Glucose catabolism via a partially cyclic pentose phosphate pathway in Gluconobacter oxydans 621H: a combined fluxomics and transcriptomics analysis

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In this study, the distribution and regulation of periplasmic and cytoplasmic carbon fluxes in *Gluconobacter oxydans* 621H with glucose were studied by $^{13}$C-based metabolic flux analysis ($^{13}$C-MFA) in combination with transcriptomics and enzyme assays. For $^{13}$C-MFA, cells were cultivated with specifically $^{13}$C-labeled glucose and intracellular metabolites were analyzed for their labeling pattern by LC-MS. In growth phase I, 90% of the glucose was oxidized periplasmically to gluconate and partially further oxidized to 2-ketogluconate. Of the glucose taken up by the cells, 9% was phosphorylated to glucose 6-phosphate, whereas 91% was oxidized by cytoplasmic glucose dehydrogenase to gluconate. Additional gluconate was taken up into the cells by transport. Of the cytoplasmic gluconate, 70% was oxidized to 5-ketogluconate and 30% was phosphorylated to 6-phosphogluconate. In growth phase II, 87% of gluconate was oxidized to 2-ketogluconate in the periplasm and 13% was taken up by the cells and almost completely converted to 6-phosphogluconate. Since *G. oxydans* lacks phosphofructokinase, glucose 6-phosphate can only be metabolized via the oxidative pentose phosphate pathway (PPP) or the Entner-Doudoroff pathway (EDP). $^{13}$C-MFA showed that 6-phosphogluconate is catabolized primarily via the oxidative PPP in both phase I and II (62% and 93%) and demonstrated a cyclic carbon flux through the oxidative PPP. The transcriptome comparison revealed an increased expression of PPP genes in growth phase II, which was supported by enzyme activity measurements and correlated with the increased PPP flux in phase II. Moreover, genes possibly related to a general stress response displayed increased expression in growth phase II.
The strictly aerobic acetic acid bacterium *Gluconobacter oxydans* is industrially applied for the production of vitamin C, L-sorbose, 2-ketogulonic acid, dihydroxyacetone and 6-amino-L-sorbose. However, *G. oxydans* grows only to low cell densities, which has been explained by an inefficient respiratory chain (20). Membrane-bound dehydrogenases enable *G. oxydans* to oxidize sugars and sugar alcohols in two or more steps in the periplasm (17, 20) (Fig. 1). Intermediates and products of these reactions accumulate in the medium. In parallel, part of the substrates or its oxidation products is taken up into the cytoplasm. In the case of glucose, uptake occurs by a yet unknown transport system and the oxidation product gluconate is imported by gluconate permease (GOX2188) (28).

As *G. oxydans* lacks phosphofructokinase, the Embden-Meyerhof-Parnas pathway (EMP) is interrupted. Intracellular glucose is either oxidized to gluconate and 5-ketogluconate by NAD(P)-linked dehydrogenases or glucose and gluconate are phosphorylated by glucose and gluconate kinases, respectively, and further catabolized via the pentose phosphate pathway (PPP) or the Entner-Doudoroff pathway (EDP) (4, 5, 14). In the absence of phosphofructokinase, the PPP is expected to operate partly cyclic, as fructose 6-phosphate formed by transaldolase or transketolase is isomerized to glucose 6-phosphate, which enters the oxidative PPP again (16, 38). Formally, this conversion can be described as follows:

\[
\text{Glucose} + \text{ADP} + \text{P}_i + \text{NAD}^+ + 6 \text{NADP}^+ \rightarrow \text{ATP} + \text{NADH} + 6 \text{NADPH} + 3 \text{CO}_2 + \text{pyruvate}^- + 8 \text{H}^+. 
\]

Due to absence of genes coding for succinate dehydrogenase, the tricarboxylic acid cycle (TCA) is also incomplete and fulfills exclusively biosynthetic functions (9, 15, 28).

Growth of *G. oxydans* on glucose proceeds in two different metabolic phases. In phase I, glucose is rapidly oxidized to gluconate by the membrane-bound glucose dehydrogenase (GdhM). In growth phase II, gluconate is further oxidized to ketogluconates. In dependence of the pH value of the culture medium, 5-ketogluconate and 2-ketogluconate are formed in the periplasm by the membrane-bound major polyol dehydrogenase and gluconate 2-
dehydrogenase, respectively (1). At pH 6, the pH value of the culture medium applied in the present work, formation of 2-ketogluconate is predominant in the periplasm (20).

In the present study, $^{13}$C-based metabolic flux analysis ($^{13}$C-MFA) was applied in order to elucidate the relative contributions of the PPP and the EDP to cytoplasmic glucose catabolism and to test the proposed cyclic flux of carbon through the oxidative PPP. The most common variant of $^{13}$C-MFA uses GC-MS to derive labeling information available in protein-bound amino acids. In this case, the utilization of a defined minimal medium is imperative to avoid erroneous interpretation of the mass isotopomer fractions (3). As a suitable minimal medium is not yet available for G. oxydans and in order to minimize influences of the medium, highly sensitive LC-MS was applied in this study for the detection of intracellular isotopomer concentrations of almost all intermediates of central metabolism down to the nanomolar range (18). Utilization of these primary metabolites for $^{13}$C-MFA rather than protein-bound amino acids prevents an increased uncertainty in the metabolic network, e.g., due to incomplete metabolic reconstruction, yet unknown carbon-atom transitions, or the need for considering putative carbon source uptake routes.

Complementary to $^{13}$C-MFA, we used genome-wide DNA microarrays to study the changes of global gene expression between growth phase I and II. Despite the long-time use in biotechnology, the regulation of carbon and energy metabolism in G. oxydans is largely unknown. Based on the genome sequence of strain 621H (DSM2343) (28), we recently applied DNA microarrays to analyze the influence of pH and oxygen limitation on global gene expression and to study mutants defective in the PPP or the EDP (11, 33).

**MATERIALS AND METHODS**

**Chemicals and enzymes.** $^{1,13}$C-Glucose and U-$^{13}$C-glucose was obtained from Deutero GmbH (Kastellaun, Germany). Other chemicals as well as auxiliary enzymes (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase from yeast) for enzyme
activity assays were purchased from Sigma-Aldrich (Taufkirchen, Germany) and Merck (Darmstadt, Germany).

**Bacterial strains, culture conditions, and bioreactor system.** *Gluconobacter oxydans* DSM 2343 (ATCC 621H) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Precultures of the strain were cultivated on complex medium containing 5 g l⁻¹ yeast extract, 2.5 g l⁻¹ MgSO₄ x 7 H₂O, 1 g l⁻¹ (NH₄)₂SO₄, 1 g l⁻¹ KH₂PO₄, 0.5 g l⁻¹ glycerol and 8% (w/v) sorbitol. The initial pH value of the medium was 6.0. *G. oxydans* possesses a natural resistance towards cefoxitin; as a precaution to prevent bacterial contaminations, cefoxitin was added to the media at a concentration of 50 μg ml⁻¹. Precultures were grown in baffled shaking flasks at 30°C and 140 rpm. For DNA microarray analysis and enzyme activity measurements, cells were cultivated in 250 ml of the same medium containing 8% glucose instead of sorbitol in a bioreactor system (DASGIP, Jülich, Germany) composed of four 400-ml vessels, each equipped with electrodes for measuring the dissolved oxygen concentration (DO) and the pH value. The system allows to constantly control these two parameters. The pH was kept at pH 6.0 by automatic titration of 2 M NaOH. The oxygen availability was kept constant at 15% DO by mixing air, O₂ and N₂. Calibration was performed by gassing with air (100% DO) and N₂ (0% DO). The agitation speed was kept constant at 900 rpm. The carbon dioxide concentration in the exhaust air was measured continuously by an infrared spectrometer and the oxygen concentration with a zirconium dioxide sensor.

Control and recording of all data was carried out by the software “Fedbatch Pro” (DASGIP, Jülich, Germany). For metabolic flux analysis cells were grown in a 200 ml volume of the same medium containing 8% glucose of the following optimized composition: 4.0% naturally labeled glucose, 7.7% 1-¹³C-glucose, and 88.3 % U-¹³C-glucose. A reference culture with 100% naturally labeled glucose was used for comparison.
**HPLC analysis and glucose determination.** Gluconate, 5-ketogluconate (5-KGA) and 2-ketogluconate (2-KGA) were analyzed by high performance liquid chromatography (HPLC). 1 ml culture was centrifuged for 5 min at 13,000 g and the supernatant was filtered through a 0.2 µm filter (Millipore, MA, USA) prior to HPLC analysis. The substances were separated using a Shodex DE 613 150 x 0.6 column (Phenomenex, Aschaffenburg, Germany) using 2 mM HClO₄ as eluant at a flow rate of 0.5 ml min⁻¹ and detected by an UV spectrophotometer at 210 nm. Glucose co-eluted with gluconate, but at this wavelength, glucose does not absorb and therefore gluconate analysis was not distorted. Glucose concentrations were determined enzymatically with glucose dehydrogenase by application of a kit (DiaSys Diagnostic Systems GmbH, Holzheim, Germany) according to the instructions of the manufacturer.

**Determination of extracellular rates.** A bioreactor model of the batch cultivation process was set up to calculate rates for growth on glucose and gluconate ($\mu_{GLC}$, $\mu_{GLCN}$), consumption and formation rates of glucose, gluconate, 2- and 5-ketogluconates as well as carbon dioxide from the glucose enzymatic test results and HPLC data (Supplement 1). This process model contains only two compartments divided by outer membrane, i.e., the extracellular compartment [ex] and the remainder, periplasm plus cytoplasm ([p]+[c]) (cf. Fig. S1.1). O₂ consumption rates and CO₂ production rates of growing cells were measured by the DASGIP bioreactor system. Because $^{13}$C-labeled carbon dioxide was not quantitatively detectable by the employed infrared spectroscopy method, carbon dioxide production rates were obtained from the reference culture grown with naturally labeled glucose. Rate estimations were then used to calculate specific extracellular rates, which were, in turn, used for $^{13}$C-MFA.

**Sampling and sample processing for LC-MS analysis.** For LC-MS analysis, 50 ml culture was harvested in the first growth phase (after 8 h, sample point I) and 15 ml culture in the second growth phase (after 14.25 h, sample point II), and mixed immediately with 150 ml or 45 ml 60% methanol at -80°C in order to stop metabolism. The mixtures were centrifuged for 5 min at 10,000 g and -20 °C and each cell pellet was resuspended in 1 ml pure methanol.
(-70°C) and 1 ml TE buffer (pH 7.0). After vortexing 2 ml chloroform (-20°C) was added. The suspension was shaken at -20°C for two hours and then centrifuged 10 min at 10,000 g at -20°C. The upper methanol phase was filtrated through a 0.2 µm filter (Millipore, MA, USA) and frozen at -80°C for subsequent LC-MS analysis.

**LC-MS analysis of intracellular metabolites.** Intracellular metabolites were extracted by a methanol/chloroform procedure described elsewhere (41). Cell extraction samples were analyzed with an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) coupled with an API4000 mass spectrometer (Applied Biosystems, Concord, Canada) equipped with a Turbolon spray source. Detailed information on separation methods have been reported previously (18, 39). The LC-MS data were analyzed as described previously (24). Briefly, the mass isotopomer fractions of the intermediates 1,3-bisphosphoglycerate, 2-phosphoglycerate, 2-oxoglutarate, 6-phosphogluconate, aconitate, citrate/isocitrate, dihydroxyacetone phosphate, erythrose 4-phosphate, fructose 1,6-diphosphate, fructose 6-phosphate, fumarate, gluconolactone, glucose 6-phosphate, glyceraldehyde 3-phosphate, malate, phosphoenolpyruvate, pyruvate, ribose 5-phosphate, ribulose 5-phosphate/xyulose 5-phosphate, sedoheptulose 7-phosphate, succinate, and the free amino acids alanine, arginine, aspartate, glutamate, histidine, leucine, lysine, methionine, phenylalanine, proline, tyrosine, and valine were determined from the respective mass spectra (Supplement 2).

**13C-metabolic flux analysis.** For 13C-labeling experiments, cells were cultivated with the 13C-labeled glucose mixture described above. Metabolic stationarity in the cells was approximately maintained. Samples taken after 8 h and 14.25 h were analyzed by LC-MS to determine the mass isotopomer patterns of the intracellular intermediates listed above. Accompanied by the estimations of consumption and formation rates, 13C-MFA enabled a detailed quantification of intracellular in vivo carbon fluxes from intracellular metabolites’ labeling patterns. 13C-MFA is a model-based approach and for more details the reader is referred to recent review papers (35, 43). Here we differentiated between three compartments:
the medium external to the cells (termed extracellular, [ex]), the cytoplasm ([c]) and periplasm ([p]).

Based on the genome information for *G. oxydans* 621H, a metabolic network model of central metabolism was formulated (Fig. S3, Table S3.1). This model included reactions of the EMP, the PPP, the EDP, and the TCA cycle, as well as all membrane-bound and cytoplasmic dehydrogenase reactions involved in glucose catabolism that are characteristic for *G. oxydans* (Fig. 1). Altogether, the model covers 57 reactions, of which 18 are reversible. Additional uptake reactions for naturally labeled fumarate (FUM), acetyl-CoA (ACCOA) and glutamate (GLUT) were added to the metabolic network allowing the utilization of components of the yeast extract in central metabolism. Aiming at a more detailed quantification of the metabolic conversion rates, a focused network without the reactions of the TCA cycle was deduced. The focused network contained 40 reactions, of which 15 are reversible (Supplement 3). In total, up to 34 model parameters (degrees of freedom) had to be estimated using 80 labeling measurements (mass isotopomers of intracellular metabolites detected by LC-MS, Fig. S2) and five carbon exchange flux values (glucose uptake, gluconate uptake, ketogluconate excretion, and carbon dioxide production rate) estimated from the process model (Table S1.1). The metabolite pools of phosphoglycerates (1,3-bisphosphoglycerate (1,3PG), 2-phosphoglycerate (2PG), and 3-phosphoglycerate (3PG) were not separable by LC-MS and, thus, lumped into a single phosphoglycerate pool (PGP). The software toolbox 13CFLUX2 (http://www.13cflux.net) (42) was used for all modeling and evaluation steps (43).

**Preparation of cell-free extracts and enzyme assays.** For *in vitro* determinations of enzyme activities, culture samples (50 ml) were taken after 8 h and 14.25 h, i.e. at the same time points used to take samples for LC-MS analysis, and centrifuged at 8,000 g for 10 min. After washing once in 0.9% NaCl, cells were resuspended in 60 mM Tris/HCl, 13 mM MgCl₂, 1 mM DTT, pH 7.5 (10 ml g⁻¹ cell wet weight). Cells were disrupted by sonification.
(3 min using cycle 0.5 and amplitude 70; UP 200s sonifier, Dr. Hielscher, Stuttgart, Germany,) in an ice bath. After centrifugation at 10,000 g for 2 min the supernatant was used as cell-free extract.

The determination of glucose kinase and gluconate kinase activity was performed with different dilutions of cell-free extract at 30°C by a coupled enzyme assay according to (8). Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities were measured at 30°C by a standard method (23). Again, different dilutions of cell-free extract were applied. Since 6-phosphogluconate dehydrogenase is an NAD⁺-preferring enzyme in *G. oxydans*, 0.1 mM NAD⁺ was used instead of NADP⁺ for activity determination (31, 40). The protein concentration of the cell extracts was determined by the method of Bradford (2) using bovine serum albumin as standard.

**DNA microarray analysis.** RNA preparation, cDNA labeling and DNA microarray analysis were performed as described recently by Hanke et al. (11). The samples for DNA microarray analysis were taken after 8 h and 14.25 h and three biological replicates were performed.

**Microarray data accession number.** The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE42223 (private status during review process).

**RESULTS**

**Substrate conversion in the periplasm, oxygen consumption and carbon dioxide production during growth of *G. oxydans* 621H with glucose.** In Fig. 1A, growth of *G. oxydans* in a medium containing 80 g l⁻¹ glucose, 0.5 g l⁻¹ glycerol, and 5 g l⁻¹ yeast extract is shown. Two independent cultures containing ¹³C-labeled glucose (for details see Materials and Methods) showed identical growth behavior and substrate oxidation rates as the reference culture containing naturally labeled glucose (Fig. 1A). Samples for carbon flux analysis were
taken after 8.0 h (sample point I) and after 14.25 h (sample point II). After 8 h, the cultures had an OD$_{600}$ of ~3.6, and used glucose as major substrate. After 14.25 h, the cultures had an OD$_{600}$ of ~7.6 and used gluconate as major substrate. With the exception of CO$_2$ production, which could be quantified only for the culture containing naturally labeled glucose, triplicate data sets were available for extracellular rates, whereas $^{13}$C-labeling data were obtained in duplicate. Because isotopic substitution will affect the distribution of vibrational and rotational energy states of a molecule, each distinct isotopomer of CO$_2$ has its own rotational-vibrational infrared spectrum (7). Therefore, $^{13}$C-labeled carbon dioxide was not quantitatively detectable by the employed infrared spectroscopy method and a reference culture with unlabeled glucose was necessary to obtain correct carbon dioxide production rates, which could be applied for flux analysis. As shown by the measurements’ standard deviations, parallel cultivation under controlled conditions allowed for collection of comprehensive and reproducible data (biological replicates) suitable for model-based $^{13}$C-MFA. The carbon balance of the averaged cultivations amounted to 101% (Table S4.1).

Extracellular rates of substrate consumption and product formation were estimated consistently with a batch bioreactor process model (Fig. S1.2A). In the first growth phase, a growth rate $\mu_{GLC}$ of 0.27 h$^{-1}$ was measured until an OD$_{600}$ of 6.6 was reached after 10 h, which corresponds to 80% of the maximal OD observed at the end of the cultivation.

At the end of growth phase I after 10 h the extracellular concentrations of glucose, gluconate and 2-ketogluconate were 49 mM, 264 mM and 63 mM, respectively. 2-Ketogluconate was formed in the periplasm by the membrane-bound gluconate 2-dehydrogenase (Gad2) (37) and accumulated in the medium. Gluconate 2-dehydrogenase has its optimum pH at 6 (37). Besides 2-ketogluconate, also 5-ketogluconate was detected (30 mM), which presumably was formed intracellularly by gluconate 5-dehydrogenase and then exported into the medium. The alternative possibility that 5-ketogluconate was formed in the periplasm by the membrane-bound major polyol dehydrogenase (GOX0854-0855) is unlikely,
as this enzyme has its optimum activity for this reaction at acidic pH values of 3.5-4.0 (1, 19, 22). The cytoplasmic formation of 5-ketogluconate is in accordance with the $^{13}$C-MFA-based prediction.

In the second growth phase, which was characterized by a very low growth rate ($\mu_{\text{GLC}} = 0.002 \, \text{h}^{-1}$), gluconate was oxidized to 2-ketogluconate. This reaction proceeded at a lower velocity than the oxidation of glucose to gluconate (Fig. 1A). The specific activity of gluconate 2-dehydrogenase indeed is 70–80% lower than that of the membrane-bound glucose dehydrogenase, as determined with intact cells using a Clark oxygen electrode (results not shown). As discussed above, the oxidation to 5-ketogluconate by the major polyol dehydrogenase was prevented by keeping the pH at 6. In both, the first and second oxidation phase, 337 mM 2-ketogluconate was formed and 432 mM O$_2$ was consumed. NADH generated in the cytoplasm probably reduced the residual 95 mM O$_2$.

Hence, the main energy supply of the cells originated from substrate oxidation in the periplasm. Biomass production in the second growth phase was only one fourth (0.38 g$_{\text{cdw}}$ l$^{-1}$) of that formed in the first growth phase (1.5 g$_{\text{cdw}}$ l$^{-1}$), although the concentration of accumulated gluconate at the beginning of the second growth phase was more than two thirds of the initial 80 g l$^{-1}$ glucose. This indicates that energy generation by gluconate oxidation to ketogluconate is much lower than by oxidation of glucose to gluconate. Growth stopped before gluconate oxidation was completed.

Parallel to biphasic growth the oxygen consumption rate also showed two maxima (Fig. 1B). In growth phase I, the cells rapidly consumed oxygen, whereas in growth phase II the oxygen consumption rate was much slower. This correlates with the fast oxidation of glucose to gluconate and the slow oxidation of gluconate to 2-ketogluconate (Fig. 1B). The carbon dioxide production rate was also biphasic (Fig. 1C), but its course was contrary to that of the oxygen consumption rate, as the rate was higher in growth phase II than in growth phase I, for which a constant rate of 6.5 mmol carbon dioxide h$^{-1}$ g$_{\text{cdw}}$ l$^{-1}$ was estimated. The total CO$_2$...
produced in phase II (213 mM) was 8-fold higher than that in phase I (27 mM) (Fig. 1B). This increase in carbon dioxide production was an outcome of an activated, cyclic PPP as shown by $^{13}$C-MFA described below.

$^{13}$C-labeling patterns of intracellular metabolites. As described before, cells were fed with specifically labeled $^{13}$C-glucose and mass isotopomer distributions of intracellular metabolites were quantified at the two sample points using LC-MS (cf. Tables S2.1, S2.2). The MS analysis showed that labeling information was predominantly distributed in intermediates of the EMP and the PPP (Fig. 2). Notably, for these metabolites, no significant changes in $^{13}$C labeling patterns between sample point I and II were observed. In contrast to EMP and PPP intermediates, almost no $^{13}$C enrichment from labeled glucose was measured for TCA cycle intermediates. Considerable amounts of unlabeled fumarate and malate were detected. This indicates formation of these metabolites from amino acids present in the yeast extract. Despite the fact that *G. oxydans* 621H lacks genes for succinyl-CoA synthetase and succinate dehydrogenase, also mainly unlabeled succinate was detected by the LC-MS analysis. It is might be formed from glutamate or glutamine present in the yeast extract by conversion to $\alpha$-ketoglutarate, which then is either oxidatively decarboxylated to succinyl-CoA, which is quite unstable and can decompose spontaneously, or by conversion of $\alpha$-ketoglutarate to succinate semialdehyde by $\alpha$-ketoglutarate decarboxylase (GOX0882) and subsequent oxidation of succinate semialdehyde to succinate by succinate semialdehyde dehydrogenase (GOX1122, GOX0499). To summarize, the isotope labeling data indicated that the TCA cycle intermediates were predominantly derived from the catabolism of amino acids in the yeast extract. All measurable amino acids were found to be purely naturally labeled in both phases (Fig. 2), which can be expected during cultivation in a medium containing yeast extract.

Although we incorporated into our model (Fig. 2) reactions in which TCA cycle intermediates were formed from naturally labeled yeast extract components, the low $^{13}$C-
labeling of citrate/isocitrate, aconitate, 2-oxoglutarate and succinate could not be reasonably explained. This discrepancy might be accounted for e.g. by assuming that part of the pyruvate is converted acetate or by assuming that labeling was not yet equilibrated in TCA intermediates at the sampling time point (8.0 h after the switch from naturally labeled glucose to $^{13}$C-labeled glucose mixture). The latter assumption is supported by the fact that most TCA cycle intermediates showed a higher labeling enrichment after 14.25 h than after 8 h. Consequently, the TCA cycle intermediates were excluded from metabolic flux analysis. Instead, a reduced model was used where effluxes from PEP and pyruvate represent fluxes to TCA, biomass and by-products like acetate (cf. Fig. 3).

**Branching of intracellular carbon fluxes between non-phosphorylated and phosphorylated compounds.** For sample points I and II intracellular fluxes from glucose to pyruvate were estimated using the extracellular flux rates determined with the bioreactor model (cf. Supplement 1) and the $^{13}$C labeling patterns of EMP and PPP intermediates of sample points I and II, respectively (Fig. 1A; Fig. 2). Repeated flux estimation with randomly chosen initial values showed a reproducibly well agreement of measurements and model predictions for all reactions of the EMP, PPP and EDP.

The small amount of glucose taken up by the cells (9.8%) at 8.0 h (sample point I) was primarily converted to gluconate (91.3%) by the cytoplasmic glucose dehydrogenase (GdhS) and gluconolactonase (Pgl) (Fig. 3A). Only 8.7% of the glucose taken up was phosphorylated to glucose 6-phosphate by glucose kinase (Glk). 69.9% of the intracellular gluconate was converted to 5-ketogluconate by the NADP-dependent gluconate 5-dehydrogenase (32) and exported out of the cell. A smaller fraction was phosphorylated by gluconokinase (GntK) to 6-phosphoglucuronate, an intermediate common to both the PPP and the EDP. Hence, the sum of fluxes through Glk and GntK is the amount of carbon captured by the cells, i.e. only 5.3% of the total glucose metabolized.
Measured *in vitro* enzyme activities revealed a Glk activity of 86 nmol min\(^{-1}\) mg\(^{-1}\), agreeing well with the value of 60 nmol min\(^{-1}\) mg\(^{-1}\) reported by Pronk et al. (27). The same authors reported activities of 4000 and 150 nmol min\(^{-1}\) mg\(^{-1}\) for GdhM and NADP-dependent GdhS, respectively, indicating that the cytoplasmic capacity for glucose dehydrogenation is only 3.8% of the membrane-bound capacity. Thus, *in vitro* determinations of enzyme activities are in agreement with our model prediction of the *in vivo* situation of carbon flux at sample point I.

At sample point II, glucose uptake almost ceased due to the depletion of glucose from the culture medium. Instead, at 14.25 h gluconate was consumed with 16.9% of the glucose consumption rate at 8.0 h, whereas the glucose consumption rate dropped to almost zero (Fig. 3B). Contrary to the situation at sample point I, almost all intracellular gluconate was captured by phosphorylation to 6-phosphogluconate (98.8%).

**Intracellular carbon fluxes of phosphorylated compounds.** High fluxes in the oxidative part of the PPP, mediated by glucose 6-phosphate dehydrogenase (Zwf), 6-phosphogluconate dehydrogenase (Gnd), transaldolase (Tal) and transketolase (Tkt) in the direction of fructose 6-phosphate formation, were indicative for a major role of the PPP in glucose catabolism of *G. oxydans*. Setting the carbon fluxes at sample point I to 100% at the level of 6-phosphogluconate, 62% of the carbon flux was directed to Gnd and 38% was directed to 6-phosphogluconate dehydratase (Edd) (Fig. 3A). Consequently, the metabolic activity of the EDP was much lower than that of the PPP at sample point I. At sample point II, the same calculation resulted in an even stronger preference for the PPP, since here 93% of the carbon flux was directed to Gnd and only 7% to Edd (Fig. 3B).

**Operation of a cyclic PPP in *G. oxydans*.** \(^{13}\)C-MFA revealed a high flux for the glucose 6-phosphate isomerase-catalyzed reaction (Pgi) in the direction from fructose 6-phosphate to glucose 6-phosphate, i.e. contrary to the direction used in glycolysis. At sample points I and II the net-negative fluxes through Pgi were 91% and 176% of the sum of fluxes via glucose
kinase and gluconate kinase. This result shows that due to the lack of phosphofructokinase, fructose 6-phosphate formed in the PPP is isomerized to glucose 6-phosphate and enters the oxidative PPP again. Consequently, a partially cyclic flow of carbon through the PPP occurs and 1 mol glucose 6-phosphate is converted to 1 mol pyruvate and three mol carbon dioxide (Fig. 3AB). Since at sample points I and II 38% and 7% of the total 6-phosphogluconate was diverted to the EDP, in which no carbon dioxide is produced, the actual CO₂ per glucose/gluconate yield should be lower than three. However, the measured amount of carbon dioxide was 20% higher than the one calculated from the cytoplasmic metabolism of glucose described above (Table S4.2). This discrepancy is most likely due to the metabolism of components present in the yeast extract and of glycerol.

In accordance with the fact that the carbon flux downstream of the PPP is continued at the level of glyceraldehyde 3-phosphate, very low fluxes were observed for the reactions of fructose 1,6-bisphosphatase (Fbp), fructose 1,6-bisphosphate aldolase (Fba) and triosephosphate isomerase (Tpi) (Fig. 3AB).

**Comparison of global gene expression and selected enzyme activities in growth phases I and II.** The results described above showed that glucose is mainly oxidized in the periplasm to gluconate (growth phase I) and subsequently to 2-ketogluconate (growth phase II). Glucose and gluconate taken up into the cytoplasm are predominantly catabolized via a partially cyclic PPP, notably in growth phase II. To correlate the carbon fluxes with global gene expression and *in vitro* activities of selected enzymes, cells were harvested for RNA isolation and preparation of cell-free extracts after 6.5 h (OD₆₀₀ = 2.2, sample point I) and after 14 h (OD₆₀₀ = 6.0, sample point II), respectively (Fig. S5). The cultivations were performed in triplicate starting from independent precultures. RNA was prepared from the six samples and used for comparative DNA microarray analysis as described recently (11). In total, 454 genes showed differential expression: 227 genes had an mRNA ratio (sample point II/sample point I) of ≥2.0 and 227 genes had an mRNA ratio of ≤0.5. These genes are listed in Table S6. Selected genes
... (including operons) are described in the following paragraphs based on a functional categorization (Table 2).

**Genes involved in respiration and energy metabolism.** Many of the genes whose expression was influenced after the transition to growth phase II are involved in respiration and ATP synthesis. Several genes encoding proteins that feed electrons into the respiratory chain showed increased mRNA levels at sample point II, such as those for a PQQ-containing myo-inositol dehydrogenase (GOX1857, mRNA ratio 12.0) (12), for the membrane-bound gluconate 2-dehydrogenase (GOX1230-1231, mRNA ratios 2.8 and 2.3), for the type II NADH dehydrogenase (GOX1675, mRNA ratio 3.0), and for the membrane-bound glycerol 3-phosphate dehydrogenase (GOX2088, mRNA ratio 4.5). One of the two terminal oxidases of the respiratory chain of *G. oxydans*, the cytochrome *bd* ubiquinol oxidase (GOX0278-0279, mRNA ratio 2.7 and 1.8), was also upregulated in growth phase II.

The genes encoding pyridine nucleotide transhydrogenase (*pntA1A2B*, GOX0310-0312, mRNA ratios 4.4-6.0) belonged to the most strongly up-regulated genes at sample point II. Transhydrogenase PntAB is located in the cytoplasmic membrane of many bacteria and couples the reduction of NADP⁺ by NADH to the import of protons across the membrane. Transhydrogenase can operate reversibly, i.e. either consumes the electrochemical proton gradient across the cytoplasmic membrane for NADP⁺ reduction or builds up an electrochemical proton gradient at the expense of NADPH oxidation (13). The genes downstream of *pntB*, GOX0313 and GOX0314, showed comparable mRNA ratios (5.5-4.8) as the *pntA1A2B* genes, suggesting that these five genes might form an operon. The two genes encode zinc-containing alcohol dehydrogenases of which one (GOX0313) was recently characterized (36)

The *G. oxydans* genome contains three gene clusters coding for subunits of F₁F₀-ATP synthases. The clusters GOX1110-1113 and GOX1310-1314 encode the subunits of the F₀ part and the F₁ part of an ATP synthase, which is an ortholog of the ATP synthases of
Acetobacter pasteurianus IFO 3283-01, Gluconacetobacter diazotrophicus PAI 5 and other α-proteobacteria. Both these clusters showed a decreased expression at sample point II (mRNA ratio 0.4-0.5). The genes of the third cluster, GOX2167-2175, might code for a Na\(^{+}\)-translocating F\(_{1}\)F\(_{0}\)-ATP synthase (6) and showed an increased expression at sample point II (mRNA ratio 1.4-3.3).

Genes involved in metabolism (Fig. 4). Differentially expressed genes encoding enzymes involved in central metabolism all showed elevated mRNA ratios during growth on gluconate in phase II: glucose 6-phosphate dehydrogenase (zwf, GOX0145, ratio 2.8), 6-phosphogluconate dehydrogenase (gnd, GOX1705, ratio 2.7), bifunctional transaldolase/phosphoglucose isomerase (pgi/tal, GOX1704, ratio 2.9), transketolase (tkt, GOX1703, ratio 2.7), and fructose 1,6-bisphosphate aldolase (fba, GOX1540, ratio 3.2). Also the genes for triosephosphate isomerase (tpi) and the four adjacent genes, encoding ribose 5-phosphate isomerase, a ribose ABC transporter and dihydroxyacetone kinase (GOX2217-2222, mRNA ratio 10.8-1.9) showed increased expression in growth phase II.

The genes encoding a glycerol facilitator (GOX 2089, mRNA ratio 3.9) and glycerol kinase (GOX3090, mRNA ratio 4.6), which presumably form an operon with GOX2088 (the membrane-bound glycerol 3-phosphate dehydrogenase, GOX2088, mRNA ratio 4.5), also showed increased mRNA levels, which could suggest that the glycerol in the cultivation medium (0.5 g l\(^{-1}\)) is metabolized preferably in the second growth phase. A model variant allowing for glycerol uptake estimated a vanishing low uptake rate of glycerol whereas the fit was not improved (data not shown). Therefore, this model variant was not regarded further. Possibly, glycerol catabolism is repressed in the presence of glucose in growth phase I, which could be due to a regulatory function of the rudimentary PTS system (see below).

Among the genes with a lowered expression level at sample point II was phosphogluconate dehydratase (edd, GOX0431, mRNA ratio 0.4), the first of the two key enzymes of the EDP. Furthermore, three genes encoding enzymes of the TCA cycle showed decreased mRNA

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ratios, aconitate hydratase (acn, GOX1335, ratio 0.4), isocitrate dehydrogenase (icd, GOX1336, ratio 0.3), and fumarate hydratase (fumC, GOX1643, ratio 0.5).

Genes involved in transport. In accordance with gluconate being the carbon source in growth phase II, the gene encoding gluconate permease (GOX2188, mRNA ratio 3.5) showed increased expression. Due to the lack of the EII$^B$ and EIIC components, the PTS system in G. oxydans is considered to be inactive as a transport system (28) and the function of the remaining components EI, HPr, EIIA is not yet clear. The genes encoding these latter proteins (GOX0812-0816) had increased expression levels (mRNA ratio 1.4-2.8) in growth phase II, showing that they are subject to transcriptional regulation.

Genes involved in regulation and signal transduction. 13 genes coding for transcriptional regulators had increased mRNA levels at sample point II, ten of them coding for one-component transcriptional regulators of different families and three coding for response regulators of two-component signal transduction systems. In addition, expression of the gene coding for sigma factor $\sigma^{32}$ (sigH, GOX0506, mRNA ratio 4.8) was increased in growth phase II. Five transcriptional regulator genes showed decreased expression. Whereas the target genes of the transcriptional regulators and the stimuli they respond to have not been identified yet, $\sigma^{32}$ of other bacteria is known to be induced under different stress conditions and to activate expression of genes required to counteract these stresses (26). As shown below, expression of several stress genes was induced during growth with gluconate in phase II, which might be due to the activity of $\sigma^{32}$.

Genes involved in stress responses. The highest expression change at all of this genome-wide transcriptional analysis was displayed by GOX2079 (mRNA ratio 31.7). The gene product possesses a GsiB domain often found in stress-induced proteins and might be related to a general starvation protein, as found in Bacillus species (21). Several other genes involved in different types of stress responses, such as heat shock (GOX1329, ratio 18.3; GOX2397, ratio 4.8; GOX1837, ratio 2.8), cold shock (GOX0833, ratio 4.7; GOX2163, ratio 3.0), or...
oxidative stress (GOX1879, ratio 3.6), the chaperons DnaK (GOX0857, ratio 2.3) and DnaJ (GOX1414, ratio 2.3), as well as two genes encoding components of Clp ATPases (GOX0608, ratio 2.4; GOX0609, ratio 1.8) were found to have increased mRNA levels at sample point II, which might be related to the increased expression of the σ^{32} gene. As the cells face neither heat nor cold stress under the cultivation conditions used, the induction of these genes might represent a kind of general stress response triggered by the decreased growth rate with gluconate.

**Genes involved in motility.** Only one of the 29 genes involved in flagella biosynthesis and function, showed an increased expression (fliL, GOX0697, mRNA ratio 2.7) and none of the 13 chemotaxis-assigned genes present in the genome of *G. oxydans* 621H were differentially regulated, suggesting that *G. oxydans* does not respond with increased motility when growing on gluconate.

**Genes involved in the transcriptional and translational machinery.** Many genes encoding proteins involved in transcription and translation showed lower expression at sample point II (Table S6). This response probably presents an adaptation to the reduced, linear growth observed after transition of the cells from glucose to gluconate as the carbon source.

**Genes with predicted and unknown functions.** 48 genes encoding proteins with predicted functions were differentially expressed, of which 30 showed an mRNA ratio ≥2.0 and 18 an mRNA ratio ≤0.5 (Table S6). 119 genes with unknown functions encoding hypothetical proteins were differentially expressed.

**In vitro enzyme assays.** The *in vitro* activities of glucose kinase (Glk), gluconate kinase (GntK), glucose 6-phosphate dehydrogenase (Zwf), and 6-phosphogluconate dehydrogenase (Gnd) were determined in cell-free extracts derived from sample points I and II (Table 1). Whereas the activity of Glk remained constant in both phases, the activity of the other three enzymes increased 2- to 3.4-fold in the second growth phase, which correlates with their
increased mRNA levels and the increased carbon flux through the PPP in growth phase II (Fig. 4).

DISCUSSION

In this study, the first $^{13}$C-based metabolic flux analysis was performed for \textit{G. oxydans} and combined with a comparative transcriptome analysis and enzyme activity assays. Although the genome sequence indicates that of \textit{G. oxydans} 621H contains all genes required for the \textit{de novo} synthesis of all nucleotides, amino acids, phospholipids and most vitamins (28), it apparently cannot provide sufficient quantities of certain building blocks, as there are several reports in the literature that growth of \textit{G. oxydans} on defined medium without yeast extract results in a very low biomass formation (25, 29, 30). Thus, in the $^{13}$C-MFA the utilization of yeast extract components had to be taken into account, which complicates the analysis. A second challenge for $^{13}$C-MFA was the low growth rate in growth phase II, which requires long experimentation times in order to reach an isotopically pseudo-equilibrated state that is characteristic for a growth phase. If cellular conditions change considerably within this period (e.g. in the case of substrate depletion), isotopic stationarity cannot be reached in less active parts of metabolism. These problems might be solved in the future by an isotope-based untargeted analysis, recently termed TARDIS (time and relative differences in systems) approach, which provides an option to address both, metabolic pathway elucidation and flux determination (44).

Despite the hurdles described above, $^{13}$C-MFA led to a number of important results with respect to the cytoplasmic sugar catabolism of \textit{G. oxydans}. Glucose taken up into the cytoplasm at sample point I was predominantly oxidized to gluconate, which then was either further oxidized to 5-ketogluconate or phosphorylated to 6-phosphogluconate. About 10% of the glucose was phosphorylated and oxidized to 6-phosphogluconate. The major route for 6-phosphogluconate metabolism was the PPP, 62% and 93% in growth phase I and II,
respectively. This preference of the PPP was also observed by us in a recent analysis of ∆gnd and ∆edd-eda mutants of *G. oxydans* during growth on mannitol (33) or on glucose (34). The prevalence of the PPP as major catabolic pathway is a feature not often observed in bacteria. In a comparative $^{13}$C-MFA of glucose metabolism in seven bacterial species (*Agrobacterium tumefaciens*, two pseudomonads, *Sinorhizobium meliloti*, *Rhodobacter sphaeroides*, *Zymomonas mobilis*, and *Paracoccus versutus*), Fuhrer and Sauer (10) showed that glucose was predominantly degraded via the EDP and the PPP had a solely anabolic function.

Another important result of $^{13}$C-MFA was the demonstration of the cyclic nature of the carbon flux through the oxidative part of the PPP, as shown by the strong net flux from fructose 6-phosphate to glucose 6-phosphate in the Pgi-catalyzed reaction. This cyclic flux is caused by the absence of 6-phosphofructokinase. Carbon dioxide production in growth phase II was in accordance with the amount calculated based on the stoichiometry of a cyclized PPP from the experimentally determined carbon uptake values. A partially cyclic operation of the PPP was expected from theoretical considerations (16) and has recently also been demonstrated for an *Escherichia coli* ∆pfkA mutant devoid of the gene encoding phosphofructokinase A (38).

The TCA cycle intermediates citrate/isocitrate, aconitate, α-ketoglutarate, succinate, fumarate and malate showed a very low $^{13}$C-label, indicating that the majority of these metabolites were derived from components of the yeast extract, in particular amino acids. The fact that bacteria stop the endogenous synthesis of amino acids (and other precursors) when external sources are available is well known. Whether these external sources are only used for protein synthesis and other biosynthetic purposes or serve as energy sources, too, depends on the particular metabolic capacity of the host and the available nutrients. In *G. oxydans*, the situation is special as its TCA cycle is incomplete due to the lack of succinyl-CoA synthetase and succinate dehydrogenase and therefore can only serve an anabolic function. Thus, even in the absence of yeast extract, the flux from citrate to α-ketoglutarate as precursor of the
glutamate family of amino acids should just be sufficient to meet the biosynthetic demands, as a higher flux would lead to the accumulation of TCA cycle intermediates. The labeling pattern of the TCA cycle intermediates suggests that the flux through citrate synthase is very low, either because the majority of pyruvate is converted to acetaldehyde and acetate rather than to acetyl-CoA, or because of a low availability of oxaloacetate.

Transition of cells from growth with glucose to growth with gluconate was accompanied by an increase of the activities of gluconate kinase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase as well as with decreased growth and oxygen consumption. These changes were paralleled by an increased expression of PPP genes and a deceased expression of the *edd* gene for 6-phosphogluconate dehydratase. The changes in gene expression and enzyme activities were in accord with the PPP being the dominant pathway for cytoplasmic gluconate catabolism in growth phase II (Fig. 4). The increased activity of gluconate kinase might explain the higher flux of gluconate into the PPP and the reduced conversion of gluconate to 5-ketogluconate in phase II.

The transcriptome comparison revealed more than 500 genes whose mRNA level was changed at least 2-fold in growth phase II. This high number presumably can be attributed to a large extent to the strongly reduced growth rate, which is expected to cause decreased expression of genes involved in transcription and translation. The reduced growth rate might also explain a significant number of similarities to the transcriptional response observed after a switch from oxygen excess to oxygen limiting conditions, which also led to slow growth (11). These similarities include e.g. the increased mRNA levels of the transhydrogenase operon (GOX0310-0314), the putative Na⁺-transporting F₁Fₒ-ATP synthase operon (GOX02167-02175), the cytochrome *bd* oxidase operon (GOX0278-0279), the PTS operon (GOX0812-0815), the sigma factor H gene (GOX0506), the transcriptional regulator gene GOX0135, and genes for several stress-related proteins (GOX0609, GOX1329, GOX1414, GOX2397) and the decreased mRNA levels of e.g. the genes coding for the H⁻-transporting
F$_1$F$_0$-ATP synthase (GOX1110-1113, GOX1310-1314), the genes for aconitase (GOX1335) and isocitrate dehydrogenase (GOX1336), and the genes for two transcriptional regulators (GOX0772, GOX0132). These changes might represent a general stress response of *G. oxydans*.

In conclusion, the present study has demonstrated by $^{13}$C-MFA, *in vitro* enzyme assays and genome-wide transcription analysis that the PPP is the predominant pathway for intracellular sugar catabolism in *G. oxydans*. In addition, $^{13}$C-MFA verified the cyclic nature of the carbon flux through the oxidative part of the PPP.

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REFERENCES


FIGURE LEGENDS

FIG 1 Biphasic growth of *G. oxydans* with 80 g l\(^{-1}\) glucose and 5 g l\(^{-1}\) yeast extract in a bioreactor at a constant pH of 6 and a constant dissolved oxygen concentration of 15%. In panel A, the concentration profiles in g l\(^{-1}\) of glucose (■, GLC, black solid line, initial concentration 80 g l\(^{-1}\)), gluconate (●, GLCN, blue dashed line), 2-ketogluconate (▲, 2KGA, red dotted line), 5-ketogluconate (○, 5KGA, magenta dotdashed line) and biomass dry weight (scaled by a factor of 10, ▼, DW, green solid line) are shown. The two vertical dashed lines indicate the times (I: 8.00 h, II: 14.25 h) at which samples were taken for \(^{13}\)C-based metabolic flux analysis. In panel B, the oxygen consumption rates of the two \(^{13}\)C-labeled cultures (-□-, -●-) and the non-labeled culture (-○-) are shown. In panel C, the carbon dioxide production rates of the \(^{13}\)C-labeled culture (-□-) and of the non-labeled culture (-●-) are indicated.

FIG 2 Mass isotopomer labeling measurements of intracellular metabolites (red bars: after 8.0 h in growth phase I, green bars: after 14.25 h in growth phase II) arranged by pathways: fractional abundance over mass in context of the metabolic network. Error bars indicate standard deviations derived from two technical replicates (phase I) and two independent biological replicates with two technical replicates each (phase II). For abbreviations of flux and metabolite names used in the model see Table S3.2 and S3.3. A switch from predominantly fully labeled mass isotopomers in the EMP and the PPP intermediates to almost naturally labeled TCA cycle intermediates is evident. Analysis of unphosphorylated glucose in the cytoplasm was rendered unfeasible by the large excess of extracellular glucose. Therefore, the labeling pattern of intracellular glucose is not included in the analysis.
FIG 3 *In vivo* flux distribution of *G. oxydans* during growth with glucose at 8.0 h (sample point I, A) and 14.25 h (sample point II, B) after switch of naturally labeled to specifically $^{13}$C-labeled glucose (GLC). The network diagram shows the metabolic pathways in the periplasm and the cytoplasm that were in the focus of the $^{13}$C-MFA study. Metabolites are represented by rectangles (white: extracellular; pink: periplasmic; orange: cytoplasmic). Flux values (yellow hexagons) at sampling times are related to 100% glucose (sample point I, A) or gluconate (sample point II, B) uptake. The width of each flux edge is scaled proportional to its underlying value; flux arrows are pointing in net flux direction. For abbreviations of flux and metabolite names used in the model see Table S3.2 and S3.3. Absolute net flux values are given in supplementary material (Table S3.4).

FIG 4 Multi-omics comparison of ratios at sampling time point II vs. sampling time point I. Arrows represent the carbon flux ratios, diamonds the enzyme activity ratios, and rounded rectangles the mRNA ratios. For abbreviations of flux and metabolite names used in the model see Table S3.2 and S3.3.