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Metabolic activity of *Corynebacterium glutamicum* grown on L-lactic acid under stress

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Abstract Respiration measurement in shake flasks is introduced as a new method to characterize the metabolic activity of microorganisms during and after stress exposure. The major advantage of the new method is the possibility to determine the metabolic activity independent of manual sampling without the necessity to change the culture vessel or the cultivation medium. This excludes stress factors, which may be induced by transferring the microorganisms to plates or respirometers. The negative influence interruptions of the shaker during sampling times which may have on the growth of microorganisms was demonstrated. The applicability of the method was verified by characterizing the behavior of *Corynebacterium glutamicum* grown on the carbon source L-lactic acid under the stress factors such as carbon starvation, anaerobic conditions, lactic acid, osmolarity, and pH. The following conditions had no effect on the metabolic activity of *C. glutamicum*: a carbon starvation of up to 19 h, anaerobic conditions, lactic acid concentrations up to 10 g/l, 3-(*N*-morpholino)propanesulfonic acid buffer concentrations up to 42 g/l, or pH from 6.4 to 7.4. Lactic-acid concentrations from 10 to 30 g/l lead to a decrease of the growth rate and the biomass substrate yield without effecting the oxygen substrate conversion. Without adaptation, the organism did not grow at pH≤5 or ≥9.

Introduction

Stress tolerance and stress adaptation of microorganisms has been characterized to understand the survival strategies of pathogenic organisms like *Mycobacterium tuberculosis* (Hu and Coates 1999; Primm et al. 2000) and *Staphylococcus aureus* (Watson et al. 1998), or food-spoiling bacteria like *Listeria monocytogenes* (Ferreira et al. 2001) and *Listeria innocua* (Houtsma et al. 1996). *Escherichia coli* was used by several researchers (Breidt et al. 2004; Ihssen and Egli 2004; Mandel and Silhavy 2005; Presser et al. 1997, 1998; Roe et al. 2002; Ross et al. 2003) to study the stress tolerance and stress response of Gram-negative organisms. Microorganisms have developed complex stress-survival and stress-response strategies to respond to various stress factors like starvation, oxygen, osmolarity, or pH. The knowledge about these strategies has increased fundamentally in the last decade due to the advances of molecular biological methods. However, the standard methods to determine the viability and activity after and during stress have remained unchanged. Viable cell counts with petri dishes and activity determination with respirometry are still the most common methods to determine the interaction between microorganisms and stress.

In this study, a new method is introduced to continuously monitor the oxygen transfer rate (OTR) of microorganisms in shake flasks during and after exposure to stress without the necessity to change the culture vessel or the medium. The OTR reflects the metabolic activity and in most cases also the growth rate of microorganisms due to the fact that almost every physiological activity in aerobic cultures is coupled to the respiratory uptake of oxygen (Anderlei et al. 2004; Anderlei and Büchs 2000; Losen et al. 2004; Stöckmann et al. 2003). The new method has three major advantages over the standard methods: (1) Cultivation and metabolic activity determination are performed in the same culture vessel and in the same medium, excluding stress factors which may be induced by transferring the microorganisms to plates or respirometers. (2) Continuous online monitoring enables the determination of the metabolic activity independent of the sampling interval. (3) The sampling times for molecular stress-response analysis can be chosen in a flexible way according to the online-monitored behavior of the culture and not according to a fixed, predetermined sampling schedule. The mentioned advantages are all part of the technical nature of the measuring technique. How important they are can only be determined through a direct comparison with alternative

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methods, which was not the focus of this study. The broad applicability of the method is demonstrated by analyzing the behavior of *Corynebacterium glutamicum* on minimal medium with L-lactic acid as sole carbon source under the stress factors such as carbon starvation, anaerobic conditions, lactic acid, osmolarity, and pH.

The physiological behavior of biological cultures can be derived from the OTR by analyzing the slope and the form of the OTR curve (Anderlei et al. 2004; Anderlei and Büchs 2000; Losen et al. 2004; Stöckmann et al. 2003); in addtion, the integral of the OTR curve can be used to control the stoichiometry and the exhaustion of essential nutrients (Stöckmann et al. 2003). In the very most cases, the OTR can be used as sole measuring parameter to study the stress response of microorganisms growing on any carbon source, if not oxygen-limited conditions are of interest. Oxygen-limited (not anaerobic) conditions can be investigated by considering the OTR and the carbon dioxide transfer rate (Anderlei et al. 2004) if the anaerobic product is consumed by diauxic growth and/or the carbon source and its anaerobic product have a different degree of reduction (e.g., glucose and ethanol). Different from most organisms, *C. glutamicum* does not show diauxic growth but coutilization if grown on mixtures of carbon sources glucose/lactate (Cocaign et al. 1993) or glucose/acetate (Wendisch et al. 2000). The applicability of the new technology to study oxygen-limited *C. glutamicum* glucose cultures is further hampered by the fact that glucose and its anaerobic product lactate have the same degree of reduction. *C. glutamicum* grown on lactic acid as carbon source does not produce an anaerobic product under oxygen-limited conditions. Thus, the stress response of *C. glutamicum* can be investigated under oxygen-unlimited and oxygen-limited conditions if the carbon source lactic acid is used. Instead of using lactic acid, it would have been possible to use glucose and limit the study to oxygen-unlimited conditions.

C. glutamicum is widely used for the industrial production of amino acids. It is a high-G+C Gram-positive soil bacterium and closely related to the Brevibacteria and Mycobacteria (Gruber and Bryant 1997; Kinoshita et al. 1957). Stress behavior and amino acid production are linked because external triggers like biotin limitation or heat stress are often required to start amino acid production (Barreiro et al. 2004, 2005; Gutmann et al. 1992; Stansen et al. 2005). To improve amino acid production, the response of *C. glutamicum* to substrate limitations/starvations has been intensively investigated with a strong focus on nitrogen (Burkovski 2003; Nolden et al. 2001; Silberbach et al. 2005) and phosphate (Ishige et al. 2003; Kočan et al. 2006). Further interest in the stress response of *C. glutamicum* comes from medical research. *C. glutamicum* as a nonhazardous organism is considered an ideal model to understand the stress response of closely related pathogenic species, such as *Corynebacterium diphtheriae*, *Mycobacterium leprae*, and *M. tuberculosis*. Physiological observations of *C. glutamicum* on lactate under standard batch and continuous conditions have been reported by Cocaign et al. (1993), Cocaign-Bousquet and Lindley (1995), and Gourdon et al. (2000). Studies of L- and D-lactate metabolism and gene expression have been mainly aimed at lactate dehydrogenase, anaplerotic reactions, coutilization of lactate and other carbon sources, and pyruvate accumulation (Cocaign et al. 1993; Gourdon et al. 2000; Koffas et al. 2003; Stansen et al. 2005).

Materials and methods

Microorganism, media, and culture conditions

Experiments were carried out with the wild type of *C. glutamicum* ATCC 13032 (Kinoshita et al. 1957). Complex medium liquid precultures were inoculated from a complex medium plate culture. The complex medium contained per liter: 20 g glucose, 10 g yeast extract, 10 g peptone, 2.5 g NaCl, and 0.25 g $MgSO_4$. The pH was adjusted to 7.2 and the glucose was sterilized separately. The precultures reached a final pH of ~7.4.

The precultures were harvested after 12 h and washed twice in 9 g/l NaCl. The pellet was resuspended in minimal medium, and the minimal medium main culture was inoculated with a biomass concentration of 0.25 g/l. The temperature for all cultivations was 30 °C.

The minimal medium contained per liter: between 1 and 29.5 g lactic acid as specified in the results part, 20 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 2 g K_2HPO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 30 mg 3,4-dihydroxybenzoic acid [$(HO)_2C_6H_3COOH$], 10 mg $CaCl_2 \cdot H_2O$, 10 mg $MnSO_4 \cdot H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 0.2 mg $CuSO_4$, 0.2 mg biotin, and 0.02 mg $NiCl_2 \cdot 7H_2O$. For one experiment, 42 g (0.2 M) 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer was added. The initial pH was adjusted with NaOH between 4 and 9.5, as specified in the results part. Trace elements, biotin, 3,4-dihydroxybenzoic acid, and lactic acid were sterilized separately. The lactic acid (80 % Karl Roth GmbH, Karlsruhe, Germany) contained =950 g/l L(+)-lactic acid. Cultures were cultivated on an orbital shaker (Lab-Shaker LS-W, Adolf Kühner AG, Birsfelden, Switzerland) with a filling volume of 10 (oxygen-unlimited), 25, 30, or 60 ml (oxygen-limited conditions), with a 50-mm shaking diameter and a 300-rpm shaking frequency. Cultivations were carried out in unbaffled 250 ml Erlenmeyer flasks with cotton plugs or in 250 ml measuring flasks of the RAMOS device (Anderlei et al. 2004; Anderlei and Büchs 2000). The maximum oxygen transfer capacity (OTR_{max}) of the different culture conditions was estimated according to the shake flask model of Maier and Büchs (2001) and Maier et al. (2004). A single inoculated medium was prepared for each experiment. This inoculated medium was then distributed to each shake flask. Only the flasks with different initial lactic acid concentrations and the flasks with MOPS buffer were inoculated separately. None of the experiments required a change of measuring device, measuring vessel, or the medium during the experiment, thus, avoiding metabolic activity losses produced by transferring the microorganisms to, e.g., respirometers or plates.

The final biomass concentration was determined by centrifuging 1.5 ml of culture broth at 14,000 rpm in a dried (24 h, 105 °C, cooled down in a dessicator) and weighed microcentrifuge tube. After removing the supernatant, the pellet was dried (24 h, 105 °C, cooled down in a dessicator) and weighed. The biomass concentration of samples taken during the experiment was calculated using an optical density (OD_{600}) dry weight correlation.

Titration curves were recorded by stepwise, adding 0.5 M NaOH to the medium, but never increasing the pH by more than 0.1. Titration curves were recorded for three different media: the standard medium at the time of inoculation with 10 g/l lactic acid and 20 g/l $(NH_4)_2SO_4$, a medium without lactic acid but with 20 g/l $(NH_4)_2SO_4$ reflecting the conditions after the consumption of the carbon source, and a medium with 10 g/l lactic acid but without $(NH_4)_2SO_4$. To increase the comparability, the titration curves were normalized to pH 7, which is the initial pH of the biological cultures. The normalization was done by subtracting the volume of 0.5 M NaOH necessary to adjust the pH to 7 from the actual titration volume giving positive volumes for pH greater than 7 and negative volumes for pH smaller than 7.

The OTR was measured with a RAMOS device (Hitec Zang, Herzogenrath, Germany). The OTR is measured by periodically repeating an automated measuring cycle. A measuring cycle is composed of a measuring phase (flask closed air-tight, 10 min) and a rinsing phase (flushed air, 20 min). The OTR is calculated from the decrease of the partial oxygen pressure in the headspace of the shake flask during the measuring phase. The rinsing phase can be operated in the one-flow or in the two-flow mode. The two-flow mode consists of a high aeration at the beginning of the rinsing phase and a low aeration for the rest of the rinsing phase. It recreates the average oxygen supply of a standard flask with a cotton plug (Anderlei et al. 2004). The variation of the partial oxygen pressure in the headspace of the shake flask caused by the measuring cycle is smaller than the differences in partial oxygen pressure produced by the variability of manually produced cotton plugs (Anderlei et al. 2004; Mrotzek et al. 2001). Thus, the stresses imposed by standard flasks and RAMOS device are comparable. The one-flow mode allows an increase of OTR_{max} in comparison to a standard flask with a cotton plug by aerating the entire rinsing phase with the high aeration.

If during the cultivation the shaker had to be stopped, e.g., to take samples, the necessary manipulation time was recorded. The manipulation time includes the sampling time for all eight RAMOS flasks or ten standard flasks and the time which the shaker needs to decelerate and re-accelerate to the set shaking frequency.

Stress induced by carbon starvation

To investigate the influence of carbon starvation on the metabolic activity of *C. glutamicum*, the microorganisms were grown in batch fermentations with 10 g/l lactic acid and then starved. The metabolic activity was tested by adding a lactic acid pulse after 28 h. After the pulse, the medium contained 5 g/l lactic acid. At the time of the pulse, the OTR of all cultures was below 0.001 mol/l/h. The pulse was added to the cultures during the rinsing phase of the RAMOS device 10 min before the start of the next measuring phase. Oxygen-unlimited and oxygen-limited culture conditions were set by using filling volumes of 10, 30, and 60 ml. The initial pH was 7. The only manipulation necessary during the experiment was to stop the shaker for ~10 min to add the lactic acid pulse.

Stress induced by anaerobic conditions

The behavior of *C. glutamicum* on lactic acid under anaerobic conditions was investigated by comparing standard batch cultures (aerated with air) with batch cultures aerated twice for 1 h with nitrogen. After starting the nitrogen aeration, it was verified with the oxygen sensor of the RAMOS device that the oxygen concentration in the flasks reached zero in less than 5 min. The anaerobic conditions were terminated by flushing the flasks with compressed air. Standard oxygen concentrations were reached again in less than 5 min. The nitrogen aeration was connected to the flask during the rinsing phase 10 min before the start of the next measuring phase and disconnected directly after the previous measuring phase. Oxygen-unlimited and oxygen-limited (not anaerobic) culture conditions were set by using filling volumes of 10, 30, and 60 ml. The lactic acid concentration was 10 g/l and the initial pH was 7. The only manipulation necessary during the experiment was stopping the shaker for ~5 min to connect and disconnect the nitrogen aeration.

The influence of a delayed agitation and aeration after inoculation on the growth of *C. glutamicum* was investigated by comparing cultures, which were directly shaken after inoculation with cultures which were not shaken after inoculation for 1, 2, and 3 h. To assure equal culture conditions of shaken and unshaken cultures, the unshaken flasks were placed directly next to the shaker in the same thermo-constant room. The filling volume was 25 ml, the lactic acid concentration was 10 g/l, and the initial pH was 7. The only manipulation necessary during the experiments was stopping the shaker for ~8 min to connect the unshaken flasks to the RAMOS device.

Stress induced by lactic acid

The stress induced by lactic acid was investigated by measuring the influence of different initial lactic acid concentrations: 1 g/l (0.51 osmol/l), 2.1 g/l (0.53 osmol/l), 4.7 g/l (0.55 osmol/l), 9.6 g/l (0.72 osmol/l), 12.4 g/l (0.78 osmol/l), 13.7 g/l (0.81 osmol/l), 18.9 g/l (0.93 osmol/l), and 29.5 g/l (1.16 osmol/l) on respiration and growth of *C. glutamicum*. The numbers in parentheses are the calculated osmolarities of the entire medium. The lactic acid concentrations were determined with high-performance liquid chromatography (HPLC) analysis

(Dionex with RI and UV–VIS detectors, Dionex Sunnyvale, CA, USA) after adjusting the pH with NaOH to 7 and before inoculation. As stationary phase, an organic acid resign (CS Chromatography, Langerwehe, Germany) was used, while as eluent, it was a 1-mM H_2SO_4 with a flow rate of 0.6 ml/min. The column temperature was maintained at 30 °C.

To avoid oxygen limitation even at higher lactic acid concentrations, the RAMOS device was operated in the one-flow mode with a filling volume of 10 ml. After terminating the experiment, the final lactic acid concentrations (HPLC), the final biomass concentrations (dry weight, OD), and the final pH were determined. During the fermentation, no manipulation was necessary.

Stress induced by osmolarity and pH

The stress induced by osmolarity and pH was investigated by comparing the respiration of two oxygen-unlimited batch cultures, one without buffering and the other with an MOPS buffer concentration of 42 g/l (0.2 M). The medium without buffer has an osmolarity of 0.73 osmol/l, which is increased by adding the buffer to 1 osmol/l. The filling volume was 10 ml, the lactic acid concentration was 10 g/l, and the initial pH was 7. The RAMOS device was operated in the two-phase mode. During the fermentation, no manipulation was necessary.

The growth characteristics of *C. glutamicum* at different initial pH was investigated by comparing respiration and biomass formation of unbuffered batch cultures with different initial pH of 4, 4.5, 5, 5.5, 7.1, 7.4, 8.5, 9, and 9.5. The biomass and the pH measurements were performed in separate shake flasks with cotton plugs so as not to interrupt the OTR measurements. The filling volume was 10 ml and the lactic acid concentration was 10 g/l. The RAMOS device was operated in the two-phase mode and stopped only twice for 10 min to take control samples. The shaker with the standard shake flasks was stopped 14 times for ~10 min to take samples (sample size 0.15 ml).

Results

The applicability of respiration measurements in shake flasks to analyze the behavior of microorganisms during and after stress exposure was verified with the model system of *C. glutamicum* grown on the carbon source lactic acid. The organism was exposed to the stress factors such as carbon starvation, anaerobic conditions, lactic acid, osmolarity, and pH. Representative results are depicted in Figs. 1, 2, 3, 4, 5, 6, and 7. All experiments have been performed at least twice and minimum triple evidence was considered necessary to draw a conclusion.

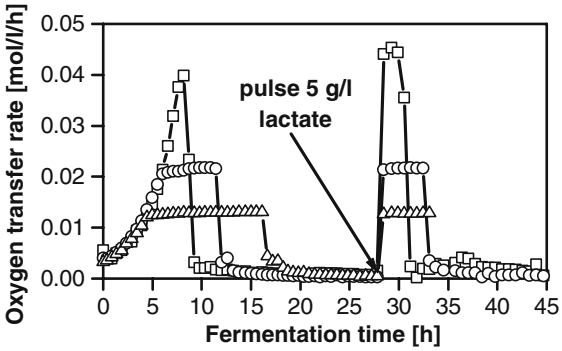

Fig. 1 Influence of carbon starvation on the metabolic activity of *C. glutamicum*. After an initial batch fermentation with a lactic acid concentration of 10 g/l, a pulse of 5 g/l lactic acid was added after 28 h. Culture conditions: 250 ml shake flask, filling volumes (before the pulse) 10 ml (□), 30 ml (○), 60 ml (△), shaking diameter 50 mm, shaking frequency 300 rpm, and initial pH 7

Stress induced by carbon starvation

The experiment was performed in three steps: first, the microorganisms were raised in a standard batch fermentation; second, they were exclusively carbon-starved (oxygen was continuously supplied) for 12–19 h depending on the culture conditions; third, the metabolic activity of the microorganisms was tested by adding a lactic acid pulse. Fig. 1 depicts the OTR over fermentation time of the entire experiment. The OTR curves reflect the experimental procedure. Initially, after inoculation, all cultures grew exponentially. The exponential growth continued until it was impaired by the OTR_{max}. The OTR_{max} depends only on the culture conditions (filling volume 10 ml oxygen-unlimited growth, and 30 and 60 ml oxygen-limited growth). After reaching OTR_{max}, the OTR stayed at a plateau until the carbon source was exhausted and then dropped sharply. With a decreasing OTR_{max}, the fermentation time increased. Then, after the drop, the OTR remained close to zero and the microorganisms were starving. Finally, after lactic acid was added, the OTR of the oxygen-limited cultures jumped to the level of the plateau before starvation. The OTR of the oxygen-unlimited culture exceeded even the maximum OTR reached in the initial batch, indicating the growth of the microorganisms after the pulse was added. After the renewed exhaustion of the carbon source, the OTR dropped again. In conclusion, a carbon starvation of up to 19 h does not influence the metabolic activity of *C. glutamicum*.

Stress induced by anaerobic conditions

The stress tolerance of *C. glutamicum* under anaerobic conditions was analyzed by comparing constantly aerobic batch cultures with temporarily anaerobic batch cultures. Fig. 2a (values are identical with the initial batch of Fig. 1) depicts the OTR of the constantly aerobic batch cultures over fermentation time. Fig. 2c depicts the OTR of the twice nitrogen-aerated batch cultures over fermentation time. The general culture characteristics were similar to the

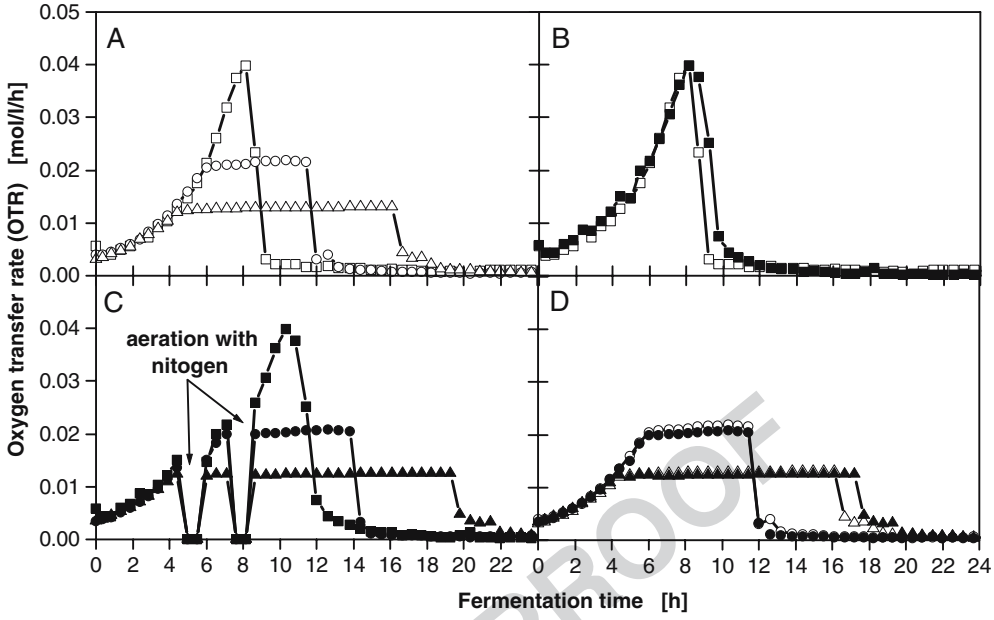

Fig. 2 Influence of anaerobic conditions on the metabolic activity of *C. glutamicum*. **a** □, ○, and △. Continuous aeration with air. **b** Comparison of oxygen unlimited cultures with and without nitrogen aeration. **c**. ■, ●, and ▲. After 4 and 7 h aeration for 1 h with nitrogen. **d** Comparison of oxygen-limited cultures with and without nitrogen aeration. **a**, **c** Actual fermentation time. **b**, **d** Nitrogen aeration time subtracted from actual fermentation time. Culture conditions: 250 ml shake flask; filling volumes 10 ml (□ and ■), 30 ml (○ and ●), 60 ml (△ and ▲); shaking diameter 50 mm; shaking frequency 300 rpm; lactic acid concentration 10 g/l; and initial pH 7

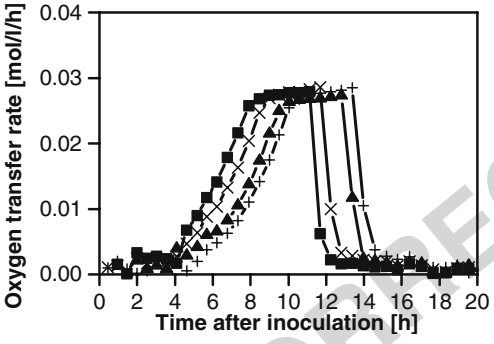

Fig. 3 Influence of a delayed aeration on the culture characteristics of *C. glutamicum*. After inoculation, the cultures were directly shaken (■), shaken after 1 h (×), shaken after 2 h (▲), and shaken after 3 h (+). Culture conditions: 250 ml shake flask, filling volume 25 ml, shaking diameter 50 mm, shaking frequency 300 rpm, lactic acid concentration 10 g/l, and initial pH 7

constantly aerobic batch. However, the fermentation time was prolonged and no growth occurred during the anaerobic periods. In a separate experiment (results not shown), it could be shown that the optical density as well of the culture did not increase during anaerobic conditions. A more detailed insight in the growth behavior of *C. glutamicum* after anaerobic stress is given in Fig 2b (oxygen-unlimited growth conditions, filling volume 10 ml) and Fig 2d (oxygen-limited growth conditions, filling volumes 30 and 60 ml). In both figures, the time of anaerobic fermentation (twice 1 h) is cut out from the data set shown in Fig. 2c to allow for a better comparison of the OTR curves. The cumulative consumed oxygen (integral of the OTR curve) was identical for oxygen-limited and oxygen-unlimited conditions, providing an oxygen substrate yield $(Y_{O_2/S})$ of 0.017 $mol_{O2}/g_{lactic\ acid}$ with a standard deviation of 0.0016 $mol_{O2}/g_{lactic\ acid}$. Also, the slope of the OTR and the whole course of the culture were not affected by the anaerobic conditions, proving that the metabolic activity of *C. glutamicum* is not affected by short periods of anaerobic conditions.

Similar observations were made with a second set of experiments. Anaerobic conditions were produced by delaying the shaking of the cultures after inoculation. Fig. 3 depicts the OTR over time after inoculation. The general course of the OTR curves was independent of the shaking conditions reflecting the tolerance of *C. glutamicum* to anaerobic conditions. However, the exponential growth phases and the exhaustion of the carbon sources were shifted for 1, 2, and 3 h reflecting the delay of aeration after inoculation. If the OTR curves of Fig. 3 are superposed by cutting out the unshaken periods, the OTR curves are nearly identical (figure not shown). In conclusion, during short anaerobic periods, *C. glutamicum* falls in a state of dormancy. Similar to sleep, we define dormancy as a state where the microorganisms have no or only very reduced metabolic activity without growth; however, the ability to be metabolically active and grow is not significantly reduced.

Stress induced by lactic acid

The stress induced by varying acid concentrations (Fig. 4) was investigated by comparing the OTR of batch cultures with initial lactic acid concentrations ranging from 1 g/l (0.01 mol/l) to 29.5 g/l (0.33 mol/l) at a constant initial pH of 7. Because the pH was always above 7 (Fig. 4d), lactic acid (pK_a 3.86) was entirely present in the dissociated form. Up to a concentration of 10 g/l (Fig. 4a), the lactic acid concentration did not influence the slope of the OTR, and at higher concentrations (Fig. 4c), the slope decreased due to lactic acid inhibition.

In aerobic cultures, almost every physiological activity is reflected by the OTR (Anderlei et al. 2004; Anderlei and

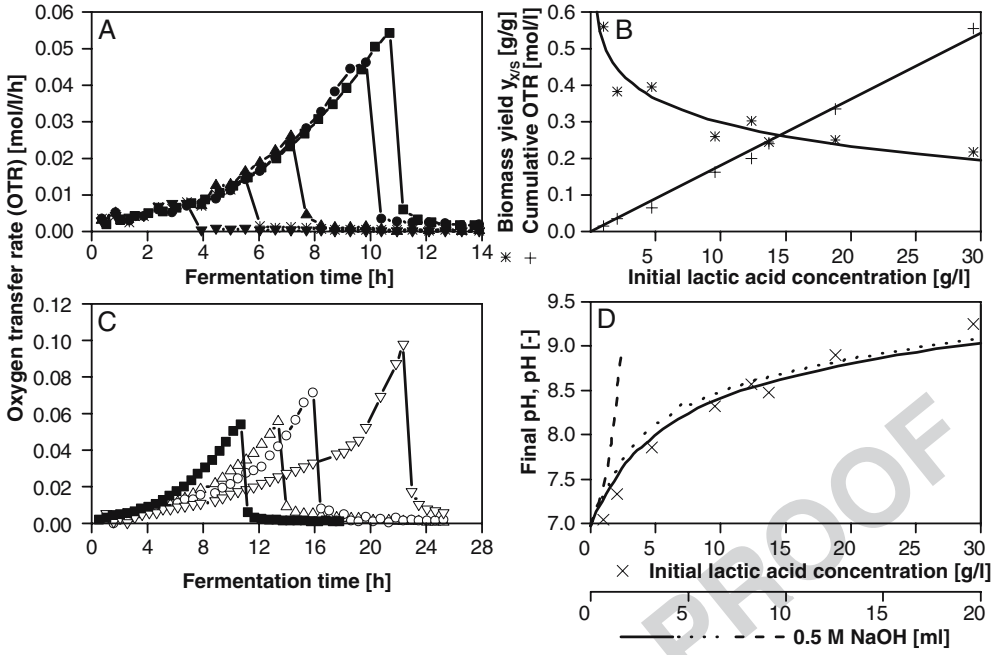

Fig. 4 Influence of different initial lactic acid concentrations on respiration and growth of *C. glutamicum*. **a** Oxygen transfer rate (OTR) over fermentation time, low initial lactic acid concentrations 1 g/l (▼), 2.1 g/l (*), 4.7 g/l (▲), 9.6 g/l (●), and 12.4 g/l (■). **b** Cumulative consumed oxygen over initial lactic acid concentration (+), and biomass substrate yield over initial lactic acid concentration (*). **c** OTR over fermentation time, high initial lactic acid concentrations 12.4 g/l (■), 13.7 g/l (▵), 18.9 g/l (○), and 29.5 g/l (▽). **d** Final pH over initial lactic acid concentration (×). Titration curves normalized to pH 7 as described in the "Materials and methods" section over added amount titrant NaOH: standard medium (——), medium without lactic acid (·····), and medium without ammonium sulfate (——). Culture conditions: 250 ml shake flask, filling volume 10 ml, shaking diameter 50 mm, shaking frequency 300 rpm, and initial pH 7

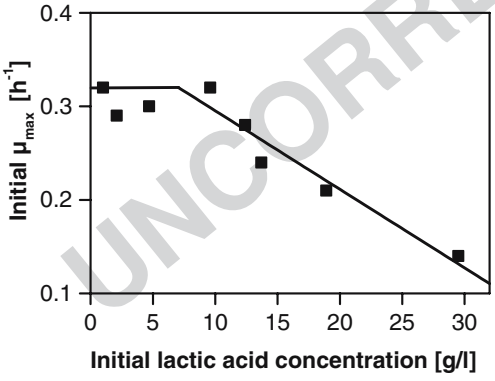

Fig. 5 Initial maximum growth rate (μ_{max}) at different initial lactic acid concentrations. The initial μ_{max} were derived from the slopes of the oxygen transfer rate (OTR) depicted in Fig. 4a,c. OTR values with a fermentation time between 2.5 and 6 h were used to calculate the initial μ_{max}

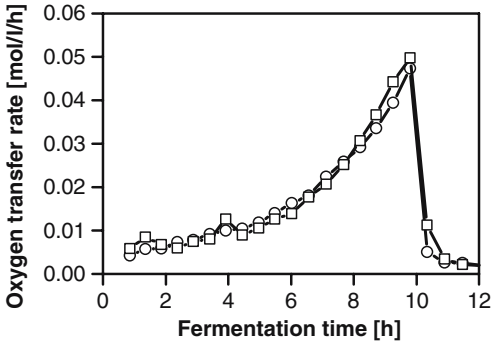

Fig. 6 Influence of buffer on the growth of *C. glutamicum*. Medium without buffer (○), and medium with 42 g/l MOPS buffer (□). Culture conditions: 250 ml shake flask, filling volume 10 ml, shaking diameter 50 mm, shaking frequency 300 rpm, lactic acid concentration 10 g/l, initial pH 7, and final pH 8.4 (unbuffered) and 7.6 (buffered)

Büchs 2000; Losen et al. 2004; Stöckmann et al. 2003). Thus, in most cases, it is possible to derive the growth rate of a biological culture from the slope of the OTR as described by Stöckmann et al. (2003). Using the slopes of the OTR curves depicted in Fig. 4a,c, it was possible to identify three growth phases. At fermentation times of less than 2.5 h, *C. glutamicum* showed an acceleration phase. As depicted in Fig. 5 at fermentation times between 2.5 and 6 h, pH values below 8, and initial lactic acid concentrations of less than 10 g/l, the microorganisms grew with the maximum growth rate of 0.3–0.32 h^{-1}. At higher initial lactic acid concentrations [$iC_3H_6O_3$ (g/l)], the growth rate [μ (h^{-1})] decreased linearly with increasing initial lactic acid concentration [μ=0.38–0.0084·$iC_3H_6O_6$, R^2=0.92, 10 g/l≤$iC_3H_6O_6$≤30 g/l]. During the late batch phase, when the lactic acid concentration was low, and the pH value was above 8, the growth rate (μ=0.235 h^{-1}, standard deviation σ=0.016 h^{-1}, $iC_3H_6O_3$≥10 g/l) was independent of the initial lactic acid concentration.

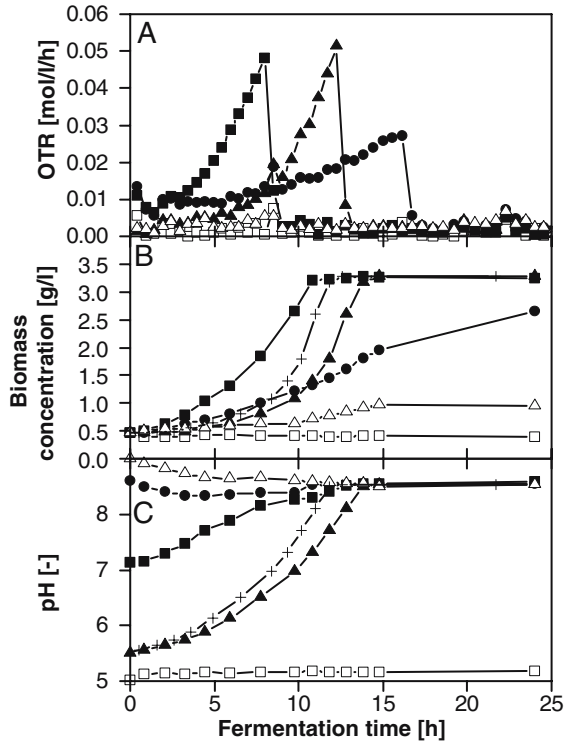

Fig. 7 Influence of different initial pH values of 5 (□), 5.5 (▲), 7.1 (■), 8.5 (●), and 9 (△) on respiration and growth of *C. glutamicum*. **a** Oxygen transfer rate (OTR), **b** Biomass concentration, **c** pH, **b**, **c** + Initial pH 5.5, the time of interruptions of the shaker is subtracted from the fermentation time. Culture conditions: 250 ml shake flask, filling volume 10 ml, shaking diameter 50 mm, shaking frequency 300 rpm, and lactic acid concentration 10 g/l

Fig. 4b depicts the biomass substrate yield coefficient ($Y_{X/S}$) and the cumulative consumed oxygen, which is the integral of the OTR curve from the time of inoculation to the time of the maximum OTR, over the initial lactic acid concentration. The biomass substrate yield decreased with increasing lactic acid concentration. The cumulative consumed oxygen, however, increased linearly with the initial lactic acid concentration giving a constant oxygen substrate yield ($Y_{O_2/S} = 0.017\ mol_{O2}/g_{lactic\ acid}$, standard deviation $\sigma = 0.0015\ mol_{O2}/g_{lactic\ acid}$).

The consumption of the acid lactic acid leads to an increase of the pH value. Fig. 4d depicts the final pH (points) reached by cultures with different initial lactic acid concentrations. The measured increase of the pH values depends on the initial lactic acid concentration and on the buffering capacity of the media. Titration curves of the medium were used as a simple tool to describe the final pH value of the biological cultures and to identify the buffering effect of the different medium components. Fig. 4d depicts the titration curves (lines) of the standard medium as described in the "Materials and methods" section, of a medium without lactic acid, and of a medium without (NH4)2SO4. The titration curves of the media with and without lactic acid are nearly identical, affirming that the consumption of lactic acid (pKa 3.86) cannot influence the buffering capacity of the medium at pH above 7. The titration curve of the medium without $(NH_4)_2SO_4$ is much steeper, showing the buffering effect of ammonium sulfate (pK_a 9.25). Titration curves of a medium without phosphates (results not shown) showed that the phosphates (pK_a 7.2) have only a small influence, proving that the excess amount of ammonium present in the medium is responsible for the relatively moderate pH increase at high initial lactate concentrations.

In conclusion, initial lactic acid concentrations up to 10 g/l do not influence the growth of *C. glutamicum*. At higher concentrations up to 30 g/l, the growth rate decreases linearly with the lactic acid concentration. With increasing lactic acid concentration, the biomass substrate yield decreases, without affecting the oxygen substrate yield. The final pH reached by batch cultures with different initial lactic acid concentrations followed the titration curve of the medium.

Stress induced by osmolarity and pH

Fig. 6 depicts the OTR over fermentation time of oxygen-unlimited unbuffered and buffered batch cultures. Both cultures had an initial pH of 7. The pH of the unbuffered and buffered cultures increased to 8.4 and 7.6, respectively. Up to a fermentation time of 8 h, both cultures had the same slope, demonstrating that the increased osmolarity does not influence the growth of *C. glutamicum*. At fermentation times exceeding 8 h, the growth rate of the unbuffered culture decreased. The buffered culture continued to grow with the maximum growth rate.

The effect of different initial pH on growth and respiration of *C. glutamicum* was investigated by comparing OTR and growth curves of unbuffered batch cultures with initial pH ranging from 4 to 9.5. Fig. 7 depicts the OTR (Fig. 7a), the biomass concentration (Fig. 7b), and the pH (Fig. 7c) over the fermentation time of cultures with different initial pH. The response of *C. glutamicum* to different initial pH values can be classified in three groups. The first group includes the initial pH values of 7.1 (6.4 and 7.4 results not shown). The OTR and growth curves of this group were nearly identical; they increased exponentially. The pH changed from its initial value to 8.5. The second group showed a reduced metabolic activity and includes the initial pH values of 5.5 and 8.5. Due to pH inhibition, the cultures of this group showed a prolonged fermentation time and a reduced growth rate. The culture with an initial pH of 5.5 had a reduced OTR and growth rate at the beginning. However, with increasing pH, the slope became equal to the first group. In contrast, the OTR curve with an initial pH of 8.5 had an equal OTR at the beginning. The slope, however, stayed low for the entire fermentation time. The third group includes the initial pH values of 5 and 9 (4, 4.5 and 9.5 results not shown). None of the cultures of this group showed any considerable metabolic activity. The OTRs remained close to zero, the biomass concentrations did not or only slightly increase, and the pH values stayed at their initial values. However, from the final pH values

reached by the cultures with a high initial lactic acid concentration (Fig. 4d), it can be concluded that *C. glutamicum* can grow with high growth rates at pH up to 9 if the microorganisms have time to adapt. Small decreases of the pH at high initial pH (Fig. 7c) are probably caused by the gassing out of NH_3 at pH close to the pK_a (9.25).

The OTR curves (Fig. 7a) reached the stationary phase earlier than the biomass (Fig. 7b) and pH (Fig. 7c) curves. During the respiration measurements, the shaker was stopped only twice for 10 min to take samples; however, the shaker for the biomass and pH determination was stopped 14 times for 10 min to take samples. If the interruptions of the shaker are subtracted from the fermentation time (Fig. 7b,c only demonstrated for the culture with a pH value of 5.5), the fermentation time of the OTR curves (Fig. 7a) match the fermentation time of the biomass and pH curves (Fig. 7b,c). Probably, stopping the shaker to take samples leads to short anaerobic periods, which freeze growth and metabolic activity. The observed influence of the interruptions of the shaker on the fermentation time confirms the conclusions drawn before about the stress induced by anaerobic conditions.

It can be concluded that the increased osmolarity of up to 1 osmol/l caused by the buffer does not affect the growth or respiration of *C. glutamicum*. Initial pH values ranging from 6.4 to 7.4 lead to uninhibited growth. Without adaptation, the organism did not grow at pH≤5 or pH≥9, but with adaptation, growth was observed up to a pH of 9.

Discussion

Stress induced by carbon starvation

With respiration measurement in shake flasks, it was shown that a starvation time of 19 h does not reduce the metabolic activity of *C. glutamicum* grown on lactic acid. The starvation time used in this study can be sufficient to reduce the viability of Gram-positive organisms. For example, Ferreira et al. (2001) observed for *L. monocytogenes* (WT) a 25% decrease in viability over a starvation time of 12 h. Other Gram-positive organisms like *M. tuberculosis* show a high resistance to carbon starvation. With plate counts, Primm et al. (2000) found no loss in viability of *M. tuberculosis* for 106 days. The high resistance against carbon starvation of *C. glutamicum* observed in this study probably results from the complex starvation-survival strategies of *C. glutamicum* (Wehmeier et al. 1998, 2001), which are similar to the ones of *M. tuberculosis* (Primm et al. 2000).

Stress induced by anaerobic conditions

In this study, it was found that during short anaerobic periods, *C. glutamicum* on lactic acid falls in a state of dormancy, without growth and without a loss in metabolic activity. The behavior of *C. glutamicum* under anaerobic conditions on lactic acid has so far not been investigated. On glucose, Inui et al. (2004) observed no growth under oxygen-deprived conditions, but a continuous formation of lactate and succinate. For *M. tuberculosis* on complex medium, Primm et al. (2000) found only a slight decrease in viability over a 23-week anaerobic period. Kim et al. (2005) describes genes involved in oxidative stress protection of *C. glutamicum*.

Stress induced by lactic acid

In this study, it was found that lactic acid present in the dissociated form (Figs. 4 and 5) had no influence on the growth of *C. glutamicum* up to an initial concentration of 10 g/l (0.11 M). At higher concentrations of up to 30 g/l (0.33 M), the growth rate decreased linearly with the lactic acid concentration. Cocaign et al. (1993) observed for *C. glutamicum* no influence of initial lactate concentrations on the growth rate between 0.035 and 0.125 M. According to Houtsma et al. (1996), the dissociated form of lactate leads to a linear decrease of the growth rate of *L. innocua* at concentrations between 0.25 and 1.1 M. Above 1.25 M, the growth of *L. innocua* is completely inhibited. According to the review of Presser et al. (1997), the growth of various organisms is completely inhibited by the dissociated form of organic acids at concentrations between 0.1 and 0.8 M.

In this study, it was observed that with increasing lactic acid concentration, the biomass substrate yield ($Y_{X/S}$) decreases. For *C. glutamicum*, decreasing growth rates combined with decreasing biomass substrate yields under stress conditions were observed by Guillouet and Engasser (1995). $Y_{X/S}$ between 0.36 and 0.62 $g_{biomass}/g_{lactate}$ depending on the growth rate and mode of operation were observed by Cocaign-Bousquet and Lindley (1995) and Cocaign et al. (1993), but were not attributed to stress conditions. The oxygen substrate yield ($Y_{O_2/S}$) of 0.017 $mol_{O2}/g_{lactic\,acid}$ observed in this study is identical to the one reported (Cocaign-Bousquet and Lindley 1995) for continuous cultures under conditions where the substrate is entirely consumed ($Y_{O_2/S}$ was calculated from the data depicted in the figures). The question on why the biomass substrate yield decreases with increasing lactic acid concentration, while the oxygen substrate yield stays constant, could not yet be explained. However, it may be caused by the formation of an undetected byproduct, or by a significant change in biomass composition.

Stress induced by osmolarity

In this study, an osmolarity of 1 osmol/l had no influence on the growth of *C. glutamicum*. This agrees with Rönsch (2000), who found the same results for osmolalities up to 2.0 osmol/kg for *C. glutamicum* on sucrose, but noticed a decreased growth rate at 2.5 osmol/kg. By increasing the osmolality from 0.4 to 2 osmol/kg for *C. glutamicum* on glucose, Guillouet and Engasser (1995) observed a reduc-

tion of the growth rate from 0.7 to 0.2 h^{-1} and a reduction of the biomass substrate yield coefficient from 0.6 to 0.3 $g_{biomass}/g_{glucose}$. The high tolerance of *C. glutamicum* against osmotic stress makes it unlikely, that the observed reduction of the growth rate with increased initial lactic acid concentration (10 g/l, 0.73 osmol/l; 30 g/l, 1.17 osmol/l) is caused by the increased osmolarity. The high tolerance of *C. glutamicum* against osmotic stress is caused by the possibilities of the organism to sense osmolarity and to take up and produce compatible solutes for protection (Guillouet and Engasser 1995, 1996; Peter et al. 1998; Skjerdal et al. 1996; Varela et al. 2003; Wolf et al. 2003).

Stress induced by pH

In this study, initial pH values ranging from 6.4 to 7.4 lead to uninhibited growth of *C. glutamicum*. Without adaptation, the organism did not grow at pH≤5 or pH≥9. With adaptation, however, growth was observed up to a pH of 9. Similar results were reported by Shah et al. (2002), who found lysine production of *C. glutamicum* from pH 6 to 9 with a maximum at pH 7.5. Bröer and Krämer (1991) analyzed the influence of external pH on lysine efflux and found a maximum at pH 7.8. The importance of adaptation for acid tolerance of Gram-positive organisms has been reported (Bodmer et al. 2000; Cotter and Hill 2003; O'Driscoll et al. 1996; Saklani-Jusforgues et al. 2000).

With the chosen measuring set-up, it is not possible to distinguish whether the observed growth inhibition of *C. glutamicum* at low pH is caused by the pH itself or by the undissociated form of the lactic acid. The undissociated form of lactic acid is believed to have a higher inhibitory effect (Cotter and Hill 2003; Salmond et al. 1984; Shelef 1994) than the dissociated form. At pH 5.5 and 5, about 0.0025 and 0.0075 M of the lactic acid (total concentration 0.11 M), respectively, were present in the undissociated form. Presser et al. (1997, 1998) observed for *E. coli* a complete inhibition of growth at undissociated lactate concentrations exceeding 0.0083 M regardless of the pH or the total lactate concentration. Houtsma et al. (1996) observed a total inhibition of *L. innocua* at pH 5.5 with a lactate concentration of 0.2 M of which 0.0045 M was undissociated.

Evaluation of the general method

In this study, we demonstrated the broad applicability of respiration measurements in shake flasks to study the behavior of *C. glutamicum* during and after exposure to stress. Experimental procedures were developed to study the following stress factors: carbon starvation, anaerobic conditions, organic acids, osmolarity, and pH. Similar procedures may be applied to study the response of microorganisms to stress factors like heat, oxygen, antibiotics, hormones, antibodies, or starvation of nitrogen, phosphorus, amino acids, or trace elements. The importance to run stress-response analysis without interferences during the cultivation was demonstrated by showing the influence, which inevitable interruptions of the shaker had when samples were taken. The short periods without agitation, necessary to transfer samples to new culture vessels or measuring devices, like respirometers, may lead to detrimental anaerobic conditions. Furthermore, if evidence exists, that the microorganisms show a different growth behavior in liquid cultures than on plates, respiration measurement in shake flasks is recommended.

The duration of the measuring cycle of the RAMOS device may limit the applicability of the method. The standard duration of the measuring cycle is 30 min, which can be reduced for strongly respiring cultures to 10 min. This is sufficient for most stress-response analysis because activity losses after exposure to stress mainly occur within hours or days. The new possibility to study the stress response of microorganisms in online-monitored shake flasks makes it possible to study several cultures simultaneously, and increases the possible number of experiments. The high throughput combined with the possibility to perform the entire experiment in the same culture vessel and in the same medium makes the respiration measurement in shake flasks an important tool for research and industrial process optimization.

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References

Anderlei T, Büchs J (2000) Device for sterile online measurement of the oxygen transfer rate in shaking flasks. Biochem Eng J 7:157–162

Anderlei T, Zang W, Papaspyrou M, Büchs J (2004) Online respiration activity measurement (OTR, CTR, RQ) in shake flasks. Biochem Eng J 17:187–194

Barreiro C, González-Lavado E, Pátek M, Martín J-F (2004) Transcriptional analysis of the *groES-groEL1*, *groEL2*, and *dnaK* genes in *Corynebacterium glutamicum*: characterization of heat shock-induced promoters. J Bacteriol 186:4813–4817

Barreiro C, González-Lavado E, Brand S, Tauch A, Martín J-F (2005) Heat shock proteome analysis of wild-type *Corynebacterium glutamicum* ATCC 13032 and a spontaneous mutant lacking *groEL1*, a dispensable chaperone. J Bacteriol 187: 884–889

Bodmer T, Miltner E, Bermudez LE (2000) *Mycobacterium avium* resists exposure to the acidic conditions of the stomach. FEMS Microbiol Lett 182:45–49

Breidt F Jr, Hayes JS, McFeeters RF (2004) Independent effects of acetic acid and pH on survival of *Escherichia coli* in simulated acidified pickle products. J Food Prot 67:12–18

Bröer S, Krämer R (1991) Lysine excretion by *Corynebacterium glutamicum* 1. Identification of a specific secretion carrier system. Eur J Biochem 202:131–135

Burkovski A (2003) I do it my way: regulation of ammonium uptake and ammonium assimilation in *Corynebacterium glutamicum*. Arch Microbiol 179:83–88

Cocaign-Bousquet M, Lindley ND (1995) Pyruvate overflow and carbon flux within the central metabolic pathways of *Corynebacterium glutamicum* during growth on lactate. Enzyme Microb Technol 17:260–267

Cocaign M, Monnet C, Lindley ND (1993) Batch kinetics of *Corynebacterium glutamicum* during growth on various carbon substrates: use of mixtures to localize metabolic bottlenecks. Appl Microbiol Biotechnol 40:526–530

Cotter PD, Hill C (2003) Surviving the acid test: responses of gram-positive bacteria to low pH. Microbiol Mol Biol Rev 67: 429–453

Ferreira A, O'Byrne CP, Boor KJ (2001) Role of σ^B in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in *Listeria monocytogenes*. Appl Environ Microbiol 67: 4454–4457

Gourdon P, Baucher M-F, Lindley ND, Guyonvarch A (2000) Cloning the malic enzyme gene from *Corynebacterium glutamicum* and role of the enzyme in lactate metabolism. Appl Environ Microbiol 66:2981–2987

Guillouet S, Engasser JM (1995) Growth of *Corynebacterium glutamicum* in glucose-limited continuous cultures under high osmotic pressure. Influence of growth rate on the intracellular accumulation of proline, glutamate, and trehalose. Appl Microbiol Biotechnol 44:496–500

Guillouet S, Engasser JM (1996) Growth of *Corynebacterium glutamicum* in ammonium- and potassium-limited continuous cultures under high osmotic pressure. Appl Microbiol Biotechnol 46:291–296

Gutmann M, Hoischen C, Krämer R (1992) Carrier-mediated glutamate secretion by *Corynebacterium glutamicum* under biotin limitation. Biochim Biophys Acta 1112:115–123

Gruber TM, Bryant DA (1997) Molecular systematic studies of eubacteria, using σ^{70}-type sigma factors of group 1 and group 2. J Bacteriol 179:1734–1747

Houtsma PC, Kant-Muermans ML, Rombouts FM, Zwietering MH (1996) Model for the combined effects of temperature, pH, and sodium lactate on growth rates of *Listeria innocua* in broth and bologna-type sausages. Appl Environ Microbiol 62:1616–1622

Hu Y, Coates ARM (1999) Transcription of two sigma 70 homologue genes, *sigA* and *sigB*, in stationary-phase *Mycobacterium tuberculosis*. J Bacteriol 81:469–476

Ihssen J, Egli T (2004) Specific growth rate and not cell density controls the general stress response in *Escherichia coli*. Microbiology 150:1637–1648

Inui M, Muratami S, Okino S, Kawaguchi H, Vertès AA, Yukawa H (2004) Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. J Mol Microbiol Biotechnol 7:182–196

Ishige T, Krause M, Bott M, Wendisch VF, Sahm H (2003) The phosphate starvation stimulon of *Corynebacterium glutamicum* determined by DNA microarray analyses. J Bacteriol 185: 4519–4529

Kim T-H, Park J-S, Kim H-J, Kim Y, Kim P, Lee H-S (2005) The *whcE* gene of *Corynebacterium glutamicum* is important for survival following heat and oxidative stress. Biochem Biophys Res Commun 337:757–764

Kinoshita S, Udaka S, Shimono M (1957) Amino acid fermentation. I. Production of L-glutamic acid by various microorganisms. J Gen Appl Microbiol 3:193–205

Kočan M, Schaffer S, Ishige T, Sorger-Hermann U, Wendisch VF, Bott M (2006) Two-component systems of *Corynebacterium glutamicum*: deletion analysis and involvement of the PhoS–PhoR system in the phosphate starvation response. J Bacteriol 188:724–732

Koffas MAG, Jung GY, Stephanopoulos G (2003) Engineering metabolism and product formation in *Corynebacterium glutamicum* by coordinated gene overexpression. Metab Eng 5: 32–41

Losen M, Lingen B, Pohl M, Büchs J (2004) Effect of oxygen-limitation and medium composition on *Escherichia coli* in small-scale cultures. Biotechnol Prog 20:1062–1068

Maier U, Büchs J (2001) Characterisation of the gas–liquid mass transfer in shaking bioreactors. Biochem Eng J 7:99–106

Maier U, Losen M, Büchs J (2004) Advances in understanding and modeling the gas–liquid mass transfer in shake flasks. Biochem Eng J 17:155–167

Mandel MJ, Silhavy TJ (2005) Starvation for different nutrients in *Escherichia coli* results in differential modulation of RpoS levels and stability. J Bacteriol 187:434–442

Mrotzek C, Andertei T, Henzler H-J, Büchs J (2001) Mass transfer resistance of sterile plugs in shaking bioreactors. Biochem Eng J 7:107–112

Nolden L, Ngouto-Nkili C-E, Bendt AK, Krämer R, Burkovski A (2001) Sensing nitrogen limitation in *Corynebacterium glutamicum*: the role of *glnK* and *glnD*. Mol Microbiol 42: 1281–1295

O'Driscoll B, Grahan CG, Hill C (1996) Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased virulence. Appl Environ Microbiol 62:1693–1698

Peter H, Weil B, Burovski A, Krämer R, Morbach S (1998) *Corynebacterium glutamicum* is equipped with four secondary carriers for compatible solutes: identification, sequencing, and characterization of the proline/ectoine/glycine betaine carrier, EctP. J Bacteriol 180:6005–6012

Presser KA, Ratkowsky DA, Ross T (1997) Modeling the growth rate of *Escherichia coli* as a function of pH and lactic acid concentration. Appl Environ Microbiol 63:2355–2360

Presser KA, Ross T, Ratkowsky DA (1998) Modeling the growth limits (growth/no growth interface) of *Escherichia coli* as a function of temperature, pH, lactic acid concentration, and water activity. Appl Environ Microbiol 64:1773–1779

Primm TP, Andersen SJ, Mizrahi V, Avarbock DA, Rubin H, Barry CE (2000) The stringent response of *Mycobacterium tuberculosis* is required for long-term survival. J Bacteriol 182: 4889–4898

Roe AJ, O'Byrne C, McLaggan D, Booth IR (2002) Inhibition of *Escherichia coli* growth by acetic acid: a problem with methionine biosynthesis and homocysteine toxicity. Microbiology 148:2215–2222

Rönsch H (2000) Untersuchung zum Einfluss der Osmoregulation auf die Aminosäureproduktion mit *Corynebacterium glutamicum*. Ph.D. Thesis, University Cologne, Germany

Ross T, Ratkowsky DA, Mellefont LA, McMeekin TA (2003) Modelling the effects of temperature, water activity, pH and lactic acid concentration on the growth rate of *Escherichia coli*. Int J Food Microbiol 82:33–43

Saklani-Jusforgues H, Fontan E, Goossens PL (2000) Effect of acid-adaptation on *Listeria monocytogenes* survival and translocation in a murine intragastric infection model. FEMS Microbiol Lett 193:155–159

Salmond CV, Kroll RG, Booth IR (1984) The effect of food preservatives on pH homeostasis in *Escherichia coli*. J Gen Microbiol 130:2845–2850

Shah AH, Hameed A, Ahmad S, Khan GM (2002) Optimization of culture conditions for L-lysine fermentation by *Corynebacterium glutamicum*. Online J Biol Sci 2:151–156

Shelef LA (1994) Antimicrobial effects of lactate: a review. J Food Prot 57:445–450

Silberbach M, Schäfer M, Hüser AT, Kalinowski J, Pühler A, Krämer R, Burkovski A (2005) Adaptation of *Corynebacterium glutamicum* to ammonium limitation: a global analysis using transcriptome and proteome techniques. Appl Environ Microbiol 71:2391–2402

Skjerdal OT, Sletta H, Flenstad SG, Josefsen KD, Levine DW, Ellingsen TE (1996) Changes in intracellular composition in response to hyperosmotic stress of NaCl, sucrose, or glutamic acid in *Brevibacterium lactofermentum* and *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 44:635–642

Stansen C, Uy D, Delaunay S, Eggeling L, Goergen J-L, Wendisch VF (2005) Characterization of a *Corynebacterium glutamicum* lactate utilization operon induced during temperature-triggered glutamate production. Appl Environ Microbiol 71:5920–5928

Stöckmann C, Maier U, Anderlei T, Knocke C, Gellissen G, Büchs J (2003) The oxygen transfer rate as key parameter for the characterization of *Hansenula polymorpha* screening cultures. J Ind Microbiol Biotechnol 30:613–622

Varela C, Agosin E, Baez M, Klapa M, Stephanopoulos G (2003) Metabolic flux redistribution in *Corynebacterium glutamicum* in response to osmotic stress. Appl Microbiol Biotechnol 60:547–555

Watson SP, Clements MO, Foster SJ (1998) Characterization of the starvation-survival response of *Staphylococcus aureus*. J Bacteriol 180:1750–1758

Wehmeier L, Schäfer A, Burkovski A, Krämer R, Mechold U, Malke H, Pühler A, Kalinowski J (1998) The role of the *Corynebacterium glutamicum rel* gene in (p)ppGpp metabolism. Microbiology 144:1853–1862

Wehmeier L, Brockmann-Gretza O, Pisabarro A, Tauch A, Pühler A, Martin JF, Kalinowski J (2001) A *Corynebacterium glutamicum* mutant with a defined deletion within the rplK gene is impaired in (p)ppGpp accumulation upon amino acid starvation. Microbiology 147:691–700

Wendisch VF, DeGraaf AA, Sahm H, Eikmanns BJ (2000) Quantitative determination of metabolic fluxes during coutilization of two carbon sources: comparative analyses with *Corynebacterium glutamicum* during growth on acetate and/or glucose. J Bacteriol 182:3088–3096

Wolf A, Krämer R, Morbach S (2003) Three pathways for trehalose metabolism in *Corynebacterium glutamicum* ATCC 13032 and their significance in response to osmotic stress. Mol Microbiol 49:1119–1134

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