Metabolic Changes in *Klebsiella oxytoca* in Response to Low Oxidoreduction Potential, as Revealed by Comparative Proteomic Profiling Integrated with Flux Balance Analysis

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Oxidoreduction potential (ORP) is an important physiological parameter for biochemical production in anaerobic or microaerobic processes. However, the effect of ORP on cellular physiology remains largely unknown, which hampers the design of engineering strategies targeting proteins associated with ORP response. Here we characterized the effect of altering ORP in a 1,3-propanediol producer, *Klebsiella oxytoca*, by comparative proteomic profiling combined with flux balance analysis. Decreasing the extracellular ORP from -150 to -240 mV retarded cell growth and enhanced 1,3-propanediol production. Comparative proteomic analysis identified 61 differentially expressed proteins, mainly involved in carbohydrate catabolism, cellular constituent biosynthesis, and reductive stress response. A hypothetical oxidoreductase (HOR) that catalyzes 1,3-propanediol production was markedly upregulated, while proteins involved in biomass precursor synthesis were downregulated. As revealed by subsequent flux balance analysis, low ORP induced a metabolic shift from glycerol oxidation to reduction and rebalancing of redox and energy metabolism. From the integrated protein expression profiles and flux distributions, we can construct a rational analytic framework that elucidates how (facultative) anaerobes respond to extracellular ORP changes.

Anaerobic and microaerobic fermentation processes are widely exploited to produce chemicals (e.g., 1,3-propanediol, succinate, lactate, pyruvate, and xylitol) (1–5) and biofuels (biogas, ethanol, and butanol) (6–10). However, while dissolved oxygen can be precisely controlled during aerobic processes, the effective physiological parameters of anaerobic fermentation are poorly understood. Consequently, anaerobic processes remain difficult to monitor and control.

A well-known physicochemical parameter is the extracellular oxidoreduction potential (ORP) or redox potential. The ORP quantifies the redox property of solutions (11) and is a primary affecter of (facultative) anaerobic growth and metabolism (4, 12–17). Generally, each microbial species optimally grows within a certain ORP range (14–16), and alteration of extracellular ORP usually leads to metabolic flux redistribution. For example, *Escherichia coli* K-12 fermented glucose to a mixture of formate, acetate, ethanol, and lactate at a molar ratio of 2.5:1:1:0.3 under high (-100 mV)-ORP conditions; decreasing the culture ORP to -320 mV alters this molar ratio to 2:0.6:1:2 (16). Low-ORP cultures of *Clostridium thermosuccinogenes* showed increased effluxes of succinate, acetate, and formate and decreased effluxes of ethanol and hydrogen (18). Decreased ORP levels also improved the xylitol yield of stationary-phase fermentation by *Candida tropicalis* (19). Therefore, the extracellular ORP is a useful parameter for monitoring and regulating microbial metabolism in anaerobic or microaerobic processes (4, 12, 13, 19–22, 24).

However, the physiological response of microbial cells to ORP alterations remains unclear. ORP changes may affect cellular physiology by altering intracellular redox levels (4), affecting the expression of individual enzymes (16, 25) and changing the oxidative states of protein thiol groups. To elucidate the underlying mechanisms of such pleiotropic responses, we require global analytic tools and methods.

Klebsiella oxytoca, a facultative anaerobe formerly classified as *K. pneumoniae* (26), can convert glycerol into 1,3-propanediol under microaerobic conditions (27, 28). Glycerol is metabolized via two routes: (i) a reductive branch through which glycerol is dehydrated and reduced to 1,3-propanediol and (ii) an oxidative branch through which glycerol is phosphorylated and subsequently catabolized. Since the ratio of metabolic fluxes through these two branches is sensitive to extracellular ORP changes (4), we adopt the microaerobic metabolism of glycerol by *K. oxytoca* as a model process. In this work, we altered the ORP in *K. oxytoca* cultures and assessed the consequent changes in protein expression and metabolic flux by combined proteomic profiling and flux balance analysis (FBA).

MATERIALS AND METHODS

Bacterial strain and culture conditions. The *K. oxytoca* YMU2 strain was derived from the M5al type strain (21, 29) but prefers a lower ORP and produces more 1,3-propanediol than the parent strain. In addition, YMU2 grows over an extended ORP range (data not shown), enabling fermentation control at a specified redox status.

A single colony of strain YMU2 was inoculated into a flask containing 50 ml Luria Bertani broth and grown aerobically (35°C, 150 rpm, 12 h).

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This preculture was inoculated (5% [vol/vol]) into a 300-ml flask containing 100 ml chemically defined medium (4) and cultured anaerobically (nitrogen in headspace, 35°C, 120 rpm, 24 h) at pH ~7.0 (buffered by adding calcium carbonate). The anaerobic flask culture was then inoculated (10% [vol/vol]) into a 7-liter BioFlo110 fermenter (New Brunswick Scientific, Edison, NJ) equipped with an ORP Automatic Controlling System (30) to initiate anaerobic fermentation (nitrogen in headspace, 35°C, 400 rpm, 66 h), with an initial working volume of 3.0 liter. Culture pH was controlled at 7.0 by automatic addition of sodium hydroxide. Given that alkali consumption and glycerol consumption are proportional (4, 29), we employed a coupling strategy to simultaneously feed both nutrients. To this end, sodium hydroxide and glycerol were mixed (140 g glycerol per 100 ml 4 M sodium hydroxide) and sterilized before addition to the cultures. The glycerol concentration in the cultures was maintained at 10 to 40 g · liter⁻¹ (see Fig. S1 in the supplemental material), which is a suitable range for fermentative growth (21).

ORP measurement and control strategy. The ORP in the fermentation broth was measured by ORP electrodes (Pt4805-DPAS-SC-K85; Mettler-Toledo, Greifensee, Switzerland) and a pH/ORP controller (PC-350; Suntex, New Taipei City, Taiwan) and corrected by referencing to a standard ($ORP = ORP_{\text{measured}} + ORP_{\text{reference}}$, where $ORP_{\text{reference}} = 210$ mV at 35°C) (31). Electrodes were precalibrated in redox standard solution (HI 7021L; Hanna Company, Lucca, Italy).

The culture ORP was controlled by an ORP Automatic Controlling System. The ORP was increased or decreased by ventilation with sterilized air or nitrogen, respectively. The ORPs around the setting points (i.e., -150 mV and -240 mV) were maintained by the following protocols: when the ORP decreased to below the setting point, the air input valve was turned on. Air was sparged into the fermenter until the ORP regained the setting point and was then turned off. When the ORP exceeded the setting value, the nitrogen input valve was turned on. Nitrogen was sparged until the ORP regained the setting points and was then turned off. Using this protocol, the broth ORP level was controlled around the setting point (± 10 mV). The ORP in sterilized medium was typically around 180 mV and was reduced to -200 mV a few hours after inoculation with strain YMU2.

Assay of biomass and metabolic products. Cell density was measured at 650 nm using a spectrophotometer (UnicoUV2802; Unico, Shanghai, China). Cell dry weight (CDW) was determined as follows: $CDW \text{ (g} \cdot \text{liters}^{-1}) = 0.255 \times OD_{650} - 0.0176$ (32). Glycerol and the secreted metabolites were quantified by high-performance liquid chromatography (HPLC) (4).

TEM analysis. In preparation for transmission electron microscopy (TEM), cells in logarithmic-growth phase (12 h postinoculation) were pelleted by centrifugation (Allegra 64R; Beckman, Fullerton, CA) at $10,000 \times g$ and 4°C for 5 min. The cells were rinsed with sterile deionized water and incubated overnight in 3% (vol/vol) glutaraldehyde. The following procedures have been previously documented (33).

Protein sample preparation. When the ORP had stabilized at 12 h postinoculation, culture samples were withdrawn from the fermenters, chilled, and centrifuged at $10,000 \times g$ and 4°C for 30 min. The pellets were then washed twice with ice-cold TE buffer (10 mM Tris-HCl, 5 mM EDTA, pH 7.5) and resuspended in lysis buffer (1.0 g cells [wet weight]-12 ml buffer) containing 8 M urea, 2 M thiourea, 4% (wt/vol) CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 50 mM dithiothreitol (DTT), and 10 mM phenylmethylsulfonyl fluoride (PMSF) and a complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Cells were lysed by sonication (Sonifier S-450D; Branson Ultrasonics Corp., Danbury, CT), and the debris was removed by centrifugation at $12,000 \times g$ and 4°C for 30 min. The supernatant was incubated with $10 \mu\text{g} \cdot \text{ml}^{-1}$ nuclease mix (GE Healthcare, Uppsala, Sweden) at ambient temperature for 30 to 45 min to degrade the nucleic acids, followed by overnight incubation in ice-cold acetone to precipitate the proteins. The precipitated proteins were washed with acetone, air dried, and then solubilized in sample lysis buffer (9.5 M urea; 4% CHAPS; 0.5%

[vol/vol] IPG buffer [pH 3 to 10] [GE Healthcare]; 1% [wt/vol] DTT). The total protein concentration was determined using a two-dimensional (2-D) Quant kit (GE Healthcare), and 1-mg aliquots were stored at -80°C in readiness for two-dimensional polyacrylamide gel electrophoresis.

Two-dimensional polyacrylamide gel electrophoresis. One-dimensional gel electrophoresis (isoelectric focusing [IEF]) was carried out using an Ettan IPGphor 3 system (GE Healthcare). Approximately 800 μg protein was solubilized in 450 μl rehydration solution (8 M urea, 2 M thiourea, 2% CHAPS, 0.5% pharmalyte [pH 3 to 10], 0.4% DTT, and 0.001% bromophenol blue) and applied to IPG strips (GE Healthcare) (pH 3 to 10 nonlinear [NL]) for in-gel rehydration. IEF was performed at 20°C under the following consecutive settings: maintaining at 200 V for 1 h, 500 V for 1 h, and 1,000 V for 1 h, increasing to 8,000 V in 30 min, and holding at 8,000 V for 10 h.

Following one-dimensional IEF, each strip was equilibrated and subjected to two-dimensional electrophoresis using SDS-PAGE, as previously described (33, 34). The protein spots were visualized by staining with Coomassie blue G-250 (Amresco, Solon, OH). Each set of samples was independently analyzed in triplicate.

The gels were scanned at 300 dots per inch (dpi) by an Image Scanner III (GE Healthcare), and spots were comparatively analyzed to determine their abundance differences using Image Master 6.0 2-D Platinum software (GE Healthcare) as previously described (33, 34). Statistically significant differences were discerned by Student's *t* test. Protein spots showing significant (>2 -fold, $P < 0.05$) changes in abundance were extracted, digested, rehydrated, and identified in an Applied Biosystems MALDI-TOF/TOF 4700 proteomics analyzer (Applied Biosystems, Framingham, MA) (33, 34). Protein annotations were automatically extracted from the NCBI database and the Kyoto Encyclopedia of Genes and Genomes database (KEGG; <http://www.genome.jp/kegg/>) by a MASCOT 2.0 search engine (Matrix Science, London, United Kingdom).

Reconstruction of the genome-scale metabolic model for *K. oxytoca*. Since the YMU2 genome has not been sequenced, we reconstructed the genome-scale metabolic model from the NCBI annotations of the M5al parent strain (27). Since *K. oxytoca* is closely related to *K. pneumoniae* MGH 78578, the reconstruction was expedited by adopting the model of *K. pneumoniae* MGH 78578, namely, iYL1228, as a template. First, orthologs of M5al and MGH 78578 were obtained by multiple sequence alignment using the Reciprocal Smallest Distance (RSD) algorithm (35). Second, orthologous enzymes in *K. oxytoca* genome were identified and the associated reactions were extracted from iYL1228. Additional reactions from the KEGG database were incorporated into a final reconstruction of the metabolic network. Third, since the biomass composition of *K. oxytoca* is not known in detail, we used the iYL1228 biomass-constituting equation instead. Finally, a readable iYZ1315 model was derived and used in subsequent analysis.

Constraint-based flux balance analysis and sampling the solution space. Metabolic flux distributions were determined by flux balance analysis (FBA) (36). The selected objective function was maximization of biomass formation in the exponential phase. The stationary phase was excluded because cells cease growing in this phase, and a variety of other fitness attributes (37–39) would seem more appropriate. First, the exchange fluxes of glycerol, ethanol, acetate, and 1,3-propanediol were strictly constrained to their experimentally determined specific rates (see Methods in the supplemental material). Second, since FBA prediction required further constraint of the oxygen uptake rate (q_{O_2}), the rate value was determined by a robustness analysis method. Adjusting the magnitude of q_{O_2} , a vector of specific growth rates (μ) was obtained and plotted as a function of q_{O_2} . To determine the estimated oxygen uptake rate $q_{O_2}^{\text{est}}$, the q_{O_2} corresponding to the experimentally determined μ^{exp} was located on the *x* axis of the plot (see Fig. S4 in the supplemental material). Finally, to characterize the solution space of the flux distributions, 5,000 points were randomly sampled by a Monte Carlo algorithm (40), assuming the growth rate to be μ^{exp} and the oxygen uptake rate as $q_{O_2}^{\text{est}}$. The samples were

FIG 1 Cell growth (B) and metabolism (C to F) under different ORP conditions. High-ORP (-150 mV) condition, open circles; low-ORP (-240 mV) condition, solid circles. The ORP was continuously and automatically recorded during fermentation. Note that calculating the averages and standard deviations may be invalidated, since not all measurements were taken at the same time points. Therefore, we present the ORP curves. High and low ORP levels in culture are denoted by H and L, respectively, in panel A. All experiments were performed in triplicate.

trimmed by applying the loopless COBRA (ll-COBRA) method (41) to remove loops. The resulting flux distributions were then averaged for illustration purposes and ratio calculation. In this study, all FBA and related analyses were performed using the COBRA Toolbox with the Gurobi 5.6.0 solver in a MATLAB R2013a environment (42).

RESULTS

Carbon flux of *Klebsiella oxytoca* shifts toward the reductive pathway under low-ORP conditions. *K. oxytoca* YMU2 grows comfortably over a wide ORP range (from -330 to -120 mV). As shown in Fig. 1, the culture ORP levels fell rapidly from above 200 to below -200 mV while cells were vigorously growing in the first few hours and then gradually stabilized at the setting points (-150 and -240 mV). Decreasing the culture ORP from -150 to -240 mV retarded cell growth, accelerated glycerol consumption, increased 1,3-propanediol production, and reduced byproduct accumulation (Fig. 1; see also Fig. S3 in the supplemental material).

Under these conditions, the average final titer of 1,3-propanediol increased by 33.1%, while those of ethanol and acetate decreased by 49.2% and 47.4%, respectively. Glycerol was consumed more rapidly at -240 than at -150 mV in the exponential-growth phase (Fig. 1C; see also Fig. S3B). Acetate and ethanol, which are pyruvate-derived products, were produced at higher rates under low-ORP conditions during the first 6 h, and the rates declined at later time points (see Fig. S3D and E). This contrasting effect might arise from the initial “lag phase,” during which the ORP did not reach the nominal -150 mV (Fig. 1A). Overall, the flux ratio of reductive to oxidative glycerol metabolism was significantly high at -240 mV (see Fig. S3F), suggesting that the carbon flux was shunted into reductive metabolism at low culture ORP.

Intracellular components in *K. oxytoca* YMU2 cells became aggregated and disordered under low-ORP conditions. The impact of low ORP on cellular morphology in the mid-log phase was

FIG 2 TEM photographs ($\times 10,000$) of *K. oxytoca* YMU2 under high-ORP conditions (-150 mV) (A) and low-ORP conditions (-240 mV) (B). Cells were harvested at 12 h postinoculation. Scale bar, 500 nm.

investigated by TEM (see Fig. 2). At high culture ORP (-150 mV), YMU2 cells were regular short rods and their cytoplasm was evenly distributed (Fig. 2A), as reported during anaerobic growth in citrate sodium media (43). In contrast, low ORP (-240 mV) induced irregularities in cell shape and aggregation of intracellular components (Fig. 2B). We speculate that reductive stress under these conditions causes cell damage, since reductive stress has been previously implicated in protein misfolding and aggregation (44, 45).

Protein expression alters in response to ORP alterations. The global proteomic response of *K. oxytoca* to ORP alterations was profiled by 2-D gel electrophoresis. In three independent experiments, protein abundance was significantly altered in 68 of approximately 650 spots (fold change ≥ 2 , $P < 0.05$) in low-ORP cultures. Among these, 61 (89.7%) spots were identified by

MALDI-TOF mass spectrometry (see Table S1 in the supplemental material and Fig. 3). The protein content in the remaining spots was either too low for valid analysis or overlapped with that of other spots on the gel. A total of 51 proteins were annotated by searching against databases and were grouped into six functional categories: carbohydrate metabolism (6 proteins), synthesis of cellular constituents (10 proteins), transportation (13 proteins), translation and posttranslation (6 proteins), antioxidation (5 proteins), and functionally uncharacterized (11 proteins). Referring to a metabolic network of *K. oxytoca*, we then located the pathways associated with the differentially expressed proteins to determine the contribution of protein expression to the metabolic shift.

In the reductive branch of glycerol metabolism, low ORP induced a 7-fold increase in levels of NADPH-dependent hypothetical oxidoreductase (HOR; EC 1.1.-.-, encoded by *yqhD*) but did

FIG 3 Comparative proteomic profiling of *K. oxytoca* YMU2 under high-ORP conditions (-150 mV) (A) and low-ORP conditions (-240 mV) (B). The differentially expressed proteins were labeled with different spot numbers. See Table S1 in the supplemental material for more details.

FIG 4 Correlation between metabolic fluxes and abundance of their associated enzymes. Color bars quantify the variation ratio of proteins and fluxes in response to ORP alterations. The average fluxes were used in the ratio calculations. Inf, infinity.

not alter expression of 1,3-propanediol oxidoreductase (PDOR; EC 1.1.1.202, encoded by *dhaT*) (46, 47). According to previous reports, HOR might be critically involved in 1,3-propanediol production from 3-hydroxypropionaldehyde (47, 48). Thus, we postulated that HOR upregulation might contribute to increased 1,3-propanediol production. Pyruvate kinase (PYK; EC 2.7.1.40) was upregulated in the glycerol oxidation branch. As such, the energy balance could be perturbed by PYK-catalyzed ATP regeneration.

In low-ORP cultures, downregulated or absent enzymes are predominantly involved in (i) synthesis of cellular constituents, e.g., amino acids, nucleotides, and vitamins; (ii) synthesis and maturation of macromolecules such as lipopolysaccharides and proteins; and (iii) the transportation and secretion system. Therefore, downregulation of these enzymes will likely result in deficient growth.

Moreover, proteins involved in stress response and function maintenance were differentially expressed in low-ORP cultures. Among these, thiol peroxidase and superoxide dismutase were largely downregulated by lack of oxidative stress. Of special interest was the upregulation of alkylhydroperoxide reductase (Ahp). Although well recognized as the primary scavenger of endogenous hydrogen peroxide (49) and essential for cell survival under oxidative stress (50), the role of Ahp in reductive stress response has been little reported. The ATP-dependent ClpB chaperon protein was upregulated 3.3-fold, suggesting a role for this protein in rescuing stress-damaged proteins from an aggregated state, thereby maintaining their functions.

Genome-scale metabolic model reconstruction for *K. oxytoca* and constraint-based flux balance analysis. To further identify proteins that are likely responsible for metabolic changes, the flux distributions were calculated in a reconstructed genome-scale metabolic model of YMU2. First, we mapped the *i*YL1228 *K.*

pneumoniae model to the genome of *K. oxytoca*. Since *K. pneumoniae* and *K. oxytoca* are closely related species, genome-wide sequence alignment yielded 1,200 orthologous genes associated with 2,242 biochemical reactions in *i*YL1228. The new model retained these reactions and adopted the same biomass formation formula and was complemented with an additional 18 reactions and 115 genes extracted from the KEGG database (see Tables S4 and S5 in the supplemental material). The final *i*YZ1315 model was constructed from 1,315 genes, 1,666 metabolites, and 2,260 reactions (see Tables S2 and S3). In contrast to *i*YL1228, the *i*YZ1315 model permits propionate production, nitrogen fixation, and indole formation and excludes pathogenic lipid A synthesis. The solution space of this model was further reduced by imposing carbon exchange constraints and estimated oxygen consumption limits. Next, 5,000 loopless feasible solutions were obtained by randomly sampling the solution space. The averages and standard deviations of each metabolic flux calculation were determined (see Table S6).

Comparisons with proteomic data revealed that most of the primary flux changes were ascribable to enzyme abundance alterations (e.g., HOR, IMPDH [inosine-5'-monophosphate dehydrogenase], DPHS [dihydropteroate synthase], KDS [3-deoxy-8-phospho-octulonate synthase], CysP, NDK [nucleoside diphosphate kinase], DapA, and ATPase). As shown in Fig. 4, correlations between flux changes and protein expression changes were observed. The glycerol reduction flux through 13PPDH was doubled over three time points, corresponding to a 7-fold increase in HOR expression. The fluxes through amino acid and nucleotide synthesis pathways were decreased by 12.3% to 55.3%, while the associated enzymes were either downregulated or completely absent in low-ORP cultures (e.g., IMPDH, DPHS, CysP, NDK, DapA, and ATPase). Of particular interest are the decreased fluxes

TABLE 1 Determined ratios based on average values of flux distribution samples

ORP level ^a	−150 mV			−240 mV		
	6 h	12 h	18 h	6 h	12 h	18 h
Respiratory quotient ($-\nu\text{CO}_2/\nu\text{O}_2$)	6.0742	10.0652	2.0917	17.7371	9.4859	6.5790
Biomass yield ($Y_{X/S}$)	0.0057	0.0047	0.0040	0.0024	0.0036	0.0023
ATP yield ($Y_{\text{ATP}/S}$)	1.5031	1.5467	2.1133	1.1834	0.9255	0.8103
NADH yield ($Y_{\text{NADH}/S}$)	0.9651	1.0371	1.3730	1.0941	0.9518	0.8720
NADPH yield ($Y_{\text{NADPH}/S}$)	0.2221	0.2690	0.3905	0.2887	0.1140	0.1207
Glycerol flux ratio calculated based on specific production rates ($q_{\text{ox}}/q_{\text{red}}$)	0.9716	1.9868	3.3395	0.8915	0.6706	0.6780
Glycerol flux ratio calculated based on FBA results ($\nu_{\text{ox}}/\nu_{\text{red}}$)	1.1984	1.5495	1.4339	0.9646	0.5673	0.4035

^a Yields of ATP, NADH, and NADPH were calculated by dividing their turnover rates by the glycerol uptake flux values. $\nu_{\text{ox}}/\nu_{\text{red}} = (\nu_{\text{GLYK}} + \nu_{\text{GLYCDX}})/\nu_{\text{GLYCDH}}$.

through the tetrahydridipicolinate succinylase (THDPS) reaction (see Table S2 in the supplemental material) and the PYK reaction during 12 to 18 h of incubation, since the corresponding enzymes, DapD and PYK, respectively, were upregulated in the proteomic profiles.

Alteration of specific metabolic fluxes may cause redox imbalance. Increased HOR abundance was expected to shunt carbon flux out of the oxidation and into the reduction pathway. In fact, the flux ratio ($\nu_{\text{ox}}/\nu_{\text{red}}$) declined from a level of 1.20 to 1.55 to a level of 0.40 to 0.96 under low-ORP conditions (Table 1). Glycerol oxidation and glycolysis are major processes for regenerating reductive equivalents (e.g., NADH/NADPH). Therefore, reduced fluxes through the oxidative branch may deplete the supply of redox equivalents. Consequently, the supply might be restored by alternative pathways, with consequent strengthening of metabolic fluxes with an equivalent ability to generate these compounds. This may explain the increased intracellular flux of pyruvate dehydrogenase complex (PDH) (Fig. 5; see also Fig. S5 in the supplemental material).

Cellular energy metabolism was also affected by changes in ORP. When the ORP was low and the ATPase component was lacking, the ATP generation flux was reduced by 11.1% to 64.6% (Fig. 5) and total ATP yield ($Y_{\text{ATP}/S}$) declined from a level of 1.50 to 2.11 to a level of 0.81 to 1.18 (mol/mol) (Table 1). Under these conditions, the available energy remaining in the presence of the non-growth-maintenance demand (which remains constant) may be insufficient to sustain nominal growth at high ORP, resulting in the observed retarded cell growth.

DISCUSSION

This study investigated the global effect of ORP alternation on microbial metabolism and characterized it by integrated comparative proteomic profiling and flux balance analysis. The working model was *Klebsiella oxytoca*. Comparative analysis of the proteomic profiles under conditions of high and low ORP revealed that differentially expressed proteins were mainly involved in glycerol catabolism, cellular constituent synthesis, redox homeostasis maintenance, and stress response. In the FBA results, flux changes were highly correlated with variable expression, shifted glycerol metabolism, and perturbed redox and energy balance. The integrated analysis connected the metabolic alteration with changes in protein abundance, enhancing our understanding of how microbes regulate their metabolisms and physiologies to cope with reducing environments.

Decreased extracellular ORP enhanced the reductive metabo-

lism of glycerol in *K. oxytoca*. One of the most interesting proteins was HOR (encoded by the *yqhD* gene), whose abundance increased more than 7-fold when the extracellular ORP decreased from −150 to −240 mV. However, in previous studies, *yqhD* overexpression did not necessarily increase the yields of reductive products (5, 11, 16, 19, 21, 34, 51–53), presumably because sufficient reductive equivalents were lacking. In this study, enhanced fluxes on PDH appeared in the FBA results (Fig. 5). This enzyme might oxidatively decarboxylate pyruvate and has been implicated in anaerobic pyruvate metabolism in cultures of *E. coli* (54), *Enterococcus faecalis* (55), and *K. pneumoniae* (56). NADH is considered to competitively inhibit PDH (57), and the increased flux on PDH might manifest from such metabolic feedback inhibition. Therefore, NADH might accumulate through downregulated NADH oxidation flux and enhanced pyruvate dehydrogenation flux, thereby becoming available to drive the HOR-catalyzed reaction. As such, HOR overexpression, accompanied by provision of a greater NAD(P)H driving force resulting from PDH overexpression, could be an effective strategy for improving 1,3-propanediol production. Mutants with robust growth performance and sufficient energy generation capability can be achieved by ORP-directed adaptive mutation and screening.

Although the association between low ORP and decreased cell growth rate has been well recognized (14, 16), the underlying mechanism remains largely unknown. In this study, comparative proteomic profiling analysis revealed that enzymes involved in biomass constituent synthesis and F_0F_1 ATPase components were underexpressed in low-ORP cultures. Flux balance analysis confirmed that low ORP also reduced the biomass and ATP yields (Table 1; see also Fig. S5 in the supplemental material). Diminished biomass synthesis and dedication of energy to maintenance are common stress responses (58–60). Low ORP may impose reductive stress, and cells may similarly respond by reducing their biomass synthesis. In *E. coli*, the total activity of cellular F_0F_1 -ATPase was sensitive to the surrounding ORP (15). The ϵ subunit of the F_1 component proved to be a vital modulator of ATPase activity (61). Microbes such as *K. oxytoca* might sense the cultural ORP alterations and consequently modulate their cellular ATP generation by altering their expression of the ϵ subunit of the F_1 component.

The upregulation of an antioxidant enzyme, the alkyl hydroperoxide reductase that catalyzes R-SH to RSSR', is of particular interest. According to previous studies, this enzyme might be the primary scavenger of endogenous hydrogen peroxide in *E. coli*

FIG 5 Schematic overview of the metabolic flux distributions in *K. oxytoca* YMU2 under high-ORP conditions (A to C) and low-ORP conditions (D to F). Upregulated and downregulated fluxes in low-ORP cultures are indicated by red and green arrows, respectively. Arrow widths indicate the averages of the results determined for 5,000 samples for each flux. The reaction identification numbers (ID), averages, and standard deviations (normalized with respect to glycerol uptake) are shown in blue.

cells (49) and participates in multiple abiotic stress response (62), but its role in reductive stress remains unknown. Disulfide bonds are crucial for maintaining protein structure and are generally formed via oxidation reactions between two thiol (RSH) groups.

Reducing environments tend to cleave these bonds, causing protein misfolding and loss of function (63) as well as aggregation. Evidence of this disruption was observed in TEM imaging in this study. To cope with reductive stress under low-ORP conditions,

K. oxytoca might upregulate alkyl hydroperoxide reductase expression to protect disulfide bonds from reduction.

A number of previous studies have shown that an appropriate ORP profile could improve the microbial production of several reductive chemicals, including 1,3-propanediol (4), ethanol (24), butanol (19, 64), succinate (22), citrate (20), and hydrogen (65). However, the physiological mechanisms of altered ORP response by microbial cells remain to be characterized. Here, we integrated comparative proteomic profiling and flux balance analysis. Our results showed that *K. oxytoca* cells might respond to ORP changes via several mechanisms: (i) converting more substrate glycerol to reductive metabolite 1,3-propanediol by upregulating HOR and strengthening NAD(P)H supply; (ii) downregulating biomass synthesis; and (iii) downregulating ATP generation. In summary, this report provides an overview of the metabolic and physiological changes that occur in facultative anaerobic cells as the environmental ORP alters from oxidative to reductive. We anticipate that such an integrated approach will greatly advance our understanding of how various (facultative) anaerobes respond to ORP changes in redox engineering.

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