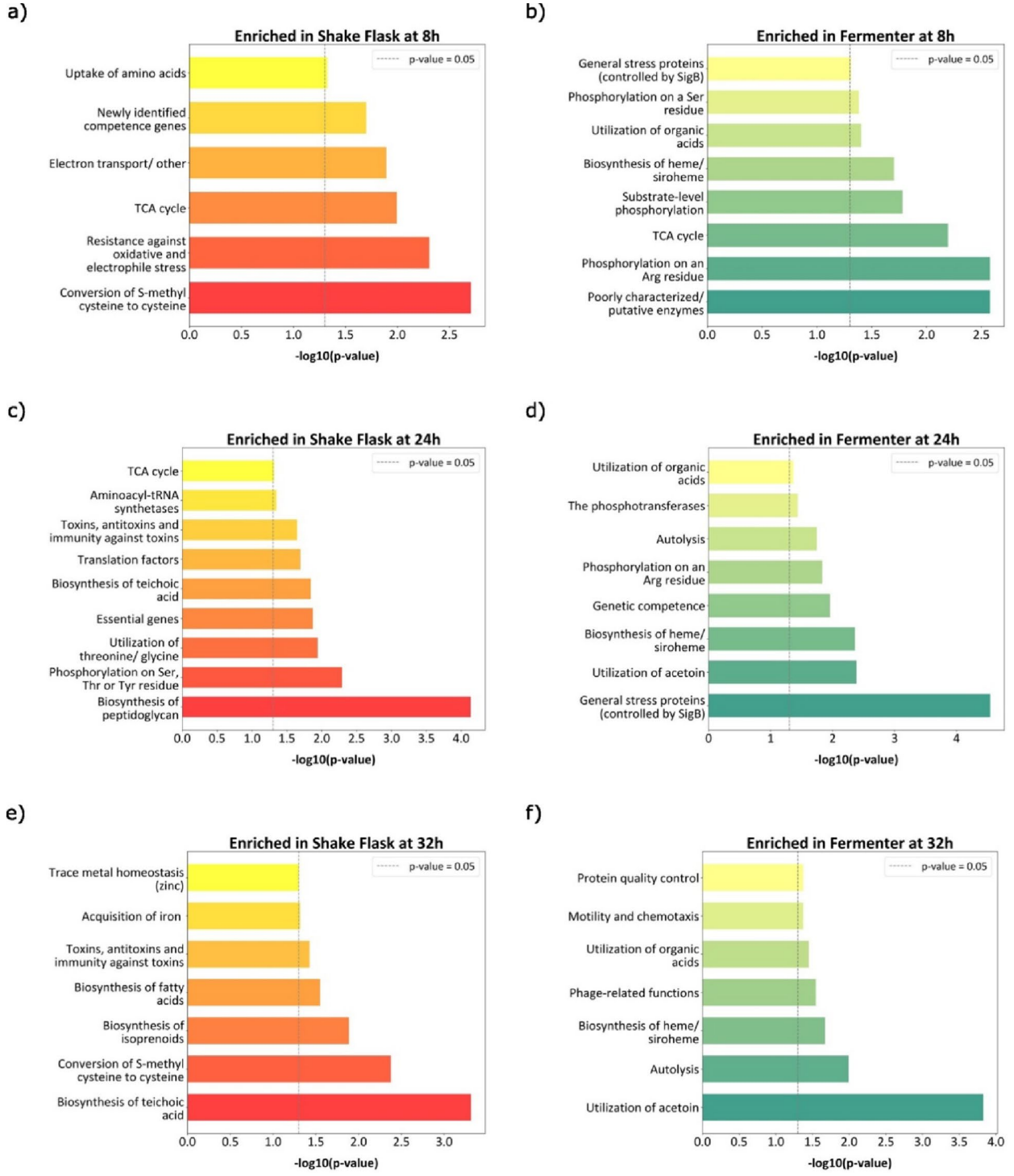

Fig. 6 Enrichment analysis of bacterial cell-associated proteins and processes based on protein functional categories. Proteins and processes in the bacterial cells that were significantly more abundant upon shake flask culturing are indicated with orange, and cellular proteins and processes that were significantly more abundant upon breathing fermenter culturing are indicated in green ($p < 0.05$ and $\log_2$fold change>|0.5|). Data from 4 independent experiments are presented

These included the YvhB (TuaB), WalR, SigX, FapR, SigV and YvrHB regulons (Fig. 8a, c and e). In particular, the YvhB, WalR and YvrHB regulons are involved in cell envelope maintenance, while the SigX and SigV regulons are important for combatting the effects of cell envelope stress inducing conditions. Both SigX and SigV are part of the extracytoplasmic function family of sigma factors. SigX is activated by YvrHB and regulates

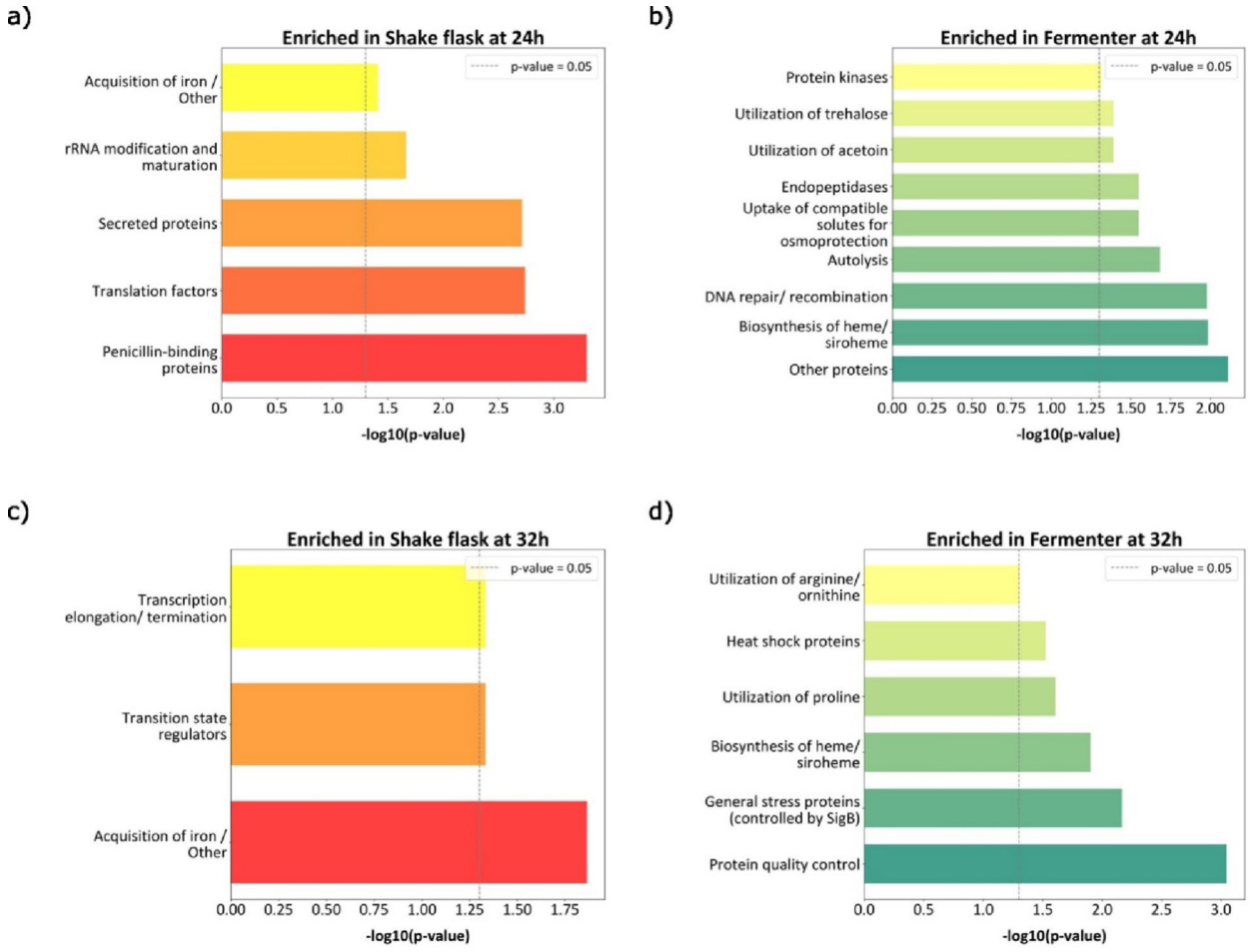

Fig. 7 Enrichment analysis of extracellular proteins and processes based on protein functional categories. Extracellular proteins and processes that were significantly more abundant upon shake flask culturing are indicated with orange, and proteins that were significantly more abundant upon breathing fermenter culturing are indicated with green ($p < 0.05$ and $\log_2$fold change>|0.5|). Data from 4 independent experiments are presented

40 genes involved in cell wall resistance. In addition to cell wall maintenance, induction of the FapR regulon indicates that fatty acid biosynthesis is stimulated. Altogether, this could indicate that the shaking causes damage to the bacterial cell wall, possibly due to sheer stress. As a consequence, the bacteria would have to replenish certain cell wall components. This type of response was not detected in the bacteria grown in the breathing fermenter, suggesting that the paddle and the screw in the breathing fermenter imposed relatively little sheer forces on the bacteria compared to the baffled shake flasks. This view is consistent with the observation that in samples from the shake flask cultures, which were collected at the mid- and late-stationary phases (T24 and T32), the LiaH and LiaG proteins were present at strongly elevated levels (Fig. 5; Supplementary Table S1). These two proteins are both induced upon cell envelope stress [44]. Here it is important to note once more that the total culture volumes in the two systems differed substantially, with a total volume of 100 mL in the shake flasks and of 1.5 L in the breathing fermenters. The shaking speed required for sufficiently aerating a 1.5 L culture in a shake flask would be much higher than what was used in our experiments. This emphasizes one aspect of why shake flask cultivations are not suitable for scale-up.

Metabolic adaptations

Interestingly, various differences were also observed for proteins and pathways related to metabolic processes when bacteria sampled from the two cultivation systems were compared, despite the fact that they were grown to comparable densities in exactly the same medium. In the bacteria grown in shake flask, proteins whose expression is controlled by the sulphur metabolism regulator AscR and its activator CymR were enriched during the early stationary phase (T8; Supplementary Table S1), which implies that sulphur metabolism was relatively enhanced upon growth in shake flasks compared to growth in the breathing fermenter (Fig. 8a). On the other hand, all proteins encoded by regulons that were relatively enriched

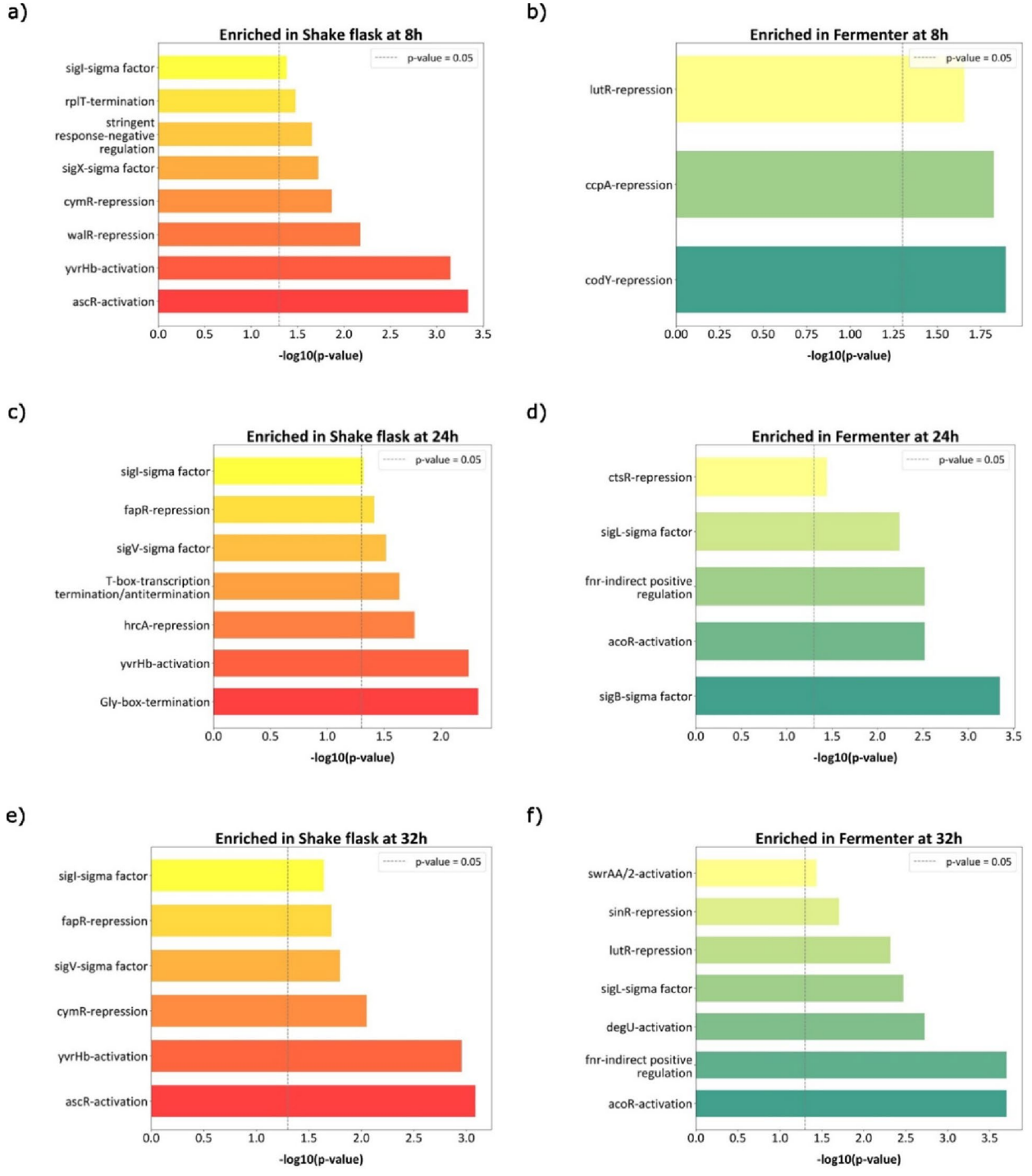

Fig. 8 Enrichment analysis based on regulons. Regulons of which encoded cellular proteins were significantly more abundant upon shake flask culturing are indicated with orange. In contrast, regulons of which the encoded proteins were significantly more abundant upon bacterial culturing in the breathing fermenter are indicated with green ($p < 0.05$ and $\log_2$fold change>|0.5|). Data from 4 independent experiments are presented

in the bacteria grown in the breathing fermenter at the early stationary phase (T8) were related to central carbon metabolism and branched chain amino acid metabolism. In particular, the results implied that the expression of proteins controlled by the LutR, CcpA and CodY repressors was relatively upregulated (Fig. 8b; Supplementary Table S1), which is indicative of a prolonged consumption of glucose and branched chain amino acids. Here, it is noteworthy that glucose consumption lasted longer by the bacteria in the breathing fermenter

to produce more acid, leading to a slightly lower pH of the growth medium compared to the pH of the medium upon shake flask culturing (Fig. 2). Furthermore, during the mid- and late-stationary phases (T24 and T32), proteins whose expression is controlled by the SigL, AcoR and Fnr regulons (i.e. AcoABCL) were enriched in bacteria grown in the breathing fermenter (Fig. 8d; Supplementary Table S1). The SigL and AcoR regulons are related to acetoin utilization and enrichment in the proteins belonging to these regulons points out a shift to acetoin catabolism as a means to obtain energy at late stages of fermentation. Fnr is involved in the bacterial response to limiting oxygen levels, which is consistent with the fact that there was no active aeration in the breathing fermenter system. Hence, the oxygen supply may have become sub-optimal during the later stages of fermentation in the breathing vessel.

Conclusions

Here, we describe a comparative study on growth behaviour, recombinant protein yield, stress responses and metabolic adaptations of the genome-reduced *Bacillus subtilis* strain IIG-Bs-27-31 cultured in a novel breathing fermenter or shake flasks. The growth characteristics of this strain were comparable in both culture systems, but bacteria grown in the breathing fermenter reached a slightly higher final OD_{600}. Additionally, the differences in the pH of the cultures indicated a differential preference for the carbon source, where proteins and/or amino acids were probably preferentially utilized by the bacteria cultured in shake flasks and sugars were a preferred carbon source for bacteria grown in the breathing fermenter. The latter view is supported by our proteome analyses. Furthermore, in this study, cells producing the heterologous amylase AmyQ were investigated in terms of enzyme production and stress responses. The productivity of the cells and the activity of the secreted AmyQ were similar in both culture conditions. However, when the culture volumes are taken into account, the breathing fermenter clearly provides higher AmyQ yields and is in principle more suitable for scale-up. An in-depth analysis of the proteomes of the cells and the culture supernatants revealed that the cells grown in the breathing fermenter exhibited various stress responses, including the general stress response regulated by SigB and a protein secretion stress response. On the other hand, in bacteria cultured in shake flasks, proteins encoded by membrane- and cell wall biogenesis-related regulons were enriched. This suggests that the shake flask shaking caused damage to the bacterial cell envelope, possibly in the form of sheer stress, and that the cells needed to replenish their cell envelopes. This was apparently not the case for bacteria growing in our breathing fermenter vessels. Finally, several different metabolic adaptations were identified using enrichment analysis. In particular, we observed enrichment of proteins involved in central carbon and branched chain amino acid metabolism in the bacteria cultured in the breathing fermenter, suggesting prolonged consumption of glucose and branched chain amino acids. Although the metabolic scenarios inferred from our proteomics analyses are plausible, they need to be followed up and substantiated by metabolomic analyses in future studies. Furthermore, enrichment of the Fnr-regulated proteins in the bacteria grown in the breathing fermenter pointed out a limitation in oxygen levels in the later stages of fermentation. This may reflect a limitation of the current design of the breathing fermenter, but this limitation can readily be bypassed by additional oxygen supply and further development of the fermenter design. In fact, optimization of the fermenter design to enhance bacterial growth and productivity is part of our ongoing research towards scale-up of the breathing vessels.

In conclusion, we show comparable growth characteristics of the genome-reduced *B. subtilis* strain IIG-Bs-27-31 and AmyQ yields per liter during parallel culturing in breathing vessels and shake flasks. However, the breathing fermenters offer the potential for scale-up, which is not the case for shake flasks. Furthermore, our proteome analyses revealed potential protein secretion bottlenecks, stresses, and nutrient or oxygen limitations experienced by the bacteria. These can be mitigated by strain engineering (e.g. overproduction of SipS or mutation of the *lonA*, *ftsH*, *htrA* and/or *htrB* genes), optimization of the fermentation broth and/or adjustments in the fermenter design. Lastly, it will be important for future implementation of breathing vessels to carefully compare their performance in bacterial fermentations, especially with respect to energy consumption, with that of conventional vessels employing sparger aeration. However, based on our present observations we can already conclude that the present breathing fermentation system provides the grounding for cost-effective and scalable new-generation alternatives to traditional cultivation systems for microbial cell factories.

Supplementary Information.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-026-02942-x.

Supplementary Material 1. Table S1. Proteomics data.

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Author contributions

A.O., K.i.Y., and J.M.v.D. conceived and designed the experiments. A.O., K.Y., Y.W. and S.I. performed the experiments. M.Y. and R.S. designed and constructed the setup of the breathing vessel. F.G. and S.M. performed proteomic analyses.

A.O., F.G., K.i.Y., and J.M.v.D. analyzed the data. K.i.Y. and J.M.v.D. supervised the project. A.O., K.i.Y., and J.M.v.D. wrote the manuscript. All authors have read and approved the manuscript.

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Data availability
The dataset supporting the conclusions of this article is available in the ProteomeXchange Consortium via PRIDE partner repository, with the dataset identifier PXD067838 (https://www.ebi.ac.uk/pride/).

Declarations

Competing interests
The authors declare no competing interests. M.Y. and R.S. are employees of Junkosha Inc.

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