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Novel Redox Potential-Based Screening Strategy for Rapid Isolation of *Klebsiella pneumoniae* Mutants with Enhanced 1,3-Propanediol-Producing Capability▽†

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This report describes a novel redox potential (oxidoreduction potential [ORP])-based screening strategy for the isolation of mutants of *Klebsiella pneumoniae* which have an increased ability to produce 1,3-propanediol (1,3-PD). This method can be described as follows: first, to determine an ORP range which is preferred for the wild-type strain to grow and to produce 1,3-PD; second, to subject a chemically mutagenized culture to a reduced ORP level which is deleterious for the wild-type strain. Colonies that showed high specific growth rates at deleterious ORP levels were selected, and their abilities to produce 1,3-PD were investigated. In an ORP-based screening experiment where the ORP was controlled at −280 mV, 4 out of 11 isolated strains were recognized as positive mutant strains. A mutant which is capable of producing higher concentrations of 1,3-PD was subjected to fed-batch fermentations for further characterization. Its preferred ORP level (−280 mV) was significantly lower than that of its parent (−190 mV). The highest 1,3-PD concentration of the mutant was 915 mmol liter^{-1}, which was 63.1% higher than that of the parent. Metabolic-flux analysis suggested that the intracellular reductive branch of the mutant was strengthened, which improved 1,3-PD biosynthesis. The procedure and results presented here provide a novel method of screening for strains with improved product formation.

In the last century, traditional random mutagenesis and selection techniques played important roles in the isolation of microbial strains with desired biosynthetic capabilities and in their subsequent improvement, which led to the emergence of the earliest industrial fermentation products such as penicillin, vitamin C, and many others. Modern strain improvement focuses mainly on the recombinant method. However, because of the complexity of the microbial metabolic network, modification of a single gene or a single pathway may not lead to a significant improvement in productivity. More recently, inverse metabolic engineering (IME) has been demonstrated as a powerful approach to strain improvement (25, 32). While the major challenge for IME is to rapidly identify the genes that underlie a naturally or laboratory-evolved phenotype, the difficulty of quickly obtaining a desired phenotype should not be underestimated. From this point of view, traditional random mutagenesis and selection technology is still a useful tool in IME, as the chance of obtaining a desired phenotype from highly randomized mutants is great. A key procedure in this area is to develop a rapid, high-throughout screening method. Once established, the method can also be used to screen mutant libraries generated by other evolutionary engineering approaches.

1,3-Propanediol (1,3-PD) is an important intermediate chemical which can be produced by bacteria belonging to the family *Enterobacteriaceae* (*Enterobacter*, *Klebsiella*, and *Citrobacter*) or to the genus *Clostridium* (1–3, 6, 22, 23, 27, 28). Levels of 1,3-PD production by wild-type strains are usually low; efforts have therefore been made, by different approaches, to improve these production levels (5). Among the attempts undertaken to improve the production of 1,3-PD by a wild-type strain, the most successful example was the selection of a *Clostridium butyricum* mutant that tolerates 90 g · liter^{-1} glycerol and 80 g · liter^{-1} 1,3-PD (1). This mutant was selected from a medium with elevated propanediol concentrations, which is not a biosynthesis mechanism-driven screening approach.

The approaches used to screen for a metabolite hyperproducer can be generally categorized on the basis of (i) a change in the absorption peak at a specific wavelength of either the target product or a derived compound, (ii) a change in the morphology of mutant colonies, and (iii) resistance to extreme conditions such as acid, alkali, or antibiotics. However, 1,3-PD does not have specific absorbance peaks in the visible-UV range. In addition, the chemical functional groups of 1,3-PD are highly similar to those of the substrate, glycerol, and the by-products, such as ethanol, lactic acid, and acetic acid. This makes it difficult to synthesize 1,3-PD derivatives without interference from the chemicals mentioned above. Moreover, tolerance to 1,3-PD does not directly correlate with 1,3-PD production. The available reports contain no description of a way to screen rapidly for mutants with improved 1,3-PD production capability.

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The aim of this study was to develop a rapid and effective screening approach to obtain *K. pneumoniae* mutants with increased 1,3-PD production capability. The parameter that we used to develop this screening approach was redox potential (oxidoreduction potential [ORP]). Recently, ORP has been used as a parameter to investigate mass and energy metabolic fluxes in several microorganisms (7, 26, 30). It has been reported that each species, or even each strain, has a preferred redox potential range. Only within this range is maximum cell growth possible, and the flux may be directed toward the target metabolite. For *K. pneumoniae*, 1,3-PD production is a requirement for anaerobic growth on glycerol, as the pathway by which glycerol is converted to 1,3-PD is the major pathway to regenerate NAD^+, which is essential for glycolysis to proceed (5, 33). In the *Klebsiella pneumoniae* M5aL fermentations performed in our previous study, the most-preferred ORP levels were −160 to −190 mV (14). Higher or lower ORP levels resulted in poor cell growth and poor 1,3-PD production. According to metabolic-flux analysis (5, 20), 1,3-PD is synthesized in the bioreductive branch, suggesting that enhancing the bioreductive branch, namely, creating a more reductive fermentation environment, would improve 1,3-PD production. The hypothesis of this study was that improved 1,3-PD productivity would be achieved in mutants whose preferred ORP levels are lower than that of the parent strain. To test this hypothesis, mutants of *K. pneumoniae* M5aL that grew relatively rapidly at unfavorable ORP levels were screened and subjected to 1,3-PD production tests. One mutant with significantly improved 1,3-PD-producing capability was further characterized at the metabolic level to elucidate the effect of an altered preferred ORP level on carbon metabolism. This study provides an important biosynthesis mechanism-driven screening approach. It can also be applied to the microbial production of other commodity chemicals whose biosynthesis is associated with oxidoreduction reactions.

MATERIALS AND METHODS

Microorganism and media. *K. pneumoniae* M5aL, which has been described in a previous study (14), was used as the parental strain in this study.

The culture medium used (17) contained the following (per liter): $K_2HPO_4 \cdot 3H_2O$, 4.45 g; KH_2PO_4, 1.3 g; $(NH_4)_2SO_4$, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot H_2O$, 0.02 g; yeast extract, 1.0 g; glycerol, 20 g (for fermentation), or glucose, 20 g (for seed culture); trace element solution, 1 ml; Fe^{2+} solution, 2 ml. Trace element solution contained the following (in milligrams per liter): $MnSO_4 \cdot 4H_2O$, 100; $ZnCl_2$, 70; $Na_2MoO_4 \cdot 2H_2O$, 35; H_3BO_3, 60; $CoCl_2 \cdot 6H_2O$, 200; $CuSO_4 \cdot 5H_2O$, 29.28; $NiCl_2 \cdot 6H_2O$, 25; HCl (37%, wt/vol), 0.9. Fe^{2+} solution contained 5 g liter^{-1} $FeSO_4 \cdot 7H_2O$ and 4 ml liter^{-1} HCl (37%, wt/vol). The pH of the culture medium was adjusted to 7.0 with 4 mol liter^{-1} NaOH. Colonies grown on LB agar (peptone, 10 g liter^{-1}; yeast extract, 5.0 g liter^{-1}; sodium chloride, 5.0 g liter^{-1}; agar powder, 15 g liter^{-1}) plates were inoculated into 500-ml flasks containing 50 ml seed culture medium and incubated at 37°C and 200 rpm for 10 h aerobically. Flask fermentations were carried out with 500-ml flasks containing 50 ml fermentation medium at 37°C and 200 rpm, where nitrogen was sparged to maintain an anaerobic environment. For a schematic overview of the anaerobic flask used in this study, see Fig. S1 in the supplemental material. Fed-batch fermentations were carried out in a 5-liter B. Braun Biostat B (B. Braun Biotech International, Germany) fermentor with a 2-liter working volume at 37°C and pH 7.0. The agitation speed was set at 400 rpm, and the nitrogen sparging rate was 1 liter/liter fermentation broth/min. The inoculum concentration was 4% (vol/vol). The glycerol concentration in the medium was maintained at around 20 g liter^{-1} by manually regulating the feeding rate of the glycerol solution (800 g liter^{-1}).

Mutagenesis. A mutation protocol combining UV light and chemical (LiCl) mutagenesis was used in this study. The cells were incubated aerobically in seed culture medium for 10 h to reach the exponential growth phase. The cell suspension was then diluted to an optical density at 650 nm (OD_{650}) of 1 with 0.9% NaCl solution and further diluted 10^6- to 10^8-fold, and then 0.1 ml was spread on LB plates (90-mm diameter, containing 2 g liter^{-1} LiCl). The plates were placed under UV light (30 W), 40 cm away, for 30 s and incubated at 37°C in the dark for 48 h. Under the above conditions, approximately 5 out of 100 cells survived.

Cell growth correlation screening method. All of the colonies derived from the parent strain were individually subjected to 50-ml aerobic flask fermentation for 8 h in seed culture medium. Mutants with a cell dry weight (CDW) higher than 1.2 g liter^{-1} were subsequently transferred into 500-ml flasks and incubated anaerobically for 36 h in fermentation culture medium with an inoculum concentration of 4% (vol/vol). Considering that *K. pneumoniae* is a facultatively anaerobic organism whose anaerobic growth may positively correlate with its aerobic growth, the aerobic biomass of mutants was determined and used as a criterion to screen mutant strains. Shake flask fermentations of isolated strains were performed in duplicate. On the basis of the data obtained from shake flask experiments (see Fig. 3 and 4), only the strain with 1,3-PD production more than 8% higher than that of the parent was recognized as a positive mutant strain.

ORP-based screening method (ORP and cell growth correlation screening method). All of the colonies derived from the parent strain were pooled, washed once with 2 ml of a 0.9% NaCl solution, and then inoculated into a 500-ml aerobic flask containing 50 ml seed culture medium. The cell suspension was incubated aerobically for 2 h and then inoculated into the B. Braun fermentor to an inoculum concentration of 4% (vol/vol). The ORP of the batch fermentation was controlled to drop evenly to −240 mV (in the first ORP-based mutagenesis trial) or −280 mV (in the second ORP-based mutagenesis trial) within 2 h. An ORP level of −240 mV was achieved by adjusting the ratio and flow rates of nitrogen and/or air, while an ORP level of −280 mV was achieved by controlled addition of a solution containing 10 g liter^{-1} $NaBH_4$ and 2 g liter^{-1} NaOH into the fermentor in addition to adjusting the ratio and flow rates of nitrogen and/or air. As a less flammable reducing agent which generates hydrogen when reacting with water, $NaBH_4$ was used to control the ORP to a relatively reductive level (31). The ORP was maintained at this value for about 6 to 8 h, until the cells reached the earlier exponential growth phase. One milliliter of fermentation broth was diluted to an OD_{650} of 1 with a 0.9% NaCl solution and then further diluted 10^6- to 10^8-fold, and 0.1 ml was spread onto LB medium plates. These plates were incubated for 48 h in the dark. All of the colonies were individually subjected to 50-ml aerobic flask fermentations for 8 h and then inoculated into a 500-ml anaerobic flask and incubated for 36 h for the examination of 1,3-PD production. Shake flask fermentations of isolated strains were performed in triplicate. The definition of a positive mutant strain was the same as described for the cell growth correlation screening method.

Analyses. Cell concentration was determined by measuring CDW with a predetermined correlation between OD_{650} (Agilent 8453 UV-visible spectrophotometer) and CDW, i.e., an OD_{650} of 1 = 0.25 g CDW liter^{-1}.

Glucose, glycerol, 1,3-PD, acetic acid, ethanol, 2,3-butanediol, and lactic acid were measured by high-performance liquid chromatography (Shimadzu HPLC-10A, RID-10A, Aminex HPX-87 H ion-exclusion column [300 mm by 7.8 mm]) under the following conditions: sample volume, 10 μl; mobile phase, 0.005 mol liter^{-1} H_2SO_4; flow rate, 0.8 ml min^{-1}; column temperature, 65°C.

Measurement of ORP. ORP was measured by a redox combination electrode that consisted of a pH electrode (HI 1131B) and a redox controller (pH 213; HANNA). The values were corrected according to the standard electrode value ($ORP = ORP_{measured} + ORP_{ref}$, where $ORP_{ref} = 210$ mV, at 37°C). Before each measurement, the electrode was treated in electrode cleaning solution (HI 7073; HANNA) for at least 2 h and then calibrated with redox solution (HI 7020; HANNA).

NAD^+/NADH assay. The intracellular concentrations of NAD^+ and NADH were determined as follows (4, 21). Samples (1 ml) were taken quickly and directly put into tubes containing precooled extractant. NADH was extracted with KOH (pH 12.3, to destroy NAD^+), and NAD^+ was extracted with HCl (pH 1.3, to destroy NADH). The KOH extract was incubated at 30°C for 10 min, while the HCl extract was incubated at 50°C for 10 min. After centrifugation at 5,000 × *g* at room temperature for 8 min, the supernatant was neutralized with HCl or KOH. The assay mixture contained 2,000 μl buffer (0.15 M glycylglycine/nicotinic acid buffer, pH 7.4), 400 μl phenazinium ethyl sulfate (4 mg ml^{-1}), 400 μl thiazolyl blue (5 mg ml^{-1}), 70 μl ethanol, and 20 μl alcohol dehydrogenase (300 U ml^{-1}). The reaction was initiated by adding 50 μl neutralized extract. The rate of increase in absorption at 570 nm was measured spectrophotometrically.

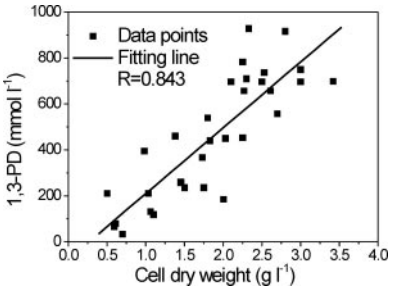

FIG. 1. Correlation of CDW and 1,3-PD production in batch and fed-batch *K. pneumoniae* fermentations in recent reports.

RESULTS

Correlation of cell growth and 1,3-PD biosynthesis. The CDWs and corresponding 1,3-PD concentrations in batch and fed-batch *K. pneumoniae* fermentations reported in recent publications are summarized in Fig. 1 (3, 8–11, 13, 14, 16, 35, 36), showing that high 1,3-PD production levels were observed in fermentations with high CDWs (for the sources of the data in Fig. 1, see Table S1 in the supplemental material). To investigate the possibility of isolating a mutant with increased 1,3-PD-producing capability by screening cells with improved growth performance, a test mutagenesis experiment was carried out. Twenty-nine mutated colonies were obtained from parent strain *K. pneumoniae* M5aL. The CDWs after 8 h of aerobic fermentation and the 1,3-PD concentrations after 36 h of anaerobic fermentation of all of these colonies are shown in Fig. 2. As the aerobic biomass of the best 1,3-PD producer was 1.2 g liter^{-1} and the average 1,3-PD concentration of the colonies with CDWs over 1.2 g liter^{-1} (79.6 mmol liter^{-1}) was threefold higher than that of the colonies with CDWs below 1.2 g liter^{-1} (26.1 mmol liter^{-1}), a growth cutoff value of 1.2 g liter^{-1} was used to select putative positive mutants in the following mutagenesis. This also enabled some potential positive mutants that had higher productivity but a lower growth ability than those of the parent strain (1.3 g liter^{-1}) to be chosen.

Cell growth correlation screening methodology. Four hundred twenty mutant colonies derived from the mutagenesis of the parent strain, 67 of which had an 8-h CDW above 1.2 g liter^{-1}, were isolated from the anaerobic fermentations. The 1,3-PD production of the top 40 strains out of the 67 strains tested is shown in Fig. 3. The average 1,3-PD concentration was only 66.1% of that of the parent strain. Only 2 of the 67 mutants selected were recognized as positive mutants. This indicated that although mutant strains with high 1,3-PD production could be obtained by the cell growth correlation method, the selection efficiency was not as high as expected; also, the procedure of this method was very laborious.

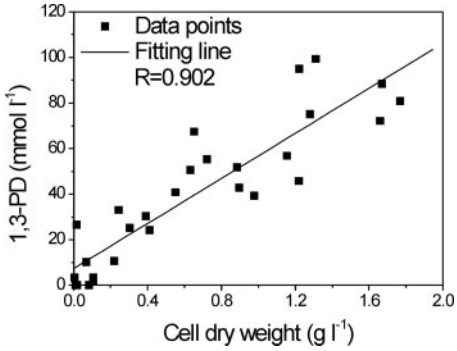

FIG. 2. Correlation of CDW and 1,3-PD production in a test mutant experiment. The CDW and 1,3-PD concentration of the parent strain used in this experiment were 1.3 g liter^{-1} and 110 mmol liter^{-1}, respectively.

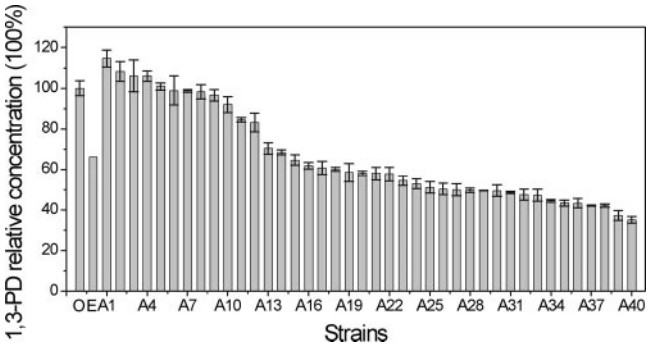

FIG. 3. Comparison of the 1,3-PD concentrations produced by the parent and mutant strains and the average of 40 mutant strains by the cell growth correlation screening method. O is the parent strain, with a 1,3-PD concentration of 122 mmol liter^{-1}, E is the average value of A1 to A40, and A1 to A40 are the mutants (average of duplicate cultures). The error was calculated by taking the difference between the average and experimental data.

ORP-based screening methodology. For improved selection efficiency, an ORP-based screening method was investigated. This method incorporated ORP tolerance as a screening parameter into the cell growth correlation screening method. On the basis of our previous study (14), the most-preferred ORP levels of the parent strain were −160 to −190 mV. As 1,3-PD is synthesized in the bioreductive branch (5, 20), enhancing the bioreductive reactions could improve 1,3-PD production. In order to obtain strains whose bioreductive branch is enhanced, ORP tolerance levels of −240 mV and −280 mV were selected.

The mutant colonies derived by UV and LiCl mutagenesis were mixed and then cultured in the 5-liter fermentor at an ORP of −240 mV. The same screening experiment was carried out at an ORP of −280 mV. Thirteen colonies that survived an ORP of −240 mV and 11 colonies that survived an ORP of −280 mV were subjected to flask fermentations to test their abilities to produce 1,3-PD. The productions of 1,3-PD by each selected mutant colony after 36 h of anaerobic fermentation is shown in Fig. 4A and B. In the screening experiment with an ORP of −240 mV, the average 1,3-PD concentration was 90.1% of that obtained with the parent strain. But no isolated strain showed significantly improved 1,3-PD production in flask fermentations. However, in the screening experiment with an ORP of −280 mV, the average 1,3-PD concentration was 103.8% of that obtained with the parent strain. The ratio of positive strains was 4 out of the total of 11 isolated mutants.

Characterization of the mutant strain with the highest 1,3-PD production. The mutant with the highest 1,3-PD production that was isolated by the cell growth correlation screening method was designated *K. pneumoniae* YC1. The mutant

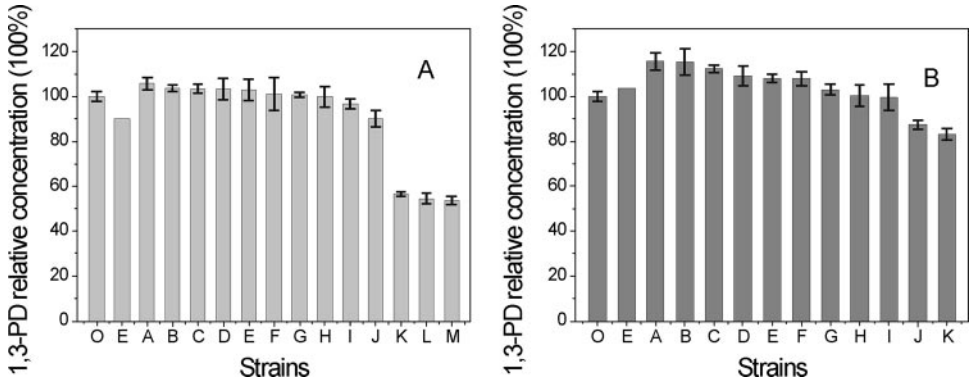

FIG. 4. Comparison of 1,3-PD production by the parent and mutant strains and the average of the isolated mutant strains by the ORP-based screening method. (A) ORP of −240 mV; (B) ORP of −280 mV. O is the parent strain, with a 1,3-PD concentration of 130 mmol liter^{-1}. E is the average value of the mutants. Strains A to M in panel A and strains A to K in panel B are isolated strains (average of triplicate cultures). The error bars represent standard deviations.

with the highest 1,3-PD production that was isolated by the ORP-based screening method at ORPs of −240 mV and −280 mV were designated *K. pneumoniae* YF1 and YMU1 (*K. pneumoniae* M5aL YMU1), respectively. These strains were cultivated in anaerobic fed-batch fermentations for 60 h (Table 1). Isolated strain B at an ORP of −280 mV (designated *K. pneumoniae* YMU2) was also subjected to anaerobic fed-batch fermentation (Table 1). Of the strains tested, *K. pneumoniae* YMU1 produced the highest 1,3-PD concentration, at 729 mmol liter^{-1}, suggesting that it was a superior 1,3-PD producer. To further understand the mechanism underlying the increase in 1,3-PD productivity, *K. pneumoniae* YMU1 was compared against the parent strain with respect to the most-preferred ORP, the metabolite flux distribution, and the reducing equivalent (NAD^+/NADH) ratio.

Preferred ORP range of *K. pneumoniae* YMU1. As mutant strain *K. pneumoniae* YMU1 was isolated from an environment where the ORP was controlled at −280 mV, its most-preferred ORP range may differ from that of the parent strain, −160 to −190 mV (14). To characterize the preferred ORP of *K. pneumoniae* YMU1, fed-batch *K. pneumoniae* YMU1 fermentations were carried out at constant ORPs of −190, −240, −280, and −320 mV. A typical result is shown in Fig. 5. In comparison to the parent strain, the preferred ORP range of *K. pneumoniae* YMU1 dropped from around −190 mV to a more reductive value of around −280 mV. Under this condition, the 1,3-PD concentration, cell concentration, and 1,3-PD specific productivity of *K. pneumoniae* YMU1 reached 915 mmol liter^{-1}, 4.52 g liter^{-1}, and 4.23 mmol g (CDW)$^{-1}$ h^{-1}. These were 63.1%, 68.0%, and 20.7% higher than those of the parent strain, respectively. Further reducing the ORP to −320 mV resulted in a decrease in the 1,3-PD concentration, suggesting that each strain has a preferred reductive environment.

Metabolic-flux analysis of mutant strain *K. pneumoniae* YMU1. To reveal the change in the metabolic pathway of the mutant strain, the distribution of metabolic flux of *K. pneumoniae* YMU1 in the fermentation at an ORP of −280 mV was analyzed (Fig. 6). The mutant strain exhibited an improved 1,3-PD-producing capacity compared to that of its parent. Lactic acid and ethanol production by the mutant strain did not change significantly. The production of 2,3-butanediol by the parent strain was below 0.1 mmol g (CDW)$^{-1}$ h^{-1}. In the mutant strain, it increased to 0.45 mmol g (CDW)$^{-1}$ h^{-1}, suggesting that the bioreductive branch had been strengthened. Interestingly, after the mutation, acetic acid production was significantly reduced (Fig. 6).

Change in the intracellular NAD^+/NADH ratio. To verify the change in intracellular redox potential, the time courses of the intracellular NAD^+/NADH ratio of the parent strain and the mutant strain were investigated. As shown in Fig. 7, the NAD^+/NADH ratio was about 4 in the parent strain. The decrease in the NAD^+/NADH ratio to 2 in the mutant strain

TABLE 1. Comparison of 5-liter fed-batch fermentation results obtained with four isolated strains and the parent strain

K. pneumoniae strain	CDW[a] (g liter^{-1})	1,3-PD[b] concn (mmol liter^{-1})	CDW yield[c] (g g^{-1} glycerol)	1,3-PD yield[d] (mol mol^{-1})	Specific productivity[e] (mmol g [CDW]$^{-1}$ h^{-1})
M5aL	2.69	558	0.026	0.499	3.45
YC1	2.82	589	0.024	0.456	3.48
YF1	3.75	684	0.030	0.511	3.03
YMU1	3.30	729	0.025	0.503	3.68
YMU2[f]	3.42	699	0.019	0.355	3.10

[a] Maximal CDWs during fermentation are shown.
[b] 1,3-PD production at the end of fermentation. For *K. pneumoniae* YMU2, the fermentation time was 62 h. For the other four strains, the fermentation time was 60 h.
[c] To calculate the CDW yield, we divided the maximal CDW by the glycerol consumption.
[d] To calculate the 1,3-PD yield, we divided the 1,3-PD production at the end of fermentation by the glycerol consumption.
[e] To calculate specific productivity, we divided the 1,3-PD production at the end of fermentation by the maximal CDW and by the hours of fermentation.
[f] The data for strain YMU2 are from our previous report (35).

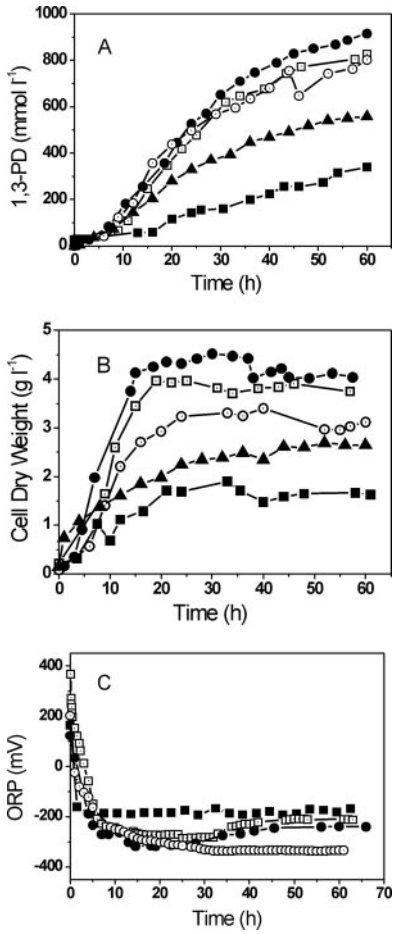

FIG. 5. Time courses of the 1,3-PD concentration (A), CDW (B), and ORP (C) of mutant strain *K. pneumoniae* YMU1 at four different ORP levels and those of the parent strain. Symbols: ■, the mutant strain at an ORP of −190 mV; □, the mutant strain at an ORP of −240 mV; ●, the mutant strain at an ORP of −280 mV; ○, the mutant strain at an ORP of −320 mV; ▲, the parent strain with the optimal ORP regulation process (adapted from our previous publication [14]).

demonstrated that the intracellular environment was more reductive in the mutant strain.

DISCUSSION

A positive correlation between the CDW of *K. pneumoniae* and its 1,3-PD biosynthesis was observed (Fig. 1 and 2), similar to that reported for *C. butyricum* (28). Metabolic network analysis (22) showed that converting 1 M glycerol to biomass would produce 1 M NADH, demonstrating that the pathway from glycerol to biomass improved 1,3-PD biosynthesis.

In this study, we investigated the possibility of using the correlation between cell growth and bioconversion to isolate a high-productivity strain. The results obtained (Fig. 3) indicated that the cell growth correlation screening method was feasible, but the selection efficiency was very low (2 out of 67) and the selection process was laborious. The reason seemed to be that 1,3-PD production is a mixed growth-associated biosynthesis process that is correlated with both biomass and specific productivity per cell. Screening for mutants with improved growth ability only affected one aspect of 1,3-PD production.

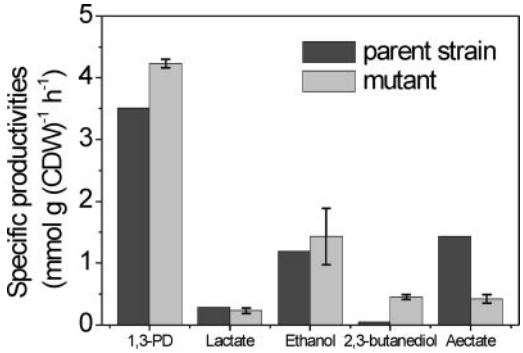

FIG. 6. Comparison of the specific production of 1,3-PD, lactate, ethanol, 2,3-butanodiol, and acetate by the parent strain and mutant strain *K. pneumoniae* YMU1 (average of triplicate cultures at an ORP of −280 mV). The error bars represent standard deviations. The CDW yield of the parent strain was 0.028 g/g, while the CDW yield of the mutant strain was 0.019 g/g.

In the present study, an ORP-based method was shown to improve screening efficiency significantly (Fig. 4B). In the ORP-based method, at an ORP of −280 mV, 4 out of 11 isolated strains were proved to be positive mutants. The screening efficiency was significantly higher than that of the cell growth correlation screening method alone. We infer that the mechanism of this method might be as follows: ORP was used as an environmental selection pressure to isolate mutants that adapted to certain ORP levels. The mutants that could not adapt to a more reduced ORP were prevented from growing and became the minority in the fermentation broth. On the contrary, the mutants that adapted to the more reduced environment propagated quickly and became dominant in the fermentation broth. By diluting and cultivating the broth, mutated strains with a high growth rate and adaptability to a specified ORP level can be isolated. In addition to shake flask fermentations, fed-batch fermentations of *K. pneumoniae* YMU1 and YMU2 both resulted in enhanced 1,3-PD production (Table 1), suggesting that 8% higher 1,3-PD production in flask fermentations may be a reliable criterion to screen positive mutant strains in these serial experiments. And the result obtained with *K. pneumoniae* YF1 in the fed-batch fermentation indi-

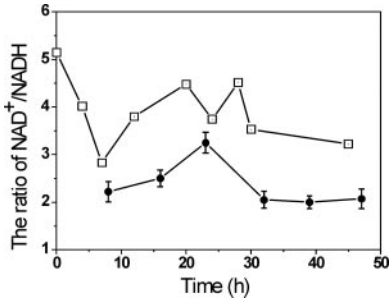

FIG. 7. Comparison of the NAD^+/NADH ratio time courses of the mutant and original strains. Symbols: □, original strain with the optimal ORP regulation process; ●, mutant strain at a constant ORP of −280 mV (average of triplicate cultures). The error bars represent standard deviations.

cated that, with 5% higher 1,3-PD production in flask fermentations, it is also possible to get a strain with increased 1,3-PD production in scale-up fermentations. The environmental-tolerance screening method has also been reported in continuous culture (chemostat) to select strains with desired traits (15, 19, 29). In continuous culture, the stains that were not diluted out by environment pressure showed a high level of tolerance for a specified environment. Compared with the continuous-culture screening method, the ORP-based screening method offered significant advantages by shortening the culture time and minimizing the instruments required and their operation.

The growth rate of mutant strain *K. pneumoniae* YMU1 was low at an ORP of −190 mV (Fig. 5), indicating that this environment was no longer suitable for the mutant strain. The preferred ORP range of the mutant shifted from around −190 mV to −280 mV (Fig. 5), suggesting not only different species or strains (7, 30, 31) but also that the mutants of the same strain had significantly different preferred ORP ranges. In comparison with the parent strain, the highest cell concentration and 1,3-PD production of the mutant strain were enhanced by 68.0% and 63.1%, respectively.

Metabolic analysis showed that the increased 1,3-PD production of the mutant strain was due to two factors. First, the cell concentration was enhanced (Fig. 5B). This was probably because the specific acetate production by mutant strain *K. pneumoniae* YMU1 was only 30% of that of the parent strain (Fig. 6). Acetate is one of the strongest *K. pneumoniae* cell growth inhibitors (34); significantly reduced acetate produced is therefore expected to improve cell growth. Second, specific 1,3-PD production was enhanced (Fig. 6). In *K. pneumoniae*, the formation of 1,3-PD from glycerol was catalyzed by glycerol dehydratase and 1,3-PD dehydrogenase consecutively (20). The first bioconversion is generally accepted as the limiting step due to inactivation of vulnerable glycerol dehydratase (18, 24). However, the inactivated enzyme could be reactivated by ATP and Mg^{2+}(18). In the mutant strain, the metabolite flux toward the energy branch was increased (data not shown), which might facilitate the reactivation of the inactivated glycerol dehydratase and thereby improved the production of 1,3-PD. Moreover, the redistribution of metabolic flux of the mutant strain, together with the altered environmental conditions (an ORP of −280 mV), resulted in a decreased intracellular NAD^+/NADH ratio (Fig. 7), which might enhance the activity of 1,3-PD dehydrogenase (12) and consequently accelerate 1,3-PD production. In short, the two bioconversion steps in the reductive branch were both improved in isolated mutant strain *K. pneumoniae* YMU1.

This paper is the first report of the use of ORP as an environmental selection pressure to isolate a high-production strain after random mutagenesis. This provides an alternative approach to the improvement of industrial microorganisms, especially in bioreductive and bio-oxidatively coupled bioconversion systems.

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REFERENCES

1. **Abbad-Andaloussi, S., C. Manginot-Durr, J. Amine, E. Petitdemange, and H. Petitdemange.** 1995. Isolation and characterization of *Clostridium butyricum* DSM 5431 mutants with increased resistance to 1,3-propanediol and altered production of acids. Appl. Environ. Microbiol. **61:**4413–4417.
2. **Barbirato, F., S. Astruc, P. Soucaille, C. Camarasa, J.-M. Salmon, and A. Bories.** 1997. Anaerobic pathways of glycerol dissimilation by *Enterobacter agglomerans* CNCM 1210: limitations and regulations. Microbiology **143:** 2423–2432.
3. **Barbirato, F., H. Himmi, T. Conte, and A. Bories.** 1998. 1,3-Propanediol production by fermentation: an interesting way to valorize glycerin from the ester and ethanol industries. Ind. Crops Prod. **7:**281–289.
4. **Barbirato, F., P. Soucaille, and A. Bories.** 1996. Physiologic mechanisms involved in accumulation of 3-hydroxyporpionaldehyde during fermentation of glycerol by *Enterobacter agglomerans*. Appl. Environ. Microbiol. **62:**4405–4409.
5. **Biebl, H., H. Menzel, A. Zeng, and W. Deckwer.** 1999. Microbial production of 1,3-propanediol. Appl. Microbiol. Biotechnol. **52:**289–297.
6. **Boenigk, R., S. Bowien, and G. Gottschalk.** 1993. Fermentation of glycerol to 1,3-propanediol in continuous cultures of *Citrobacter freundii*. Appl. Microbiol. Biotechnol. **38:**453–457.
7. **Bourel, G., S. Hennini, C. Divies, and D. Garmyn.** 2003. The response of *Leuconostoc mesenteroides* to low external oxidoreduction potential generated by hydrogen gas. J. Appl. Microbiol. **94:**280–288.
8. **Chen, X., D. Zhang, W. Qi, S. Gao, Z. Xiu, and P. Xu.** 2003. Microbial fed-batch production of 1,3-propanediol by *Klebsiella pneumoniae* under micro-aerobic conditions. Appl. Microbiol. Biotechnol. **63:**143–146.
9. **Cheng, K., D. Liu, Y. Sun, and W. Liu.** 2004. 1,3-Propanediol production by *Klebsiella pneumoniae* under different aeration strategies. Biotechnol. Lett. **26:**911–915.
10. **Cheng, K., H. Liu, and D. Liu.** 2005. Multiple growth inhibition of *Klebsiella pneumoniae* in 1,3-propanediol fermentation. Biotechnol. Lett. **27:**19–22.
11. **Cheng, K., J. Zhang, D. Liu, Y. Sun, M. Yang, and J. Xu.** 2006. Production of 1,3-propanediol by *Klebsiella pneumoniae* from glycerol broth. Biotechnol. Lett. **28:**1817–1821.
12. **Daniel, R., R. Boenigk, and G. Gottschalk.** 1995. Purification of 1,3-propanediol dehydrogenase from *Citrobacter freundii* and cloning, sequencing, and overexpression of the corresponding gene in *Escherichia coli*. J. Bacteriol. **177:**2151–2156.
13. **Du, C., C. Li, D. Yang, Y. Zhang, and Z. Cao.** 2004. Biocatalysis-coupled cell growth process in 1,3-propanediol production by *Klebsiella pneumoniae*. J. Chem. Ind. Eng. (China) **55:**505–508.
14. **Du, C., H. Yan, Y. Zhang, Y. Li, and Z. Cao.** 2006. Use of oxidoreduction potential as an indicator to regulate 1,3-propanediol fermentation by *Klebsiella pneumoniae*. Appl. Microbiol. Biotechnol. **69:**554–563.
15. **Dykhuizen, D. E., and D. L. Hartl.** 1983. Selection in chemostats. Microbiol. Rev. **47:**150–168.
16. **Hartlep, M., W. Hussmann, N. Prayitno, I. Meynial-Salles, and A. Zeng.** 2002. Study of two-stage processes for the microbial production of 1,3-propanediol from glucose. Appl. Microbiol. Biotechnol. **60:**60–66.
17. **Homann, T., C. Tag, H. Biebl, W. Deckwer, and B. Schink.** 1990. Fermentation of glycerol to 1,3-propanediol by *Klebsiella* and *Citrobacter* strains. Appl. Microbiol. Biotechnol. **33:**121–126.
18. **Kajiura, H., K. Mori, T. Tobimatsu, and T. Toraya.** 2001. Characterization and mechanism of action of a reactivating factor for adenosylcobalamin-dependent glycerol dehydratase. J. Biol. Chem. **276:**36514–36519.
19. **Lane, P., S. Oliver, and P. Butler.** 1999. Analysis of a continuous-culture technique for the selection of mutants tolerant to extreme environmental stress. Biotechnol. Bioeng. **65:**397–406.
20. **Lin, E.** 1976. Glycerol dissimilation and its regulation. Annu. Rev. Microbiol. **36:**535–578.
21. **Menzel, K., K. Ahrens, A. Zeng, and W. Deckwer.** 1998. Kinetic, dynamic, and pathway studies of glycerol metabolism by *Klebsiella pneumoniae* in anaerobic continuous culture. IV. Enzymes and fluxes of pyruvate metabolism. Biotechnol. Bioeng. **60:**617–626.
22. **Menzel, K., A. Zeng, and W. Deckwer.** 1997a. Enzymatic evidence for an involvement of pyruvate dehydrogenase in the anaerobic glycerol metabolism of *Klebsiella pneumoniae*. J. Biotechnol. **56:**135–142.
23. **Menzel, K., A. Zeng, and W. Deckwer.** 1997b. High concentration and productivity of 1,3-propanediol from continuous fermentation of glycerol by *Klebsiella pneumoniae*. Enzyme Microb. Technol. **20:**82–86.
24. **Mori, K., and T. Toraya.** 1999. Mechanism of reactivation of coenzyme B_{12}-dependent diol dehydratase by a molecular chaperone-like reactivating factor. Biochemistry **38:**13170–13178.
25. **Ohnishi, J., S. Mitsuhashi, M. Hayashi, S. Ando, H. Yokoi, K. Ochiai, and M. Ikeda.** 2002. A novel methodology employing *Corynebacterium glutamicum* genome information to generate a new L-lysine-producing mutant. Appl. Biochem. Biotechnol. **58:**217–223.

26. **Ouvry, A., Y. Wache, R. Tourdot-Marechal, C. Divies, and R. Cachon.** 2002. Effects of oxidoreduction potential combined with acetic acid, NaCl and temperature on the growth, acidification, and membrane properties of *Lactobacillus plantarum*. FEMS Microbiol. Lett. **214:**257–261.
27. **Papanikolaou, S., M. Fick, and G. Aggelis.** 2004. The effect of raw glycerol concentration on the production of 1,3-propanediol by *Clostridium butyricum*. J. Chem. Technol. Biotechnol. **79:**1189–1196.
28. **Papanikolaou, S., P. Ruiz-Sanchez, B. Pariset, F. Blanchard, and M. Fick.** 2000. High production of 1,3-propanediol from industrial glycerol by a newly isolated *Clostridium butyricum* strain. J. Biotechnol. **77:**191–208.
29. **Parekh, S., V. A. Vinci, and R. J. Strobel.** 2000. Improvement of microbial strains and fermentation processes. Appl. Microbiol. Biotechnol. **54:**287–301.
30. **Riondet, C., R. Cachon, Y. Wache, G. Alcaraz, and C. Divies.** 1999. Changes in the proton-motive force in *Escherichia coli* in response to external oxidoreduction potential. Eur. J. Biochem. **262:**595–599.
31. **Riondet, C., R. Cachon, Y. Wache, G. Alcaraz, and C. Divies.** 2000. Extracellular oxidoreduction potential modifies carbon and electron flow in *Escherichia coli*. J. Bacteriol. **182:**620–626.
32. **Stafford, D., K. Yanagimachi, P. Lessard, S. Rijhwani, A. Sinskey, and G. Stephanopoulos.** 2002. Optimizing bioconversion pathways through systems analysis and metabolic engineering. Proc. Natl. Acad. Sci. USA **99:**1801–1806.
33. **Zeng, A., and H. Biebl.** 2002. Bulk chemicals from biotechnology: the case of 1,3-propanediol production and the new trends. Adv. Biochem. Eng. Biotechnol. **74:**240–259.
34. **Zeng, A., A. Ross, H. Biebl, C. Tag, and W. Deckwer.** 1994. Multiple product inhibition and growth modeling of *Clostridium butyricum* and *Klebsiella pneumoniae* in glycerol fermentation. Biotechnol. Bioeng. **44:**902–911.
35. **Zhang, Y., Y. Li, C. Du, M. Liu, and Z. Cao.** 2006. Inactivation of aldehyde dehydrogenase: a key factor for engineering 1,3-propanediol production by *Klebsiella pneumoniae*. Metab. Eng. **8:**578–586.
36. **Zheng, P., K. Wereath, J. Sun, J. van den Heuvel, and A. Zeng.** 2006. Overexpression of genes of the dha regulon and its effects on cell growth, glycerol fermentation to 1,3-propanediol and plasmid stability in *Klebsiella pneumoniae*. Process Biochem. **41:**2160–2169.