

Genome Shuffling in *Clostridium diolis* DSM 15410 for Improved 1,3-Propanediol Production

Burkhard Otte, Eike Grunwaldt, Osama Mahmoud and
Stefan Jennewein

Appl. Environ. Microbiol. 2009, 75(24):7610. DOI:
10.1128/AEM.01774-09.

Published Ahead of Print 23 October 2009.

Updated information and services can be found at:
<http://aem.asm.org/content/75/24/7610>

These include:

REFERENCES

This article cites 29 articles, 5 of which can be accessed free at:
<http://aem.asm.org/content/75/24/7610#ref-list-1>

CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new
articles cite this article), [more»](#)

Information about commercial reprint orders: <http://journals.asm.org/site/misc/reprints.xhtml>
To subscribe to to another ASM Journal go to: <http://journals.asm.org/site/subscriptions/>

Genome Shuffling in *Clostridium diolis* DSM 15410 for Improved 1,3-Propanediol Production[▽]

Burkhard Otte, Eike Grunwaldt, Osama Mahmoud, and Stefan Jennewein*

Fraunhofer Institut für Molekularbiologie und Angewandte Ökologie, Forckenbeckstrasse 6, 52074 Aachen, Germany

Received 27 July 2009/Accepted 8 October 2009

Several microorganisms are known for their efficient anaerobic conversion of glycerol to 1,3-propanediol, with *Clostridium diolis* DSM 15410 as one of the better performers in terms of molar yield and volumetric productivity. However, this performance is still insufficient to compete with established chemical processes. Previous studies have shown that high concentrations of 1,3-propanediol, glycerol, and fermentation side products can limit the productivity of *C. diolis* DSM 15410. Here, we describe the use of genome shuffling for improved 1,3-propanediol fermentation by the strict anaerobe *C. diolis* DSM 15410. By using chemical mutagenesis, strains with superior substrate and product tolerance levels were isolated and higher product yields were obtained. These superior strains were then used for genome shuffling and selection for 1,3-propanediol and organic acid tolerance. After four rounds of genome shuffling and selection, significant improvements were observed, with one strain attaining a 1,3-propanediol volumetric yield of 85 g/liter. This result represents an 80% improvement compared to the yield from the parental wild-type strain.

The use of biomass instead of petrochemical feedstock could facilitate the sustainable production of many chemicals, but this approach has proven economically feasible in only a few cases (4, 17, 20, 21). The microbial production of 1,3-propanediol (1,3-PD) provides an interesting case study because this monomer is used to produce several plastics, including the relatively new and highly versatile polytrimethylene terephthalate, which has significantly increased demand for 1,3-PD (12, 23, 24). Polytrimethylene terephthalate is currently produced from petrochemical feedstock in a process that involves the conversion of ethylene oxide into 3-hydroxypropionaldehyde by hydroformylation under high pressure and then further into 1,3-PD by hydrogenation using a nickel or rubidium catalyst (21).

Several companies have investigated the sustainable production of 1,3-PD from biomass. For example, DuPont and Genencor transferred the relevant 1,3-PD biosynthetic genes from *Klebsiella pneumonia* into *Escherichia coli* and further modified carbohydrate metabolism and transport so that 1,3-PD could be synthesized from glucose (10, 11; M. Emptage, S. L. Haynie, L. A. Laffend, J. Pucci, and G. M. Whited, 2001, Patent Cooperation Treaty international application WO 2001/01/12833). Large-scale production of 1,3-PD by this approach is likely to be too expensive due to the high input costs of vitamin B₁₂ and antibiotics, so the use of glycerol as an alternative feedstock has been investigated, although this strategy requires additional enzymes and a shift from aerobic to anaerobic conditions (15, 25, 30).

Clostridium diolis DSM 15410 (formerly *C. butyricum* DSM 5431) can produce 1,3-PD from glycerol under anaerobic conditions and is therefore a desirable alternative to *E. coli* given

the relative costs of industrial aerobic and anaerobic fermentation (6, 8, 9, 22). However, the efficiency of conversion is not yet high enough for an industrial process. The production of 1,3-PD by *C. diolis* is limited by inhibition from both substrates and products, as well as organic acids produced as fermentation by-products (13).

Classical strain improvement has significantly increased 1,3-PD production, but this is a slow process and the mutations are predominantly neutral or detrimental (2). We have therefore approached the problem using genome shuffling, which is more efficient and reliable for engineering complex phenotypes, as demonstrated in several other examples of microbial strain development (16, 26, 31). Genome shuffling offers the advantages of accumulated beneficial mutations and the removal of unnecessary mutations due to simultaneous changes at different positions throughout the genome and, therefore, yields microbes of superior fitness (29). We applied both the classical approach and genome shuffling to *C. diolis* DSM 15410 to improve the production of 1,3-PD, which is a necessary prerequisite for the fermentation process. To our knowledge, this study is the first example of genome shuffling in a strictly anaerobic microorganism.

MATERIALS AND METHODS

Bacteria and media. *C. diolis* DSM 15410 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). Spores were stored at 4°C in reinforced clostridial medium (RCM; Carl-Roth, Germany). Standard cultivation was carried out in a Bactron IV anaerobic chamber (Omni Life Sciences, Hamburg) at 34°C. The preculture medium (improved minimal medium [IMM]) contained the following components per liter of distilled water: 18 g glycerol, 13.6 g KH₂PO₄, 17.4 g K₂HPO₄, 2 g (NH₄)₂SO₄, 0.2 g MgSO₄, 20 mg CaCl₂, 5 mg FeSO₄, 1 g yeast extract, and 2 ml trace element solution SL7 (7). For high-throughput screening in 96-deep-well plates, IMM was used with the following additions or changes: 60 g/liter glycerol, 0.1 g/liter K₂HPO₄, 100 mM MES [2-(N-morpholino)ethanesulfonic acid], 1 g/liter CaCO₃, 5 g/liter yeast extract, and bromothymol blue as a pH indicator. Cultivation in 96-deep-well plates was carried out for 3 days. The medium for bioreactor fermentation was the same as IMM except for the use of 1 g/liter K₂HPO₄, 0.5 g/liter KH₂PO₄, and 5 g/liter yeast extract.

An exponential-phase culture grown in preculture medium was used as the

* Corresponding author. Mailing address: Fraunhofer Institut für Molekularbiologie und Angewandte Ökologie, Forckenbeckstrasse 6, 52074 Aachen, Germany. Phone: 49 241 6085 12121. Fax: 49 241 6085 10000. E-mail: Stefan.jennewein@ime.fraunhofer.de.

[▽] Published ahead of print on 23 October 2009.

inoculum at 6% (vol/vol) for 96-deep-well plate fermentation and 5% (vol/vol) for batch or fed-batch fermentation in a 250-ml bioreactor vessel (Ochs GmbH, Bovenden/Lengler, Germany). The pH and feed were coupled by using a ratio of 1.5 M KOH to 3.75 M glycerol. The pH was monitored and maintained at 7.0 with a T50 titrator (Mettler Toledo GmbH, Giessen, Germany). Feed consisting of 20 g/liter yeast extract, 6 g/liter NH_4SO_4 , 60 mg/liter CaCl_2 , 20 mg/liter FeSO_4 , 6 ml/liter trace element solution SL7, and 8 M glycerol was manually added during the fermentation to maintain the glycerol concentration between 20 and 25 g/liter and to refresh the medium composition. The growth temperature for 96-deep-well plate fermentations was 34°C, whereas that for fed-batch fermentations was 28°C. The working volume for deep-well fermentations was 1 ml, and that for fed-batch fermentations was 220 ml.

Crude glycerol. Crude glycerol was kindly provided by M. Menner (Fraunhofer Institute IVV, Freising, Germany) from a biodiesel production plant in Mainburg (Germany). It was at pH 11 and contained the following components: glycerol, 70% (wt/vol); potash, 15%; fatty acids, 5 to 10%; and methanol, <1%. For the fed-batch fermentations, the fatty acids were removed by acidification and subsequent extraction. Glycerol was concentrated by a rotary evaporator.

Mutagenesis and selection. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was used for mutagenesis in *C. diolis* at the initial exponential phase: 10-ml aliquots of cells were treated with different concentrations of NTG for at least 1 h at 34°C. The cells were then centrifuged for 10 min at $4,000 \times g$, washed twice with anaerobic potassium phosphate buffer, resuspended in IMM, and regenerated for at least 1 h. Cells were spread onto agar plates containing different concentrations of 1,3-PD or glycerol. For high-throughput screening, the mutated cells were spread onto IMM agar plates. Colonies were picked from 96-deep-well plates.

Analytical methods. The cell concentrations were estimated as the dry weight of cells by using a predetermined correlation between A_{600} (measured with a BioPhotometer [Eppendorf, Hamburg, Germany]) and the dry weight of cells. For the analysis of fermentation metabolites, either high-performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry (GC-MS) was applied. For HPLC, we used a Shimadzu Prominence HPLC system equipped with a refractive index detector. For the HPLC separation, we used a Rezex RFO-Fast Acid column (100 by 7.8 mm) from Phenomenex (Aschaffenburg, Germany), and metabolites were detected by a refractive index detector. The following operating conditions were employed: oven temperature, 60°C; mobile phase, 5 mM sulfuric acid; and flow rate, 1 ml/min. As an internal standard, 48 mM 1,5-pentanediol was used. For GC-MS analysis, a Shimadzu GCMS-QP2010S system was used. For sample preparation prior to analyses of 1,3-PD and glycerol concentrations, deep-well plates were centrifuged for 10 min at $3,000 \times g$ and 25 μl of biomass-free supernatant was transferred into 975 μl ethanol containing 11 mM 1,4-butanediol as an internal standard. Additional centrifugation was carried out for 10 min at $3,000 \times g$. A total of 600 μl of the sample was transferred into a new deep-well plate. A Shimadzu GCMS-QP2010S system equipped with an Rtx Wax GC column (internal diameter, 0.18 mm; length, 10 m [Restek, Bad Homburg, Germany]) was used for metabolite analysis. For separation of the compounds, the following temperature program was used: a split injection temperature of 250°C and a temperature gradient program starting at 130°C for 1 min, with heating of the column at 40°C per min to 250°C. Mass detection was done with electric ionization at 1 keV by using the selected-ion monitoring/scan mode for masses of m/z 50 to 100. Standards for the fermentation broth products were purchased from Sigma-Aldrich.

Genome shuffling and selection. Cells were cultivated in RCM until they reached mid-log phase and harvested by centrifugation at $3,000 \times g$ for 10 min. For protoplast formation, 500 μl of the culture was mixed with clostridium protoplasting buffer (CPB), which consisted of RCM with 500 mM sucrose, 25 mM MgCl_2 , 25 mM CaCl_2 , and 20 mg/liter lysozyme. Enzyme treatment was continued for 1 h at 35°C. The appearance of spherical cells as judged by light microscopy was used as an indicator of protoplast formation. Protoplasts were centrifuged twice for 5 min at $2,000 \times g$ and washed with CPB lacking lysozyme. To generate fusions between protoplast preparations, approximately equal numbers of protoplasts from different populations were mixed and the mixture was centrifuged and resuspended in 100 μl CPB lacking lysozyme. Nine volumes of 30 to 60% polyethylene glycol 6000 in CPB were added, and the protoplast mix was incubated for 15 to 30 min at room temperature. For regeneration of protoplasts, 5 ml CPB lacking lysozyme was added and the protoplasts were centrifuged, washed, and resuspended in CPB containing 100 mM *N*-acetylglucosamine. Protoplasts were incubated for 5 h at 34°C and spread onto regeneration plates consisting of RCM plus 12.5 mM MgCl_2 , 12.5 mM CaCl_2 , 10 g/liter casein hydrosylate, and 40 g/liter gelatin. The plates were scraped, and the cells were spread onto selection medium consisting of IMM and different concentrations of glycerol, 1,3-PD, and butyric and acetic acids.

The formation of protoplasts and protoplast fusion, subsequent regeneration, and selection were repeated four times, with the pooled regenerated cells from one fusion serving as the inoculum for the subsequent protoplast culture. Controls were prepared by protoplast formation and regeneration without exposure to polyethylene glycol.

RESULTS

Improvement of glycerol tolerance through chemical mutagenesis. *C. diolis* DSM 15410 cannot grow on agar plates containing concentrations of glycerol greater than 1 M. In order to obtain mutants with the ability to grow at higher concentrations of glycerol, *C. diolis* DSM 15410 was subjected to treatment with NTG, followed by selection on agar plates with increasing concentrations of glycerol. We used a concentration of NTG that allowed 1% of cells to survive, and this protocol has turned out to be effective for strain improvement. The starting concentration for the following rounds of mutagenesis was 900 mM glycerol. The isolated mutants were used for subsequent mutagenesis at higher concentrations. In total, five rounds of mutagenesis and selection were carried out for the improvement of glycerol tolerance. Five mutants with enhanced abilities to grow on glycerol concentrations of up to 1.5 M were isolated (Fig. 1). All mutants produced more biomass than the wild type and showed similar behaviors with regard to glycerol tolerance.

Improvement of 1,3-PD tolerance through chemical mutagenesis. Growth of *C. diolis* is hampered by inhibition from 1,3-PD at concentrations greater than 600 mM. Chemical mutagenesis was employed to obtain a mutant that can tolerate more than 1 M 1,3-PD. The mutagenesis protocol was equivalent to that of the glycerol tolerance improvement approach. After four rounds of mutagenesis, four mutants that were able to grow on 1,3-PD at concentrations of up to 1.2 M were isolated. Figure 2 shows a representative characterization of the two best selected mutants. All mutants yielded significantly more biomass than the wild-type strain.

Batch fermentations with *C. diolis* DSM 15410 and selected glycerol mutants. In order to test the performances of the *C.*

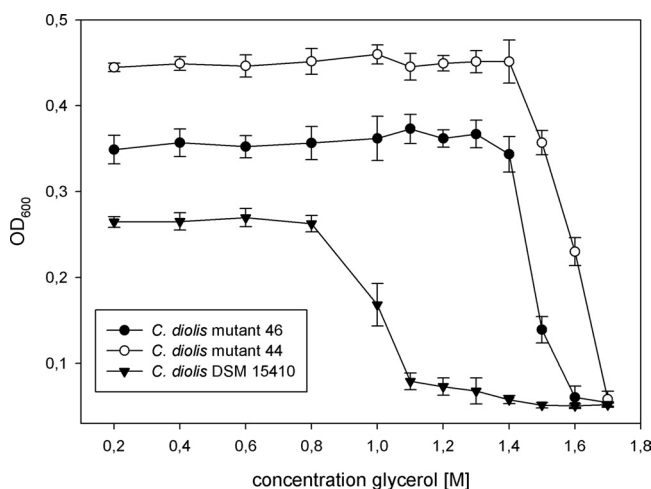


FIG. 1. Comparison of levels of inhibition of *C. diolis* DSM 15410 and mutants grown for 3 days at 35°C in media with increasing glycerol concentrations. Standard deviations were calculated from the results of at least six independent experiments. OD₆₀₀, optical density at 600 nm.

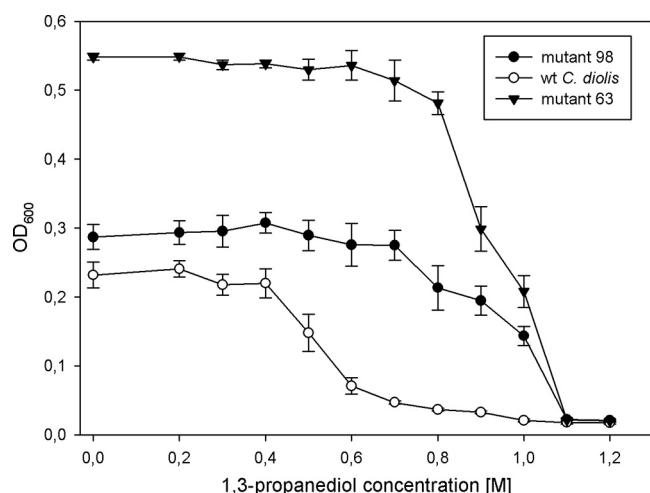


FIG. 2. Comparison of levels of inhibition of *C. diolis* wild-type (wt) strain DSM 15410 and mutants grown for 3 days at 35°C in media with increasing 1,3-PD concentrations. Standard deviations were calculated from the results of at least six independent experiments. OD₆₀₀, optical density at 600 nm.

diolis wild type and the selected glycerol-tolerant mutants in 1,3-PD formation, batch fermentations with these strains were carried out. Glycerol concentrations were chosen according to the highest concentrations tolerated by the wild type and mutant 98. Starting glycerol concentrations of 673 mM for the wild-type strain and 1.19 M for the mutant were used. Figure 3 shows the levels of 1,3-PD production by the wild-type strain and mutant 98. Mutant 98 produced 597 mM 1,3-PD, and the wild-type strain produced 328 mM 1,3-PD. For mutant 98, the ratio of moles of 1,3-PD produced to moles of glycerol consumed ($Y_{1,3-PD}$) was 0.50, and the $Y_{1,3-PD}$ for the wild-type strain was 0.48.

High-throughput screening for a superior 1,3-PD producer.

Due to the lack of direct selection methods for improved productivity, a mutagenesis approach with subsequent screening was performed. For this purpose, different concentrations of NTG were applied to the wild-type strain. Mutants were randomly picked and then tested for their productivity in a 96-deep-well small-scale fermentation format. A severe problem that was partially overcome was rapid acidification of the medium and subsequent growth inhibition due to the generation of organic acids as metabolic side products. We have tested various buffer conditions and found that a dynamic buffering system using MES and calcium carbonate attenuated the fast acidification. For analysis of product concentrations, 1,3-PD was extracted from the fermentation broth and analyzed by a fast yet accurate GC-MS method. In Fig. 4, a GC chromatogram and the corresponding MS spectra from the high-throughput screening approach are shown. Because of different growth conditions in the deep-well plates, mutants that showed improved productivity were retested for superior 1,3-PD production. This retesting was carried out by statistical analysis of the mutants and the wild type. At least eight independent small-scale fermentations were performed. This procedure was done in iterative rounds, and mutants with a modest but reproducible improvement in 1,3-PD production were obtained. In Fig. 5, a representative result for the final 1,3-PD

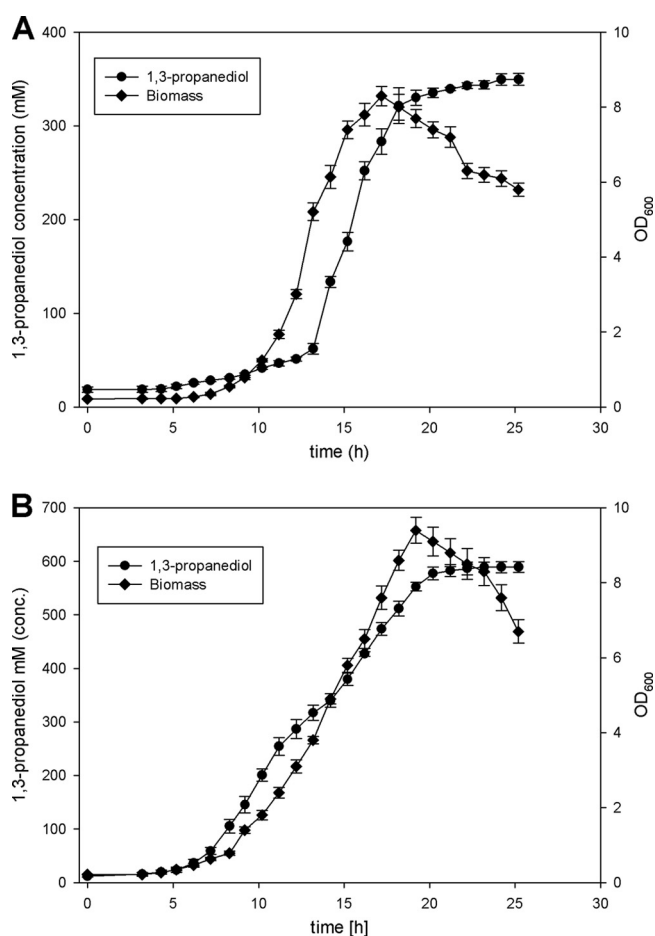


FIG. 3. (A) Results from batch fermentation with *C. diolis* DSM 15410. For the wild type, 62 g/liter glycerol was used as a starting concentration. Standard deviations were calculated from the results of at least four independent experiments. (B) Results from batch fermentation with the glycerol-tolerant mutant 98. A glycerol concentration of 112 g/liter was used as the starting concentration. Standard deviations were calculated from the results of at least four independent experiments. OD₆₀₀, optical density at 600 nm; conc., concentration.

yield from a small-scale fermentation is shown. Differences in product formation are clearly demonstrated. The wild-type strain *C. diolis* DSM 15410 produced up to 150 mM 1,3-PD. Among the screened mutants, PSM 7 produced up to 242 mM 1,3-PD under the applied screening conditions. In total, more than 4,000 mutants were screened for superior 1,3-PD production. Altogether, four mutants with superior 1,3-PD production in the high-throughput screening were isolated, with PSM 7 as the most productive one.

Genome shuffling for improvement of 1,3-PD production.

Successful formation of protoplasts from several different *Clostridium* spp. has been demonstrated previously (28), and the approach was slightly modified. Genome shuffling in mutants possessing enhanced tolerance for 1,3-PD and glycerol, as well as mutants with improved 1,3-PD productivity, was performed. The colonies that appeared after regeneration and subsequent selection were scraped from agar plates, pooled, and used for the next iterative cycle of genome shuffling with higher concentrations of 1,3-PD and butyric and acetic acids. A total of

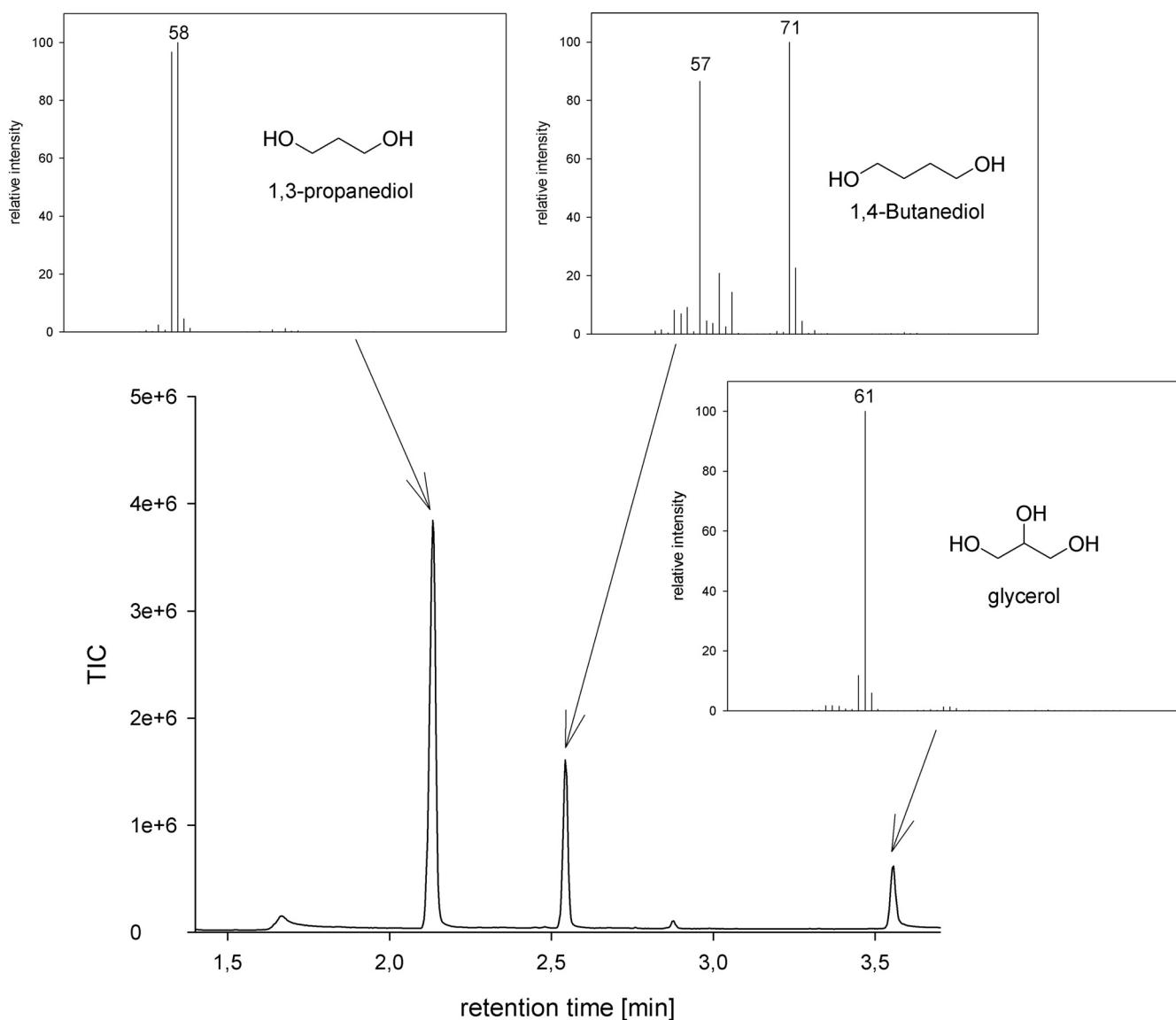


FIG. 4. GC-MS analysis of fermentation broth showing the total ion chromatogram (TIC) together with the mass spectra of 1,3-PD, 1,4-butanediol, and glycerol.

four rounds of genome shuffling were performed. Two strains, GSHM 2 and GSHM 4, that were able to grow at high 1,3-PD concentrations (1.05 M) and organic acid concentrations greater than 150 mM were isolated. These strains were further tested in fed-batch fermentations for their productivity using pharmaceutical or crude glycerol as the carbon source.

Comparative fed-batch fermentations with *C. diolis* DSM 15410 and strains obtained by high-throughput screening and genome shuffling. In order to test the performances of the strains obtained from the high-throughput screening campaign and genome shuffling, fed-batch fermentations using pharmaceutical glycerol as the carbon source were conducted. Here, the wild-type strain produced a mean $\pm$ standard deviation of 624 ± 38 mM 1,3-PD, and the mutant PSM 7 produced 746 ± 55 mM 1,3-PD (Table 1). PSM 7, which was isolated from a high-throughput screening for elevated 1,3-PD production, of-

fered a better yield than the wild type and a mildly improved specific production rate. The strains derived from genome shuffling showed better volumetric productivity and specific production rates and reached higher yields than the wild type and PSM 7. In total, strain GSHM 2 produced $1,032 \pm 79$ mM 1,3-PD and strain GSHM 4 produced $1,114 \pm 86$ mM 1,3-PD. These values represent an 80% improvement in the yield of 1,3-PD. Moreover, the strains showed a different acid profile from that of the wild type. GSHM 2 produced more acetic acid than butyric acid, whereas strain GSHM 4 generated more butyric acid. Both strains generated more biomass than the wild type.

Comparative fed-batch fermentations with *C. diolis* DSM 15410 and GSHM 2 using crude glycerol as a carbon source. Crude glycerol obtained as a side product in biodiesel production is a more attractive carbon source than pharmaceutical

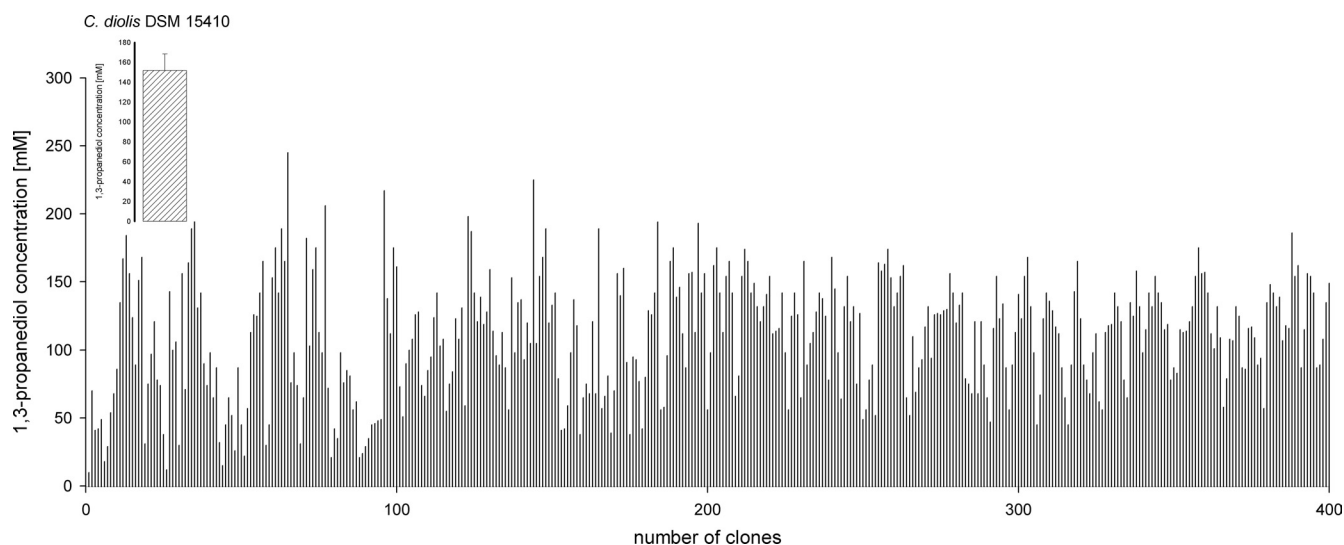


FIG. 5. Overview of 1,3-PD product formation after mutagenesis. Screening for a mutant with increased 1,3-PD production was performed in small-scale fermentations using 96-deep-well plates. The standard deviation in levels of 1,3-PD production by *C. diolis* DSM 15410 was calculated from the results of least eight independent experiments.

glycerol due to significant cost reduction and the recirculation of a waste product from biodiesel production. However, crude glycerol products often differ greatly in terms of quality, the glycerol amount, and possible components that may inhibit the growth of *C. diolis*. Crude glycerol was kindly provided by M. Menner (Fraunhofer Institute IVV) and contained large amounts of fatty acids that may hamper the growth of *C. diolis*; these were therefore removed by liquid extraction. This crude glycerol was used for comparative fed-batch fermentations with the wild-type strain and the generated strain GSHM 2.

Both *C. diolis* DSM 15410 and GSHM 2 produced less 1,3-PD when crude glycerol was used than when pharmaceutical glycerol was used as a carbon source. The wild-type strain reached a final yield of 448 mM, and GSHM 2 yielded 706 mM 1,3-PD (Fig. 6). Both strains displayed a longer lag phase with crude glycerol than with pharmaceutical glycerol. The molar yields of 1,3-PD were 0.46 for the wild-type strain and 0.49 for GSHM 2, lower than those obtained by fed-batch fermentation with pharmaceutical glycerol.

DISCUSSION

Although the classical strain improvement approach has been successful in the development of industrial fermentation strains, it is a time-consuming process that often generates only

incremental improvements, as seen in our attempts to select for both glycerol and PD tolerance (Fig. 1 and 2). This characteristic reflects practical limitations such as the simultaneous accumulation of desirable and undesirable mutations and the inability to introduce heterologous genes (29). Classical strain improvement competes with current rational metabolic engineering approaches, which can specifically address shortcomings in the natural producer or even establish entire pathways in a better-suited heterologous host (3). However, such rational methods rely on biochemical models of the relevant metabolic pathways and a detailed understanding of the genes involved and their regulation. For complex phenotypes such as tolerances to certain inhibitory molecules, multiple genes distributed throughout the genome are often involved (26). This pattern raises serious obstacles for the application of genetic engineering to complex strain improvement and favors the employment of combinatorial approaches such as genome shuffling.

The factors limiting 1,3-PD production by *C. diolis* are not clearly defined, and a multitude of factors related to membrane effects, metabolic feedback regulation, transport, and competing metabolic pathways seem to be involved (1, 5, 14). Recursive protoplast fusion of improved strains leads to homologous recombination and allows the generation of a com-

TABLE 1. Fermentation data for *C. diolis* DSM 15410 and mutants grown in fed-batch cultures

Strain	Time (h)	Biomass (g liter ⁻¹)	Concn (mM) of glycerol used ^a	Concn (mM) of product ^a :			$Y_{1,3-PD}$	$Q_{1,3-PD}^b$	$q_{1,3-PD}^c$
				Acetate	Butyrate	1,3-PD			
Wild type	22	1.84	989 (56)	63 (4)	96 (4)	624 (38)	0.586	2.147	1.17
PSM 7	27	1.76	1,204 (75)	73 (5)	92 (5)	746 (55)	0.614	2.099	1.19
GSHM 2	28	1.95	1,695 (95)	160 (14)	50 (2)	1,032 (79)	0.642	2.821	1.44
GSHM 4	31	2.10	1,815 (114)	58 (3)	240 (18)	1,114 (86)	0.632	2.745	1.30

^a Values are the averages of four independent determinations. Standard deviations are given in parentheses.

^b $Q_{1,3-PD}$, 1,3-PD volumetric productivity (g liter⁻¹ h⁻¹).

^c $q_{1,3-PD}$, 1,3-PD specific formation rate (g g of cells [dry weight]⁻¹ h⁻¹).

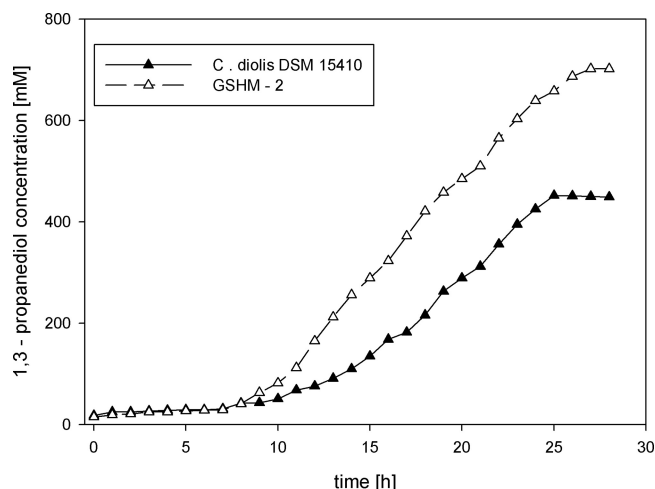


FIG. 6. Results from fed-batch fermentations with *C. diolis* and GSHM 2 using crude glycerol as a carbon source. Levels of 1,3-PD product formation by the different strains are shown.

binatorial library. This approach provides for simultaneous changes at different positions throughout the genome, even in the absence of detailed genetic information. Beneficial mutations can accumulate, whereas neutral or detrimental mutations are weeded out, thus increasing fitness rapidly (29).

In the present study, we demonstrated the application of genome shuffling to the strict anaerobic microorganism *C. diolis* for high-volume 1,3-PD production. Industrial 1,3-PD production by *C. diolis* is hampered primarily by inhibition from fermentation products (14). By using a classical strain improvement approach, mutants that showed an 80% improvement in terms of 1,3-PD tolerance and a 15% increase in the product yield were generated. For generating genetic diversity in the genome shuffling approach, natural 1,3-PD producers isolated from other sources, e.g., environmental samples, could have been used instead of a well-characterized 1,3-PD producer derived from the classical approach. However, isolation of 1,3-PD producers from environmental samples can result in the coisolation of potentially pathogenic organisms (19). Genome shuffling in the mutants derived from chemical mutagenesis led to a dramatic increase in the volumetric productivity and specific production rate, demonstrating the power of applying of these methods to a strict anaerobe. These results are consistent with those of previous studies in which notable improvements in *Lactobacillus* and *Streptomyces* species were shown (26, 31). An indication of the mechanism underlying the improved productivity is provided by the data in Table 1, which show that the mutants produced more biomass than the wild type and that higher biomass may confer tolerance. The enhanced growth rate may reflect mutations that allow the cells to use nutrients in the medium more effectively or to export inhibitory molecules more rapidly, as suggested in previous studies (2).

Crude glycerol, which is an abundant by-product from biodiesel production, is an attractive substrate for the production of high-value chemicals such as 1,3-PD. Previous investigations showed that crude glycerol can be used as the sole carbon source for 1,3-PD production by *C. butyricum* (18, 27).

The results of our fed-batch fermentation with crude glycerol showed reduced productivity and a prolonged lag phase for both the mutants and the wild type, thus indicating inhibitory or missing compounds in the fermentation medium. However, the quality and composition of crude glycerol from biodiesel production plants vary. Additional rounds of genome shuffling may be required to further improve the utilization of crude glycerol. For an industrial strain under process conditions, tolerance of substrates and fermentation products is desired, in addition to high-volume productivity. The medium composition must also be taken into account. A major cost factor is the use of yeast extract, which should be replaced with a synthetic medium if possible. A previous study gave some indications that *p*-aminobenzoic acid and biotin are important vitamins for *C. butyricum* (18). Based on the characteristics of the mutants isolated in the present study, further improvements with regard to volumetric productivity, fermentation cost, and the use of crude glycerol can be expected to be realized by further optimization of the fermentation process.

ACKNOWLEDGMENTS

This work was financially supported by the German Federal Ministry of Education and Research (BMBF; through grant no. 0315046A). We thank N. Koch for excellent technical assistance.

REFERENCES

1. Abbad-Andaloussi, S., E. Guedon, E. Spiesser, and H. Petitdemange. 1996. Glycerol dehydratase activity: the limiting step for 1,3-propanediol production by *Clostridium butyricum* DSM 5431. *Lett. Appl. Microbiol.* **22**:311–314.
2. Abbad-Andaloussi, S., C. Manginot-Durr, J. Amine, E. Petitdemange, and H. Petitdemange. 1995. Isolation and characterization of *Clostridium butyricum* DSM 5431 and mutants with increased resistance to 1,3-propanediol and altered production of acids. *Appl. Environ. Microbiol.* **61**:4413–4417.
3. Alper, H., K. Miyaoku, and G. Stephanopoulos. 2005. Construction of lycopene-overproducing *E. coli* strains by combining systematic and combinatorial gene knockout targets. *Nat. Biotechnol.* **23**:612–616.
4. Berovic, M., and M. Legisa. 2007. Citric acid production. *Biotechnol. Annu. Rev.* **13**:303–343.
5. Biebl, H. 1991. Glycerol fermentation of 1,3-propanediol by *Clostridium butyricum*. Measurement of product inhibition by use of a pH-auxostat. *Appl. Microbiol. Biotechnol.* **35**:701–705.
6. Biebl, H., S. Marten, H. Hippe, and W. D. Deckwer. 1992. Glycerol conversion to 1,3-propanediol by newly isolated clostridia. *Appl. Microbiol. Biotechnol.* **36**:592–597.
7. Biebl, H., and N. Pfennig. 1981. Isolation of members of the family Rhodospirillaceae, p. 267–273. In M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (ed.), *The prokaryotes*. Springer-Verlag KG, Berlin, Germany.
8. Biebl, H., and C. Sproer. 2002. Taxonomy of the glycerol fermenting clostridia and description of *Clostridium diolis* sp. nov. *Syst. Appl. Microbiol.* **25**:491–497.
9. Biebl, H., A. P. Zeng, K. Menzel, and W. D. Deckwer. 1998. Fermentation of glycerol to 1,3-propanediol and 2,3-butanediol by *Klebsiella pneumoniae*. *Appl. Microbiol. Biotechnol.* **50**:24–29.
10. Cameron, D. C., N. E. Altaras, M. L. Hoffmann, and A. J. Shaw. 1998. Metabolic engineering of propanediol pathways. *Biotechnol. Prog.* **14**:116–125.
11. Christopher, A. T., S. B. Tummala, and E. T. Papoutsakis. 2005. Metabolic engineering of solventogenic clostridia, p. 814. In P. Dürre (ed.), *Handbook on clostridia*. CRC Press, Boca Raton, FL.
12. Chuah, H. 1996. Corterra poly(trimethylene terephthalate)—new polymeric fiber for carpets. *Chem. Fibers Int.* **46**:424–428.
13. Colin, T., A. Bories, C. Lavigne, and G. Moulin. 2001. Effects of acetate and butyrate during glycerol fermentation by *Clostridium butyricum*. *Curr. Microbiol.* **43**:238–243.
14. Colin, T., A. Bories, and G. Moulin. 2000. Inhibition of *Clostridium butyricum* by 1,3-propanediol and diols during glycerol fermentation. *Appl. Microbiol. Biotechnol.* **54**:201–205.
15. Coombs, A. 2007. Glycerin bioprocessing goes green. *Nat. Biotechnol.* **25**:953–954.
16. Dai, M., and S. D. Copley. 2004. Genome shuffling improves degradation of the anthropogenic pesticide pentachlorophenol by *Shingobium chlorophenolicum* ATCC 39723. *Appl. Environ. Microbiol.* **70**:2391–2397.

17. Datta, R., S. P. Tsai, P. Bonsignore, S. H. Moon, and J. R. Frank. 1995. Technological and economical potential of poly(lactic acid) and lactic acid derivatives. *FEMS Microbiol. Rev.* **16**:221–231.
18. Gonzalez-Pajuelo, M., J. C. Andrade, and I. Vasconcelos. 2004. Production of 1,3-propanediol by *Clostridium butyricum* VPI 3266 using a synthetic medium and raw glycerol. *J. Ind. Microbiol. Biotechnol.* **31**:442–446.
19. Hao, J., R. Lin, Z. Zheng, H. Liu, and D. Liu. 2008. Isolation and characterization of microorganisms able to produce 1,3-propanediol under aerobic conditions. *World J. Microbiol. Biotechnol.* **24**:1731–1740.
20. Hermann, B. G., K. Blok, and M. K. Patel. 2007. Producing bio-based bulk chemicals using industrial biotechnology saves energy and combats climate change. *Environ. Sci. Technol.* **41**:7915–7921.
21. Hermann, B. G., and M. Patel. 2007. Today's and tomorrow's bio-based bulk chemicals from white biotechnology. *Appl. Biochem. Biotechnol.* **136**:361–388.
22. Homann, T., C. Tag, H. Biebl, W. D. Deckwer, and B. Schick. 1990. Fermentation of glycerol to 1,3-propanediol by *Klebsiella* and *Citrobacter* strains. *Appl. Microbiol. Biotechnol.* **33**:121–126.
23. Kurian, J. V. 2005. A new polymer platform for the future—Sorona from corn derived 1,3-propanediol. *J. Polym. Environ.* **13**:159–167.
24. Lligadas, G., J. C. Ronda, M. Galia, and V. Cadiz. 2007. Poly(ether urethane) networks from renewable resources as candidate biomaterials: synthesis and characterization. *Biomacromolecules* **8**:686–692.
25. O'Brien, J. R., C. Raynaud, C. Croux, L. Girbal, P. Soucaille, and W. N. Lanzilotta. 2004. Insights into the mechanism of the B12 independent glycerol dehydratase from *Clostridium butyricum*: preliminary biochemical and structural characterization. *Biochemistry* **43**:4635–4645.
26. Patnaik, R., S. Louie, V. Gavrilovic, K. Perry, W. P. Stemmer, C. M. Ryan, and S. B. del Cardayre. 2002. Genome shuffling of *Lactobacillus* for improved acid tolerance. *Nat. Biotechnol.* **20**:707–712.
27. Petitdemange, E., C. Durr, S. A. Andaloussi, and G. Raval. 1995. Fermentation of raw glycerol to 1,3-propanediol by new strains of *Clostridium butyricum*. *J. Ind. Microbiol. Biotechnol.* **15**:498–502.
28. Stal, M. H., and H. P. Blaschek. 1985. Protoplast formation and cell wall regeneration of *Clostridium perfringens*. *Appl. Environ. Microbiol.* **50**:1097–1099.
29. Stephanopoulos, G. 2002. Metabolic engineering by genome shuffling. *Nat. Biotechnol.* **20**:666–668.
30. Tang, X., Y. Tan, H. Zhu, K. Zhao, and W. Shen. 2009. Microbial conversion of glycerol to 1,3-propanediol by an engineered strain of *Escherichia coli*. *Appl. Environ. Microbiol.* **75**:1628–1634.
31. Zhang, Y. X., K. Perry, V. A. Vinci, K. Powell, W. P. Stemmer, and S. B. del Cardayre. 2002. Genome shuffling leads to rapid phenotypic improvement in bacteria. *Nature* **415**:664–666.