Isolation and Physiological Characterization of Butanol-Tolerant Bacteria

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Contents

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Abstract

Although *n*-butanol (referred to as butanol) is considered to be a promising biofuel, biological production is very limited due to the high toxicity of butanol to microorganisms. Recently, heterologous production of butanol and 2-methylpropan-1-ol (referred to as isobutanol) has become realized by the development of genetic engineering, and thus butanol-tolerant bacteria have been proposed as alternative hosts overcoming growth interruption problem during butanol production. To date, only a few butanol-tolerant bacteria have been screened mainly from microbes deposited in the culture collections. Since there have been few attempts for isolating butanol-tolerant bacteria from natural environments, very little is known about the butanol-tolerant bacteria that are present in nature.

Here, I extensively explored butanol-tolerant microorganisms from various environments and characterized their physiological traits associated with butanol tolerance. I succeeded in isolating 10 aerobic and 6 anaerobic bacteria that could grow in greater than 2.0% (vol/vol) butanol and isobutanol. None of the isolates assimilated or degraded butanol and isobutanol. Among them, ten isolates were phylogenetically distinct from previously identified butanol-tolerant bacteria, and three of them were phylogenetically novel microbes with low 16S rRNA gene sequence similarities (<94%) to any other species. Of the isolates, *Enterococcus faecalis* strain CM4A (aerobe) and strain GK12 (anaerobe) showed a relatively higher tolerance, with the ability to grow in 3.5% and 3.0% butanol, respectively. Therefore, I selected these strains for further analyses.

Phenotypic, phylogenetic and chemotaxonomic methods were used to taxonomically characterize strain GK12, which was isolated from an anaerobic digester. The 16S rRNA gene sequence analysis revealed that the strain was affiliated with the family *Erysipelotrichaceae* in the phylum *Firmicutes* and showed a 91.8% sequence similarity to the most closely related species, *Faecalicoccus acidiformans*. DNA G+C content, fatty acid content, cell wall murein

type, and biochemical characteristics of strain GK12 were distinctive from those of the closely related species. Then, I propose that strain GK12 represents a new genus and species in the family *Erysipelotrichaceae* as a type strain (=NBRC 108915^T=DSM 25799^T), with the name *Catenisphaera adipataccumulans* gen. nov., sp. nov.

To elucidate the underlying mechanism of butanol tolerance, I characterized strains CM4A and GK12 by examining cell surface structure, fatty acid compositions, and genes associated with butanol tolerance. Strain CM4A decreased the cell surface hydrophobicity by increasing extracellular capsule thickness in the presence of butanol, which may function as a physical defense against butanol. In contrast to strain CM4A, such a change in morphology was not observed in strain GK12. When the cells were grown with butanol, strains CM4A and GK12 increased the proportion of saturated and cyclopropane fatty acids, and long-chain fatty acids, respectively, which can serve for enhancing its butanol tolerance. Indeed, heterologous expression of the cyclopropane fatty acid synthase gene derived from strain CM4A improved solvent tolerance of the recombinant *E. coli* strain. I also found that only strain GK12 could improve its tolerance after successive subcultures with butanol. The butanol-adapted cells did not exhibit membrane modification, indicating that yet unknown mechanisms may contribute to this ability. These results suggested that the organisms perhaps maintain their structural integrity by increasing the extracellular capsule thickness, altering the membrane fatty acid components, and adaptation via unknown mechanisms.

I further investigated how culture pH affects microbial butanol tolerance using strain CM4A. The strain grew over a broad pH range (pH 4.0-12.0) and preferred alkaline pH (pH 8.0 and 10.0) in the absence of butanol. However, in the presence of butanol, strain CM4A grew better under acidic and neutral pH conditions (pH 6.0 and 6.8). Membrane fatty acid analysis revealed that the increase in the proportion of cyclopropane and saturated fatty acids, which contribute to butanol tolerance by decreasing membrane fluidity, was little observed in the cells grown under alkaline pH with butanol. Meanwhile, the strain grown under alkaline

pH without butanol increased short chain fatty acids, which is involved in increasing membrane fluidity for alkaline adaptation. Such a change was not observed in the cells grown under alkaline pH with butanol. These results suggested that strain CM4A simultaneously exposed to butanol and alkali stresses was not likely able to properly adjust membrane fluidity due to the opposite response to each stress. To our knowledge, this is the first report describing the effect of pH on microbial butanol tolerance.

I also found that strain GK12 accumulated free fatty acids (FFAs) consisting of myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$) and arachidic acid ($C_{20:0}$) under stress conditions, including butanol exposure and higher-than-optimum temperatures that affect cell membrane integrity. The chain lengths of both membrane lipids and FFAs, and the amounts of FFAs increased in a butanol dose-dependent manner. The gene expression profiles involved in fatty acid and phospholipid metabolism suggested that FFA accumulation may be as a result of imbalance between excess membrane fatty acid biosynthesis and limited β-oxidation activity. FFA accumulation did not have a positive affect for butanol tolerance of this strain. Strain GK12 is a first organism that intrinsically accumulates significant amounts of FFAs, which may provide useful strategies for biodiesel production without generating by-product glycerol.

In conclusion, this study demonstrated that a wide variety of butanol-tolerant bacteria are present in the environments and have various mechanisms to maintain structural integrity against butanol. These findings provide further strategies for developing potential solvent-tolerant platforms for microbial fuel production.

Chapter 1

General Introduction

1.1 Butanol as a promising biofuel

Due to increasing environmental concerns and depletion of fossil fuels, microbial fuel production from renewable feedstock has become a global priority. Indeed, the US Department of Energy has established the goal of replacing 30% gasoline consumption to cellulosic ethanol by 2030 (Herrera, 2006). Bioethanol from sugarcane and biodiesel from oil crops or microalgae are the two potential renewable fuels that have attracted the most attention (Chisti, 2008). Meanwhile, *n*-butanol (hereafter referred to as butanol) is considered to be a good alternative to the traditional biofuel ethanol due to the advantages of butanol's high energy content, high octane rating, low volatility, and miscibility with gasoline and diesel oil (Lee *et al*., 2008; Schwarz & Gapes, 2006). Like ethanol, it can be produced fermentatively as well as petrochemically. In order to use butanol as renewable fuel, industrial development of microbial production is now ongoing. In fact, Butamax Advanced Biofuels LLC was established in 2009 as a joint-venture company from DuPont and BP, and several patents have been applied (Buelter *et al*., 2007; Brekke, 2007; Anthony *et al*., 2009).

1.2 Problems in microbial production of butanol

Butanol is produced biologically through an acetone-butanol-ethanol (ABE) fermentation process by obligate anaerobic clostridia (Jones & Woods, 1986). ABE industrial technology was developed by Chaim Weizmann in 1916, and the regulation of solvent formation has been intensively investigated (Huang *et al*., 2010). However, these fermentations are still economically unfavorable due to the high toxicity of butanol to

microorganisms (Li *et al*., 2010; Liu & Qureshi, 2009). Butanol is known to intercalate into the cell membrane and break the lipid hydrogen bonds, resulting in a disruption of membrane structure and cell death (Huffer *et al*., 2011). In batch fermentations, most of clostridia are unable to grow in the presence of greater than 2.0% (vol/vol) of butanol (Woods, 1995; Qureshi *et al*., 2007; Knoshaug & Zhang, 2009), and the maximum production of butanol was reported to be 2.4% by using butanol-tolerant *Clostridium beijerinckii* mutant (Qureshi & Blaschek, 2001).

Recently, vigorous efforts have been made to develop alternative platform microorganism to generate C3- to C5-chain alcohols, including butanol and 2-methylpropan-1-ol (hereafter referred to as isobutanol) through genetic manipulation. The butanol synthetic pathway has been introduced into *Escherichia coli* (Atsumi *et al*., 2008a; Inui *et al*., 2008), *Saccharomyces cerevisiae* (Steen *et al*., 2008), *Pseudomonas putida*, *Bacillus subtilis* (Nielsen *et al*., 2009), *Corynebacterium glutamicum* (Smith *et al*., 2010) and *Lactobacillus brevis* (Berezina *et al*., 2009), and all these strains could produce a certain amount of butanol. For example, *E. coli* introduced with a butanol synthetic pathway could produce 0.15% butanol and 2.8% isobutanol (Atsumi *et al*., 2008b; Inui *et al*., 2008). However, these microbes do not seem to be suitable for butanol-producing hosts due to their low tolerance to butanol.

1.3 History of the study of butanol-tolerant bacteria

Butanol-tolerant bacteria have attracted increased attention as alternative butanol production hosts which were able to overcome the end product inhibition. To date, several

studies have been performed to screen butanol-tolerant bacteria that can grow in the presence of greater than 2.0% butanol (Li *et al*., 2010; Knoshaug & Zhang, 2009; Huffer *et al*., 2011; Kataoka *et al*., 2011; Ruhl *et al*., 2009; Ting *et al*., 2012). Although a number of microorganisms tolerating other organic solvent (e.g. toluene, benzene, etc) have been isolated from natural environments, there were only a few butanol-tolerant bacterial species reported. The previously identified butanol-tolerant microorganisms were predominantly screened from microbes deposited in the culture collections, which are, therefore, taxonomically limited, such as the genera *Clostridium*, *Pseudomonas*, *Zymomonas*, *Bacillus, Lactobacillus*, and *Enterococcus* (Li *et al*., 2010; Knoshaug & Zhang, 2009; Huffer *et al*., 2011; Kataoka *et al*., 2011; Ruhl *et al*., 2009; Ting *et al*., 2012). Furthermore, limited studies have been done in order to understand the mechanisms of butanol tolerance, which extensively focused on solventogenic clostridia and *Escherichia coli* (Borden & Papoutsakis, 2007; MacDonald & Goldfine, 1991; Rutherford *et al*., 2010). Since few attempts for isolating butanol-tolerant bacteria from natural environments have been made, very little is known about butanol-tolerant bacteria present in the natural environments.

1.4 Outline of this thesis

The aim of the research presented in this thesis is to newly isolate untapped butanol-tolerant microbes from the environments, and to investigate their mechanisms to tolerate butanol.

The **Chapter 2** describes extensive screening of butanol- and isobutanol-tolerant microorganisms that can grow in greater than 2.0% solvents from various environmental

samples and investigated the phylogenetic positions and tolerance to butanol and isobutanol of the microorganisms.

In the **Chapter 3**, the taxonomic studies of the obligate anaerobic butanol-tolerant bacterium, designated strain GK12, were conducted. A novel species and genus in the family *Erysipelotrichaceae* of the phylum *Firmicutes* is proposed for this isolate based on the results of phenotypic, phylogenetic and chemotaxonomic analyses.

In the **Chapter 4**, two representative isolates, aerobic strain CM4A and anaerobic strain GK12, were characterized to elucidate the underlying mechanism of butanol tolerance. At first, cell surface structures and fatty acid compositions were investigated. Next, selected gene was heterologously expressed in *E. coli* to confirm its association with butanol tolerance. Additionally, butanol-adapted characteristic of strain GK12 is described in detail.

The **Chapter 5** addresses the effect of culture pH on microbial butanol tolerance using strain CM4A. The response of cell membrane to both butanol and pH stresses was further characterized to discuss the interactive effects of butanol and alkali stresses.

In the **Chapter 6**, a novel phenomenon, free fatty acids accumulation of strain GK12 caused by butanol exposure, is reported. Although this response does not have a positive affect for butanol tolerance of this strain, the accumulation mechanism is discussed by integrating the data of lipid analysis and gene expression analysis.

Finally, the findings presented in this thesis are summarized in the **chapter 7**.

References

Anthony LC, Dauner M, Donaldson GK, Paul BJ. 2009. Carbon pathway optimized production hosts for the production of isobutanol. U.S. Patent Application. 12/606,410.

Atsumi S, Cann AF, Connor MR, Shen CR, Smith KM, Brynildsen MP, Chou KJY, Hanai T, Liao JC. 2008a. Metabolic engineering of *Escherichia* coli for 1-butanol production. Metabolic engineering. 10:305-311.

Atsumi S, Hanai T, Liao JC. 2008b. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature. 451:86-89.

Berezina OV, Brandt A, Yarotsky S, Schwarz WH, Zverlov VV. 2009. Isolation of a new butanol-producing *Clostridium* strain: High level of hemicellulosic activity and structure of solventogenesis genes of a new *Clostridium saccharobutylicum* isolate. Syst. Appl. Microbiol. 32:449-459.

Borden JR, Papoutsakis ET. 2007. Dynamics of genomic-library enrichment and identification of solvent tolerance genes for *Clostridium acetobutylicum*. Appl. Environ. Microbiol. 73:3061-3068.

Brekke K. 2007. Butanol, an Energy Alternative? Ethanol Today. March. 36-39.

Buelter T, Hawkins AC, Kersh K, Meinhold P, Peters MW, Subbian E. 2007. Engineered microorganisms for producing *n*-butanol and related methods. U.S. Patent Application. 11/949,724.

Chisti Y. 2008. Biodiesel from microalgae beats bioethanol. Trends in biotech. 26:126-131.

Herrera S. 2006. Bonkers about biofuels. Nature biotech. 24:755-760.

Huang H, Liu H, Gan YR. 2010. Genetic modification of critical enzymes and involved genes in butanol biosynthesis from biomass. Biotech. Advances. 28: 651-657.

Huffer S, Clark ME, Ning JC, Blanch HW, Clark DS. 2011. The role of alcohols in growth, lipid composition, and membrane fluidity of yeast, bacteria, and archaea. Appl. Environ. Microbiol. 77:6400-6408.

Inui M, Suda M, Kimura S, Yasuda K, Suzuki H, Toda H, Yamamoto S, Okino S, Suzuki N, Yukawa H. 2008. Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*. Appl. Microbiol. Biotechnol. 77:1305-1316.

Jones DT, Woods DR. 1986. Acetone-butanol fermentation revisited. Microbiol. Mol. Biol. Rev. 50:484-524.

Kataoka N, Tajima T, Kato J, Rachadech W, Vangnai A. 2011. Development of butanol-tolerant *Bacillus subtilis* strain GRSW2-B1 as a potential bioproduction host. AMB Express 1:10-20.

Knoshaug EP, Zhang M. 2009. Butanol tolerance in a selection of microorganisms. Appl. Biochem. Biotechnol. 153:13-20.

Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, Jung KS. 2008. Fermentative butanol production by Clostridia. Biotechnol. Bioeng. 101:209-28.

Li J, Zhao JB, Zhao M, Yang YL, Jiang WH, Yang S. 2010. Screening and characterization of butanol-tolerant micro-organisms. Lett. Appl. Microbiol. 50:373-379.

Liu S, Qureshi N. 2009. How microbes tolerate ethanol and butanol. New Biotechnol. 26:117-121.

MacDonald DL, Goldfine H. 1991. Effects of solvents and alcohols on the polar lipid composition of *Clostridium butyricum* under conditions of controlled lipid chain composition. Appl. Environ. Microbiol. 57:3517-3521.

Nielsen DR, Leonard E, Yoon SH, Tseng HC, Yuan C, Prather KLJ. 2009. Engineering alternative butanol production platforms in heterologous bacteria. Metabolic engineering. 11:262-273.

Qureshi N, Blaschek HP. 2001. Recent advances in ABE fermentation: hyper-butanol producing *Clostridium beijerinckii* BA101. J Ind. Microbiol. Biotechnol. 27:287-291.

Qureshi N, Saha BC, Cotta MA. 2007. Butanol production from wheat straw hydrolysate using C*lostridium beijerinckii*. Bioprocess. Biosyst. Eng. 30:419-427.

Ruhl J, Schmid A, Blank LM. 2009. Selected *Pseudomonas putida* strains can grow in the presence of high butanol concentrations. Appl. Environ. Microbiol. 75:4653-4656.

Rutherford BJ, Dahl RH, Price RE, Szmidt HL, Benke PI, Mukhopadhyay A, Keasling JD. 2010. Functional genomic study of exogenous *n*-butanol stress in *Escherichia coli*. Appl. Environ. Microbiol. 76:1935-1945.

Schwarz WH, Gapes JR. 2006. Butanol-rediscovering a renewable fuel. BioWorld Europe 1:16-19.

Smith KM, Cho KM, Liao JC. 2010. Engineering *Corynebacterium glutamicum* for isobutanol production. Appl. Microbiol. Biotechnol. 87:1-11

Steen EJ, Chan R, Prasad N, Myers S, Petzold CJ, Redding A, Ouellet M, Keasling JD. 2008. Metabolic engineering of *Saccharomyces cerevisiae* for the production of *n*-butanol. Microbial Cell Factories. 7:36.

Ting CNW, Wu J, Takahashi K, Endo A, Zhao H. 2012. Screened butanol-tolerant *Enterococcus faecium* capable of butanol production. Appl. Biochem. Biotech. 168: 1672-1680.

Woods DR, 1995. The genetic engineering of microbial solvent production. Trends in Biotechnol. 13:259-264.

Chapter 2

Isolation of Butanol-Tolerant Bacteria from Environmental Samples

Abstract

Toxicity of butanol and isobutanol is one of the most profound issues in microbial production of these solvents as biofuels. To date, only a very limited number of butanol-tolerant bacteria have been identified. Here, I extensively explored butanol- and isobutanol-tolerant bacteria from various environmental samples. Enrichment cultures of environmental samples such as grease-contaminated soils and thermophilic anaerobic digester sludges amended with 2.0~9.0% (vol/vol) of butanol were performed under aerobic or anaerobic conditions. Eventually, I succeeded in isolating a total of 16 different aerobic or obligatory anaerobic, mesophilic or thermophilic bacterial strain able to grow in more than 2.0% butanol and isobutanol. 16S rRNA gene sequencing analysis revealed that the isolates were phylogenetically distributed over at least nine genera; *Bacillus*, *Lysinibacillus*, *Rummeliibacillus*, *Brevibacillus*, *Coprothermobacter*, *Caloribacterium*, *Enterococcus*, *Hydrogenoanaerobacterium*, and *Cellulosimicrobium*, within the phyla *Firmicutes* and *Actinobacteria*. Importantly, ten isolates were phylogenetically distinct from the previously known butanol-tolerant bacteria, and three of them were phylogenetically novel microbes with low 16S rRNA gene sequence similarities (<94%) to any other described species. The results demonstrate that a wide variety of butanol- and isobutanol-tolerant bacteria exist in the environment.

Introduction

Butanol is produced biologically through an acetone-butanol-ethanol (ABE) fermentation process by obligate anaerobic clostridia (Jones & Woods, 1986). Recently, vigorous efforts have been made to develop alternative processes to generate C3- to C5-chain alcohols, including butanol and 2-methylpropan-1-ol (hereafter referred to as isobutanol), through metabolic engineering using *Escherichia coli* as the platform microorganism (Atsumi *et al*., 2008). However, production is very limited due to the high toxicity of butanol and isobutanol to microorganisms (Li *et al*., 2010; Liu & Qureshi, 2009). These solvents are known to cause an increase in plasma membrane fluidity by intercalating into the membrane and breaking the hydrogen bonds between lipid tails, resulting in a loss of membrane potential and a decline in cell growth (Huffer *et al*., 2011; Ingram & Buttke, 1984; Vollherbst-Schneck *et al*., 1984). In fact, the known butanol-producing microorganisms including clostridia, genetically engineered *E. coli* and *Saccharomyces cerevisiae*, are highly sensitive to butanol (Knoshaug & Zhang, 2009; Qureshi *et al*., 2007; Woods, 1995), and this toxicity has long been a critical issue in practical biobutanol production.

To date, only a few butanol-tolerant bacterial species with the ability to grow in greater than 2.0% (vol/vol) butanol have been found (Li *et al*., 2010; Knoshaug & Zhang, 2009; Kataoka *et al*., 2011; Ruhl *et al*., 2009; Ting *et al*., 2012). The previously identified butanol-tolerant microorganisms were predominantly screened from culture collections that included well-known strains that tolerate other organic solvents (e.g., toluene and benzene) (Li *et al*., 2010; Knoshaug & Zhang, 2009; Ruhl *et al*., 2009). This strategy was effective but resulted in the collection of taxonomically limited butanol-tolerant bacteria, such as the genera *Clostridium*, *Pseudomonas*, *Zymomonas*, *Bacillus, Lactobacillus*, and *Enterococcus*

(Li *et al*., 2010; Knoshaug & Zhang, 2009; Huffer *et al*., 2011; Kataoka *et al*., 2011; Ruhl *et al*., 2009; Ting *et al*., 2012). Furthermore, as there have been very few attempts to isolate butanol-tolerant bacteria from natural environments, very little is known about the butanol-tolerant bacteria that are present in nature.

In this study, I sought to extensively screen butanol- and isobutanol-tolerant microorganisms that can grow in greater than 2.0% butanol from various environmental samples and investigated the phylogenetic positions and tolerance levels of the microorganisms.

Materials and Methods

Sampling, enrichment, and isolation. Butanol- and isobutanol-tolerant microorganisms were enriched in the presence of these solvents. Cultivation was performed under both aerobic and strictly anaerobic conditions. To prevent evaporation of the solvents, a 50-ml, tightly capped conical tube (for aerobic cultivation) and 50-ml serum vial sealed with a butyl rubber stopper and aluminum crimp (for strictly anaerobic cultivation) were used. Evaporation was negligible during the cultivation period under both the aerobic and anaerobic conditions and thus did not affect the evaluation of solvent tolerance.

To enrich and isolate aerobic bacteria, samples were collected from freshwater sediments, grease-contaminated soils, cabbage-field soils, vegetable wastes, and composts in Ibaraki Prefecture, Japan. After the samples were sonicated in water and centrifuged at 400 x *g* for 5 min, the supernatant was inoculated into a butanol-containing (0.1-3.0% (vol/vol)) fresh medium consisting of (per liter) 5 g glucose, 1 g tryptone, 0.5 g yeast extract, 1 g $(NH_4)_2SO_4$, 0.75 g KH_2PO_4, and 0.78 g K_2HPO_4 supplemented with 7 ml basal salt solution and 1 ml vitamin solution, as described previously (Hanada *et al*., 1997). Two different aerobic enrichments, short- and long-term cultivations, were performed. For short-term cultivation, 50 µl of inoculum was added to 2 ml of fresh medium containing 0.5-3.0% butanol. After incubation at 30 °C with shaking for 48 h, the cultures were spread onto agar plates with the same medium containing 2.0% butanol, and colonies were isolated and purified. For long-term cultivation (3-9 months in total), 1 ml of each inoculum was added to 50 ml of fresh medium containing 0.1% butanol. After incubation at room temperature with shaking for 48-72 h, the 2-3% of the culture fluid was transferred into fresh medium containing 1.0% butanol and incubated for 5 days. The culture was then repeatedly transferred

into fresh medium with increasing concentrations of butanol of up to 9.0% in a stepwise manner. After consecutive transfers, the cultures were spread onto agar plates, as described above.

To enrich and isolate strictly anaerobic bacteria, samples for enrichment were collected from oil-contaminated soils, thermophilic and mesophilic anaerobic digesters, bovine rumens, hot springs, and bovine manure composts in Hokkaido Prefecture. The oil-contaminated soil or bovine manure compost samples were mixed well with 10 mM phosphate-buffered 150 mM NaCl. A portion of a 5-ml sample or slurry was inoculated into fresh medium containing 2.0% or 5.0% butanol or isobutanol. The medium was prepared based on a modified Widdel medium (Pfennig *et al*., 1981) with the following composition (per liter), as described previously (Sekiguchi *et al*., 2000): 5 g glucose, 1 g yeast extract, 0.53 g NH_4Cl, 0.14 g KH_2PO_4, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.15 g $CaCl_2 \cdot 2H_2O$, and 2.52 g $NaHCO_3$ supplemented with 1 ml selenium and tungsten solution, 1 ml trace-element solution, and 2 ml vitamin solution. Cultivation was performed in 50-ml serum vials containing 20 ml of medium under an atmosphere of N_2/CO_2 (80:20) at 37 °C or 55 °C. The cultures were transferred to fresh medium five times at intervals of approximately 1 month. The Hungate roll-tube technique (Hungate, 1969) was used to isolate single colonies using the above-mentioned medium solidified with 2% noble agar. Each colony was inoculated into a liquid medium containing 2.0% butanol to verify the tolerance to butanol.

Phylogenetic analysis based on 16S rRNA gene sequences. The 16S rRNA gene sequences (*E. coli* positions 28-1491) of each isolate were determined as previously described (Tamaki *et al*., 2009). All sequences were aligned with their relatives using ARB software

(Ludwig *et al*., 2004) and the SILVA database (Pruesse *et al*., 2007). A phylogenetic tree was constructed by the neighbor-joining method (Saitou & Nei, 1987) using the MEGA 4.0 program (Tamura *et al*., 2007). The robustness of the tree's topology was assessed by a bootstrap analysis (Felsenstein, 1985) based on 1,000 replications.

Butanol and isobutanol tolerance assay. To obtain better growth for determining the solvent tolerance and physiological traits, the media for the aerobic and anaerobic solvent-tolerant isolates were altered as follows. For the aerobic strains, 5 g/l glucose, 20 g/l tryptone, 5 g/l yeast extract, 1.5 g/l KH_2PO_4, and 1.56 g/l K_2HPO_4 were supplemented with 7 ml basal salt solution and 1 ml vitamin solution (Hanada *et al*., 1997). For the anaerobic strains, 10 g/l glucose, 5 g/l yeast extract, 0.53 g/l NH_4Cl, 0.14 g/l KH_2PO_4, 0.2 g/l $MgCl_2 \cdot 6H_2O$, 0.15 g/l $CaCl_2 \cdot 2H_2O$, and 2.52 g/l $NaHCO_3$ were supplemented with 1 ml selenium and tungsten solution, 1 ml trace-element solution, and 2 ml vitamin solution (Sekiguchi *et al*., 2000). The cultivation temperature for each isolate was the same as the temperature used in the enrichment.

The tolerance to butanol and isobutanol of the isolates was assessed based on cellular growth in the presence of the solvents, as previously described (Li *et al*., 2010; Knoshaug & Zhang, 2009; Kataoka *et al*., 2011; Ting *et al*., 2012). In particular, butanol-tolerant bacteria were defined in this study as those that could grow in greater than 2.0% butanol, as all of the previously identified butanol-tolerant microorganisms can grow in at least 2.0% butanol (Li *et al*., 2010; Knoshaug & Zhang, 2009; Kataoka *et al*., 2011; Ruhl *et al*., 2009; Ting *et al*., 2012). The maximum concentrations of butanol and isobutanol that allowed the isolates to grow were determined. Cells grown without solvent were inoculated into fresh liquid media

amended with butanol or isobutanol at a concentration of 1.0-5.5% in increments of 0.5%. The optical density at 600 nm (OD_{600}) was monitored using a spectrophotometer, and the specific growth rates were calculated from the linear range of exponential growth. The concentration of butanol or isobutanol in culture was measured by high-performance liquid chromatography equipped with a cation-exchange column and a refractive index (RI) detector.

Nucleotide sequence accession numbers. The GenBank/EMBL/DDBJ accession numbers of the 16S rRNA gene sequences of the isolates are AB669587 to AB669596 and AB537978 to AB537983.

Results and Discussion

Isolation of butanol- and isobutanol-tolerant bacteria. After enrichment in the presence of butanol under aerobic or anaerobic condition, a total of 16 microorganisms that could grow in the presence of greater than 2.0% butanol and isobutanol were isolated from various environmental samples (Table 2.1). All isolates comprised heterotrophic mesophilic bacteria, except for a thermophilic bacterium, strain GK5. No thermophilic microorganisms tolerating butanol have been found to date. The obligate anaerobic isolates (GK3, GK5, GK12, YN1, YN3, and YN5) were obtained from either thermophilic anaerobic digesters or oil-contaminated soil. In contrast, aerobic strains (CM4A, CM9A, SK4A, SK4D, SK5A, SK7A, SK9A, FW5A, CP3A, and CY2C) were isolated from all the samples used in this study, even freshwater sediments and cabbage-field soils in which organic solvents were

likely not present.

Phylogenetic identification of the obtained butanol-tolerant microorganisms. The phylogenetic analysis based on 16S rRNA gene sequencing indicated that the obtained isolates were affiliated with the genera *Bacillus*, *Lysinibacillus*, *Brevibacillus*, *Rummeliibacillus*, *Coprothermobacter*, *Caloribacterium*, *Enterococcus*, and *Hydrogenoanaerobacterium*, within the phylum *Firmicutes*, and the genus *Cellulosimicrobium*, within the phylum *Actinobacteria* (Table 2.1). Strains CM4A, SK4D, and SK5A, belonging to the genus *Enterococcus*, exhibited a relatively higher tolerance than did the other isolates. This finding is consistent with the results of a previous study showing that many lactic acid bacteria, including *Enterococcus* species, can tolerate butanol (Li *et al*., 2010; Ting *et al*., 2012). Similarly, strains SK4A, FW5A, and CP3A were closely related to the known butanol-tolerant bacterium *Bacillus subtilis* (Kataoka *et al*., 2011). In contrast, the other 10 isolates were phylogenetically distinct from the previously identified butanol-tolerant bacteria (Fig. 2.1). In particular, strains GK3, GK12, and YN5 were phylogenetically novel, showing less than 94% sequence similarity to the most closely related species.

Solvent tolerance of the isolates. To further evaluate the ability of the isolates to tolerate butanol and isobutanol, the maximum concentrations of solvents that allowed the isolates to grow were determined. Most of the isolates (all of the aerobic strains and three anaerobic strains [GK3, GK12, and YN5]) grew slightly better with isobutanol than with butanol (Table 2.1). None of the tested isolates assimilated or degraded butanol and isobutanol since the

concentration of them in culture did not change during cultivation.

Of the isolates, the aerobic strain CM4A exhibited the highest tolerance, growing in the presence of 3.5% butanol and 4.0% isobutanol (Fig. 2.2). The anaerobic isolate, strain GK12, also showed a relatively higher tolerance, with the ability to grow in 3.0% butanol and 3.5% isobutanol (Fig. 2.3). The tolerance levels of the two strains were comparable or even greater than the tolerance of previously identified butanol-tolerant microorganisms (Li *et al*., 2010). More importantly, both strains showed clear exponential growth and reached high cell densities, even in the presence of 2.5% butanol (Fig. 2.2 and 2.3), which was not observed for the previously reported butanol-tolerant bacteria (Li *et al*., 2010; Ting *et al*., 2012).

Taken together, the results indicate that there is a wider variety of butanol- and isobutanol-tolerant bacteria than previously recognized. I selected strains CM4A and GK12 that showed a relatively high tolerance for further analyses, as described in the following chapters.

Figures and Tables

Table 2.1. Characteristics of the isolates.

Strain	Origin	Enrichment conditions [a]	Phylum	Closest relative species (Accession no.)	Similarity (%)	Tolerance (%) [b]	
						Butanol	Isobutanol
Butanol-tolerant bacteria isolated under aerobic conditions							
CM4A	grease-contaminated soil	30 °C / 4 months / 4.0%	*Firmicutes*	*Enterococcus faecalis* (AB012212)	99.6	3.5	4.0
CM9A	grease-contaminated soil	30 °C / 9 months / 9.0%	*Firmicutes*	*Rummeliibacillus pycnus* (AB271739)	98.4	2.0	2.5
SK4A	freshwater sediment	30 °C / 3 months / 4.0%	*Firmicutes*	*Bacillus amyloliquefaciens* (AB255669)	99.6	2.0	2.5
SK4D	freshwater sediment	30 °C / 4 months / 4.0%	*Firmicutes*	*Enterococcus faecalis* (AB012212)	99.6	2.5	3.0
SK5A	freshwater sediment	30 °C / 7 months / 5.0%	*Firmicutes*	*Enterococcus faecalis* (AB012212)	99.5	3.0	3.5
SK7A	freshwater sediment	30 °C / 9 months / 7.0%	*Firmicutes*	*Lysinibacillus xylanilyticus* (FJ477040)	98.5	2.5	3.0
SK9A	freshwater sediment	30 °C / 9 months / 9.0%	*Firmicutes*	*Brevibacillus reuszeri* (AB112715)	99.8	2.0	2.5
FW5A	vegetable waste	30 °C / 2 days / 0.5%	*Firmicutes*	*Bacillus amyloliquefaciens* (AB255669)	97.9	2.0	2.5
CP3A	cabbage-field soil	30 °C / 2 days / 3.0%	*Firmicutes*	*Bacillus mycoides* (AB021192)	98.3	2.5	3.0
CY2C	compost	30 °C / 2 days / 2.0%	*Actinobacteria*	*Cellulosimicrobium cellulans* (X83809)	98.1	2.0	2.5
Butanol-tolerant bacteria isolated under anaerobic conditions							
GK3	thermophilic anaerobic digester	37 °C / 5 months / 2.0%	*Firmicutes*	*Garciella nitratireducens* (AY176772)	92.8	2.0	2.5
GK5	thermophilic anaerobic digester	55 °C / 4 months / 2.0%	*Firmicutes*	*Coprothermobacter proteolyticus* (X69335)	98.8	2.0	2.0
GK12	thermophilic anaerobic digester	37 °C / 5 months / 2.0%	*Firmicutes*	*Eubacterium cylindroides* (L34617)	91.2	3.0	3.5
YN1	oil-contaminated soil	37 °C / 5 months / 2.0%	*Firmicutes*	*Caloribacterium cisternae* (JF262044)	96.7	2.0	2.0
YN3	oil-contaminated soil	37 °C / 5 months / 2.0%	*Firmicutes*	*Hydrogenoanaerobacterium saccharovorans* (EU158190)	98.0	2.0	2.0
YN5	oil-contaminated soil	37 °C / 4 months / 2.0%	*Firmicutes*	*Clostridium pasteurianum* (FR870440)	93.6	2.5	3.0

[a]The conditions of the enrichment cultures (temperature / culture period / final butanol concentration) are given. [b]The values represent the maximum butanol or isobutanol concentration allowing growth and were based on three independent replicates. The temperature at which tolerance was tested was the same as for enrichment.

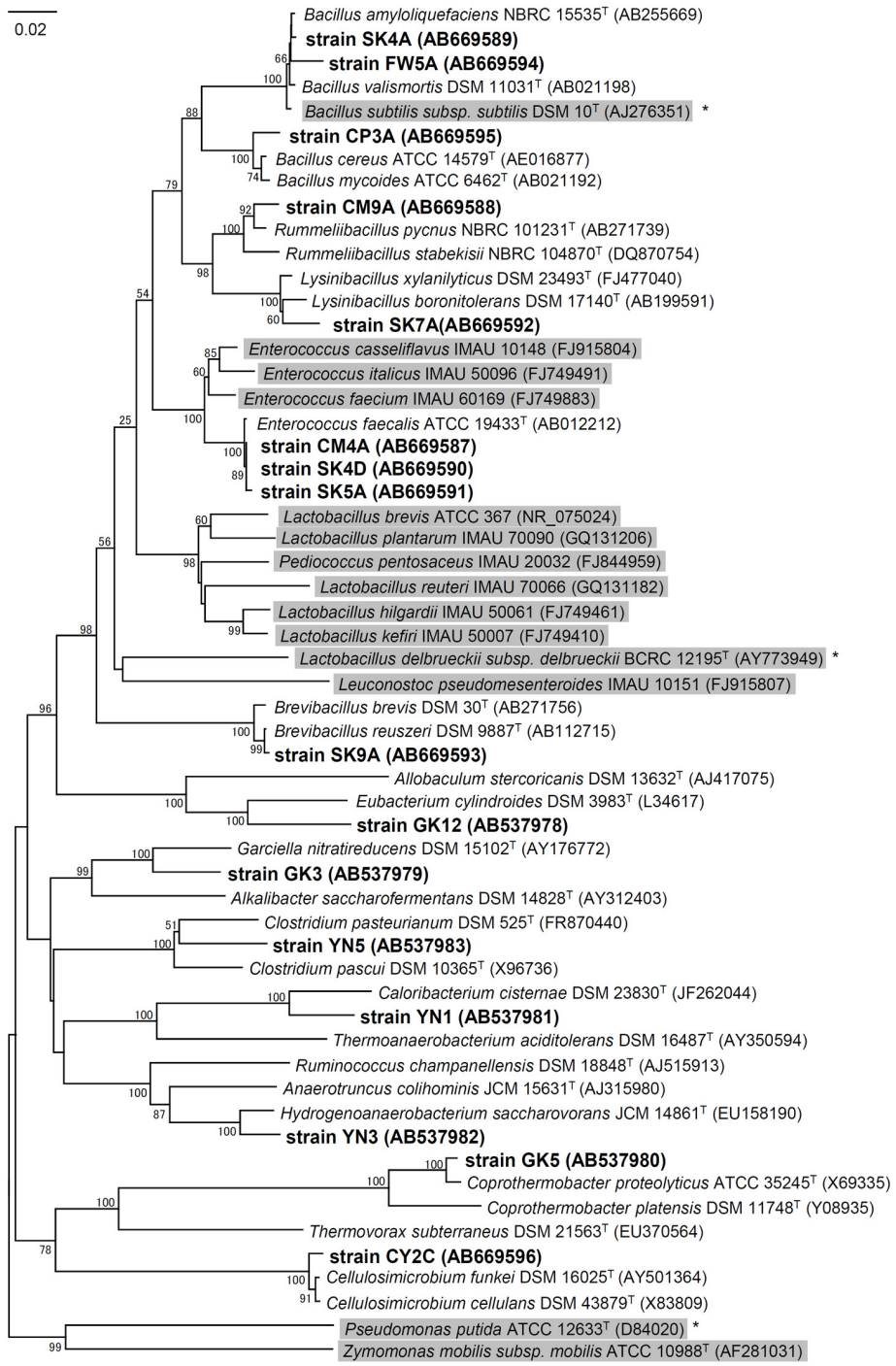

Figure 2.1. Neighbor-joining tree based on 16S rRNA gene sequences, showing the relationship between the isolates (bold), their relatives, and other butanol-tolerant bacteria (shaded clusters). Due to the lack of sequences for butanol-tolerant *Bacillus subtilis* (13), *Lactobacillus delbrueckii* (10), and *Pseudomonas putida* (14) strains, as indicated by asterisks, their type strains are shown in the tree. The bootstrap values that were above 50% are shown at the nodes. Bar, 0.02 substitutions per nucleotide position.

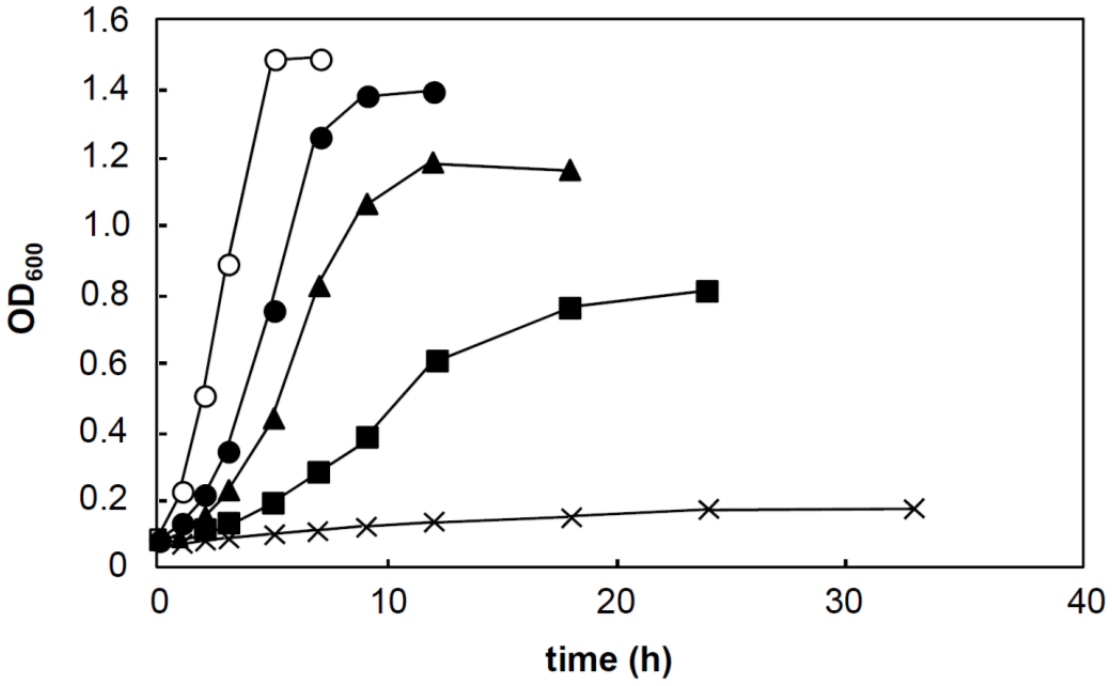

Figure 2.2. Effect of butanol on the growth of strain CM4A. The values represent the mean of triplicate experiments. Butanol concentrations: (○) 0%, (●) 2%, (▲) 2.5%, (■) 3.0%, and (×) 3.5%.

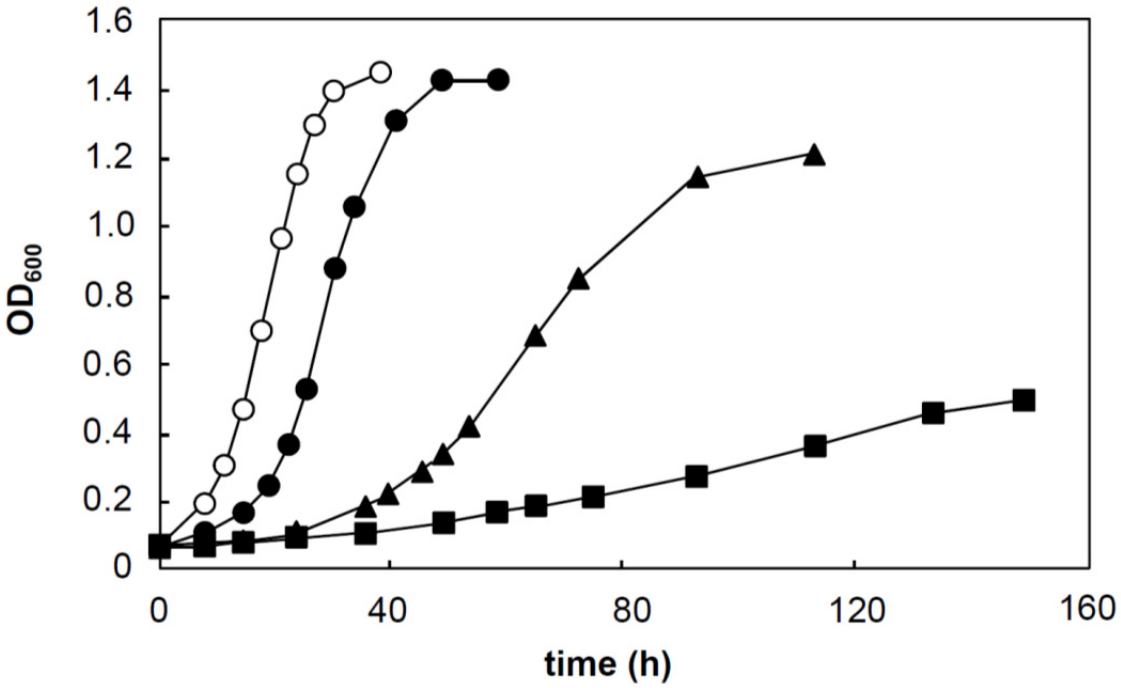

Figure 2.3. Effect of butanol on the growth of strain GK12. The values represent the mean of triplicate experiments. Butanol concentrations: (○) 0%, (●) 2%, (▲) 2.5%, and (■) 3.0%.

References

Atsumi S, Hanai T, Liao JC. 2008. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature 451:86-89.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783-791.

Hanada S, Kawase Y, Hiraishi A, Takaichi S, Matsuura K, Shimada K, Nagashima KVP. 1997. *Porphyrobacter tepidarius* sp. nov., a moderately thermophilic aerobic photosynthetic bacterium isolated from a hot spring. Int. J. Syst. Evol. Microbiol. 47:408-413.

Huffer S, Clark ME, Ning JC, Blanch HW, Clark DS. 2011. The role of alcohols in growth, lipid composition, and membrane fluidity of yeast, bacteria, and archaea. Appl. Environ. Microbiol. 77:6400-6408.

Hungate R. 1969. A roll tube method for cultivation of strict anaerobes. Method. Microbiol. 3:117-132.

Ingram LO, Buttke TM. 1984. Effects of alcohols on micro-organisms. Adv. Microb. Physiol. 25:253-300.

Jones DT, Woods DR. 1986. Acetone-butanol fermentation revisited. Microbiol. Mol. Biol. Rev. 50:484-524.

Kataoka N, Tajima T, Kato J, Rachadech W, Vangnai A. 2011. Development of butanol-tolerant *Bacillus subtilis* strain GRSW2-B1 as a potential bioproduction host. AMB Express 1:10-20.

Knoshaug EP, Zhang M. 2009. Butanol tolerance in a selection of microorganisms. Appl. Biochem. Biotechnol. 153:13-20.

Li J, Zhao JB, Zhao M, Yang YL, Jiang WH, Yang S. 2010. Screening and characterization of butanol-tolerant micro-organisms. Lett. Appl. Microbiol. 50:373-379.

Liu S, Qureshi N. 2009. How microbes tolerate ethanol and butanol. New Biotechnol. 26:117-121.

Ludwig W, Strunk O, Westram R, Richter L, Meier H. 2004. ARB: a software environment for sequence data. Nucleic Acids Res. 32:1363-1371.

Pfennig N, Widdel F, Truper HG. 1981. The dissimilatory sulfate-reducing bacteria, p 926-940. *In* Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (ed), The Prokaryotes, vol 1. Springer, New York, NY.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glöckner FO. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35:7188-7196.

Qureshi N, Saha BC, Cotta MA. 2007. Butanol production from wheat straw hydrolysate using *Clostridium beijerinckii*. Bioproc. Biosyst. Eng. 30:419-427.

Ruhl J, Schmid A, Blank LM. 2009. Selected *Pseudomonas putida* strains can grow in the presence of high butanol concentrations. Appl. Environ. Microbiol. 75:4653-4656.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.

Sekiguchi Y, Kamagata Y, Nakamura K, Ohashi A, Harada H. 2000. *Syntrophothermus lipocalidus* gen. nov., sp. nov., a novel thermophilic, syntrophic, fatty-acid-oxidizing anaerobe which utilizes isobutyrate. Int. J. Syst. Evol. Microbiol. 50:771-779.

Tamaki H, Hanada S, Sekiguchi Y, Tanaka Y, Kamagata Y. 2009. Effect of gelling agent on colony formation in solid cultivation of microbial community in lake sediment. Environ. Microbiol. 11:1827-1834.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596-1599.

Ting CNW, Wu J, Takahashi K, Endo A, Zhao H. 2012. Screened butanol-tolerant *Enterococcus faecium* capable of butanol production. Appl. Biochem. Biotech. 168: 1672-1680.

Vollherbst-Schneck K, Sands JA, Montenecourt BS. 1984. Effect of butanol on lipid composition and fluidity of *Clostridium acetobutylicum* ATCC 824. Appl. Environ. Microbiol. 47:193-194.

Woods DR. 1995. The genetic engineering of microbial solvent production. Trends Biotechnol. 13:259-264.

Chapter 3

Taxonomic Characterization of an Anaerobic Butanol-Tolerant Strain GK12

Abstract

An obligate anaerobic bacterium, designated as strain GK12, was isolated from an anaerobic digester in Fukagawa, Hokkaido Prefecture, Japan. The cells of strain GK12 were non-motile, non-spore-forming cocci that commonly occurred in chains. The 16S rRNA gene sequence analysis revealed that strain GK12 was affiliated with the family *Erysipelotrichaceae* in the phylum *Firmicutes* and showed a 91.8% sequence similarity to the most closely related species, *Faecalicoccus acidiformans*. The strain grew at 30-50 °C (optimally at 40 °C) and at pH 5.5-8.5 (optimally at pH 7.5). The main end product of glucose fermentation was lactate. Yeast extract was required for growth. The strain contained $C_{14:0}$, $C_{14:0}$ 1,1-dimethoxy alkane (DMA), $C_{16:0}$ DMA and $C_{18:0}$ DMA as the major cellular fatty acids (>10% of the total). The polar lipid profile was composed of phosphatidylglycerol, phosphatidylinositol and an unidentified phospholipid. The whole-cell sugars were galactose, rhamnose and ribose. The cell wall murein contained alanine, glutamic acid, lysine, serine and threonine, but not diaminopimelic acid. The G + C content of the genomic DNA was 47.7 mol%. Based on phenotypic, phylogenetic and chemotaxonomic properties, a new genus and species, *Catenisphaera adipataccumulans* gen. nov., sp. nov., was proposed for strain GK12 (=NBRC 108915^T=DSM 25799^T).

Introduction

Members of the family *Erysipelotrichaceae* in the phylum *Firmicutes* were once regarded as clostridia and relatives related to members of the order *Mycoplasmatales* (Collins *et al*., 1994). In 2004, the family *Erysipelotrichaceae* was established with *Erysipelothrix* as the type genus, and the *Clostridium* subphylum clusters XVI, XVII and XVIII were placed in this family (Verbarg *et al*., 2004). The family now consists of the following thirteen genera: *Allobaculum* (Greetham *et al*., 2004), *Bulleidia* (Downes *et al*., 2000), *Catenibacterium* (Kageyama & Benno, 2000a), *Coprobacillus* (Kageyama & Benno, 2000b), *Eggerthia* (Salvetti *et al*., 2011), *Erysipelothrix* (Skerman *et al*., 1980; Takahashi *et al*., 1987; Migula, 1990; Verbarg *et al*., 2004), *Faecalicoccus* (Maesschalck *et al*., 2014), *Faecalitalea* (Maesschalck *et al*., 2014), *Holdemanella* (Maesschalck *et al*., 2014), *Holdemania* (Willems *et al*., 1997), *Kandleria* (Salvetti *et al*., 2011), *Solobacterium* (Kageyama & Benno, 2000c) and *Turicibacter* (Bosshard *et al*., 2002).

A phylogenetic analysis based on the 16S rRNA gene sequence revealed that a number of species belonged to the genera *Clostridium*, *Eubacterium*, *Lactobacillus* or *Streptococcus* formed a cluster with the members of the family *Erysipelotrichaceae* and that these organisms were in need of taxonomic reconsideration (Stackebrandt, 2009). In 2011, two of the misplaced organisms in this family were reclassified as novel genera, *Eggerthia* and *Kandleria* (formerly *Lactobacillus catenaformis* and *Lactobacillus vitulinus*) (Salvetti *et al*., 2011). Recently, another three organisms were also reclassified as novel genera, *Faecalicoccus*, *Faecalitalea* and *Holdemanella* (formerly *Streptococcus pleomorphus*, *Eubacterium cylindroides* and *Eubacterium biforme*) (Maesschalck *et al*., 2014). At the time of writing, the other six species belonging to the genera *Clostridium* and *Eubacterium* have

continued to be included as members of the family *Erysipelotrichaceae*.

I isolated an anaerobic butanol-tolerant bacterium, designated strain GK12 (Kanno *et al*., 2013). A phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain GK12 obviously belonged to the family *Erysipelotrichaceae* but was distant from other members of this family (Katayama *et al*., 2014). In order to taxonomically characterise strain GK12, I conducted phenotypic, phylogenetic and chemotaxonomic methods and based on these results I propose a novel species and genus in the family *Erysipelotrichaceae* for this isolate.

Materials and Methods

Culture condition. Cell cultures of strain GK12 used for all the experiments were prepared in a medium with the following ingredients (per liter): 10 g of glucose, 5 g of yeast extract, 0.53 g of NH_4Cl, 0.14 g of KH_2PO_4, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.15 g of $CaCl_2 \cdot 2H_2O$, and 2.52 g of $NaHCO_3$, supplemented with 1 ml of selenium and tungsten solution, 1 ml of trace elements solution, and 2 ml of vitamin solution. The selenium and tungsten solution consisted of 2 mg l^{-1} $NaSeO_3 \cdot 5H_2O$ and 3 mg l^{-1} $Na_2WO_4 \cdot H_2O$. The trace elements solution was based on medium 318 of DSMZ (1983) with elimination of NaCl. The vitamin solution was based on the vitamin mixture in medium 141 of DSMZ (1983) with the modification that all seven components were mixed at a concentration of 20 μmol l^{-1}. The cell culture was grown under an atmosphere of N_2/CO_2 (80:20, vol/vol) at 40 °C unless indicated otherwise.

Phylogenetic analysis based on 16S rRNA gene sequences. The 16S rRNA gene sequence of strain GK12 was previously determined, as described in the Chapter 2. The sequence was aligned with its relatives using the Mothur software package (Schloss *et al*., 2009) with reference to the SILVA database (Pruesse *et al*., 2007). Neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) trees were constructed as described previously (Takeuchi *et al*., 2014).

Phenotypic characterization. Cell morphology in the late exponential growth phase was examined under a phase-contrast microscope (AX80; Olympus) and a transmission electron

microscope (H-7600; Hitachi). Samples used for transmission electron microscope were treated as described previously (Katayama *et al*., 2014). Gram staining was performed according to Tamaki *et al*. (2003). Cell growth was determined based on an increase in optical density at 600 nm. Assimilation of carbohydrates was tested using a medium containing 10 g l^{-1} each carbohydrate as the main carbon source; this medium also contained 2 g l^{-1} yeast extract due to its requirement for growth. Catalase and cytochrome oxidase activities were determined by bubble formation in 3% H_2O_2 solution and by use of an oxidase test paper (Nissui Pharmaceutical), respectively. Other biochemical and enzyme activities were tested using API ZYM and rapid ID 32 A (bioMérieux) test strips according to the manufacturer's instructions. Fermentation products in the liquid and gas phases were analyzed using HPLC with conductive detection (LC-20A; Shimadzu) and GC with flame ionization detection (GC-2014, Shimadzu), respectively. Sensitivity to antibiotics was determined by the growth in the presence of the following compounds (50 mg l^{-1}): ampicillin, chloramphenicol, kanamycin, neomycin, rifampicin and vancomycin.

Chemotaxonomic analyses. Crude lipids of the cells grown at 37 °C medium were extracted by the method of Bligh & Dyer (1959). Polar lipids, including phospholipids, were separated from free fatty acids by TLC, and were subsequently methanolyzed. The fatty acid methyl esters were analyzed using GC-MS as described previously (Katayama *et al*., 2014). For the identification of phospholipid species, crude lipid extracts were analyzed by two-dimensional TLC using HPTLC Silica gel 60 plates (Merck Millipore). Chloroform/methanol/7 M ammonium hydroxide (65:25:4, by vol.) and chloroform/methanol/acetic acid/water (170:25:25:2, by vol.) were used for first and second

direction of development, respectively (Nichols & James, 1964). Each spot was identified by spraying with primuline reagent (for all lipids; Katayama *et al*., 2014), Dittmer-Lester reagent (for phospholipids; Dittmer & Lester, 1964), Dragendorff reagent (for choline-derived lipids; Sigma-Aldrich), naphthol reagent (for glycolipids; Jacin & Mishkin, 1965) and ninhydrin reagent (for aminolipids; Wako). Spots corresponding to phospholipids were scraped off the plate and subsequently loaded on one-dimensional TLC together with authentic standards (Sigma). Presence of diaminopimelic acid in the cell wall was determined by TLC. Whole-cell sugars and amino acid composition of murein were determined using HPLC (L-2000; Hitachi) and an automatic amino acid analyser (L-8900; Hitachi) according to the method of Komagata & Suzuki (1987). DNA G+C content of strain GK12 was determined by HPLC with a UV detector (LC-6A; Shimadzu) as described previously (Kamagata & Mikami, 1991).

Results and Discussion

Phylogenetic characteristics. A phylogenetic analysis based on the 16S rRNA gene sequence revealed that strain GK12 was assigned to the *Clostridium* subphylum cluster XVI in the family *Erysipelotrichaceae* (Fig. 3.1). The *Clostridium* subphylum cluster XVI was clearly distant from the *Erysipelothrix* species, the type genus in this family, and formed a distinct clade, which was supported by a high bootstrap value. Within this subphylum cluster, five type strains, *Allobaculum stercoricanis*, *Faecalicoccus acidiformans*, *Faecalicoccus pleomorphus*, *Faecalitalea cylindroides* and *Holdemanella biformis*, formed a tight cluster together with strain GK12 (Fig. 3.1). Strain GK12 exhibited the highest sequence similarity to

F. acidiformans DSM 26963^T (91.8%) followed by *F. cylindroides* DSM 3983^T (91.2%), *F. pleomorphus* DSM 20574^T (90.6%), *H. biformis* DSM 3989^T (89.7%) and *A. stercoricanis* DSM 13633^T (86.5%). These low sequence similarities of strain GK12 with the described species (< 92%) were most likely sufficient to warrant the creation of a new genus and species in the family *Erysipelotrichaceae*.

Phenotypic characteristics. The cells of strain GK12 were non-motile and spherical (0.5-0.7 μm in diameter) and commonly occurred in chains (Fig. 3.2). Spore formation was not observed, and the Gram-stain results were positive. Cell growth occurred at 30-50 °C but not at 25 °C or 55 °C. The pH range for growth was pH 5.5-8.5. The optimum growth occurred at 40 °C and at pH 7.5. Strain GK12 did not grow in the presence of oxygen (20%, v/v, in the gas phase). The following substrates were used as energy and carbon sources: fructose, glucose, mannose, ribose, maltose, sucrose, mannitol and sorbitol. The following substrates did not support significant growth: arabinose, galactose, xylose, cellobiose, lactose, trehalose, inositol, starch, aesculin and gelatine. Yeast extract was required for growth (2 g l^{-1}). The catalase and cytochrome oxidase tests were negative. In API tests, strain GK12 showed positive enzymatic activity for acid phosphatase, alkaline phosphatase and naphtol-AS-BI-phosphohydrolase. The end products of glucose (1 mol) fermentation were lactate (0.9 mol), acetate (0.05 mol), butyrate (0.03 mol), formate (0.02 mol), hydrogen (0.1 mol) and carbon dioxide. Strain GK12 did not utilize the following electron acceptors (5 mM each): nitrate, sulphate, or Fe(III) nitrilotriacetate. The strain was sensitive to ampicillin, chloramphenicol, neomycin and vancomycin, but resistant to kanamycin and rifampicin.

Chemotaxonomic characteristics. The membrane fatty acids were composed of $C_{14:0}$ (25.2%), $C_{16:0}$ (8.3%), $C_{18:0}$ (3.5%), $C_{14:0}$ DMA (13.0%), $C_{16:0}$ DMA (28.0%) and $C_{18:0}$ DMA (22.0%). DMAs have previously been found in *Holdemania filiformis*, which belongs to this family (Willems *et al*., 1997). The polar lipid profile of strain GK12 contained phosphatidylglycerol, phosphatidylinositol and one unidentified phospholipid (Fig. 3.3). Galactose, rhamnose and ribose were detected as the whole cell sugars of strain GK12. Diaminopimelic acid was absent in the cell wall. In addition, the purified murein contained alanine, glutamic acid, lysine, serine and threonine at a molar ratio of 2.4:1.0:0.9:0.4:1.5, respectively. Members of some genera of the family *Erysipelotrichaceae* contained the group B type of peptidoglycan (Stackebrandt, 2009). The genera *Erysipelothrix* (Schubert & Fiedler, 2001; Verbarg *et al*., 2004) and *Holdemania* (Willems *et al*., 1997) in this family were previously reported to possess murein type B1δ. The B1δ type of murein includes alanine in the peptide subunit without diaminopimelic acid (Schleifer & Kandler, 1972), which was consistent with the results of strain GK12. However, threonine was detected in the isolate as an component but was not found in the above species. Strain GK12 most likely has a new type of murein different from the hitherto type of B1δ. The G+C content of genomic DNA was 47.7 mol% (standard deviation 0.09 mol%).

Differentiation of isolate from other taxa. Based on the phenotypic, phylogenetic and chemotaxonomic properties, I propose that strain GK12 represents a new genus and species in the family *Erysipelotrichaceae* as a type strain, with the name *Catenisphaera adipataccumulans* gen. nov., sp. nov. The novel species and other members of this family

possess several physiological features in common: they undergo mesophilic growth, lack motility, test negative for catalase, and are fermentative anaerobes that produce lactic, acetic, or butyric acids as end products (Stackebrandt, 2009). However, strain GK12 has some unique characteristics that differentiate it from the five closely related species, *Allobaculum stercoricanis*, *Faecalicoccus acidiformans*, *Faecalicoccus pleomorphus*, *Faecalitalea cylindroides* and *Holdemanella biformis* in the *Clostridium* subphylum cluster XVI (Table 3.1). The strain shows a higher DNA G+C content (47.7%) than all the other species (< 41%). The presence of DMA and the absence of branched-chain and unsaturated fatty acids are found only in strain GK12. The cell walls of this strain contain type B murein, whereas *A. stercoricanis* has type A. The various biochemical characteristics of strain GK12 are also different (Table 3.1). By these clear differential characteristics and the low 16S rRNA gene sequence similarities of less than 92%, this new strain can be easily differentiated from other related genera of the family *Erysipelotrichaceae*. In addition, there was an obvious difference in the isolation source between strain GK12 and its relatives: the new strain was isolated from an anaerobic digester, whereas other members of this family have been isolated from the gastrointestinal tracts or faeces of animals, including humans (Stackebrandt, 2009).

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Figures and Tables

Table 3.1. Differential characteristics of strain GK12 and related species in the family *Erysipelotrichaceae*.

Characteristic	1	2	3	4	5	6
Isolation source	Anaerobic digester	Intestines of human	Caeca of bird	Caeca of bird	Human faeces	Dog faeces
Growth at 45 °C	+	+	ND	+	−	−
Cell shape	Spherical	Rod	Spherical	Spherical	Spherical	Rod
DNA G+C content (mol%)	47.7	31.0	40.4	38.8	33.8	37.9
Fatty acid profile	Sa, DMA	Sa, Un, Br	Sa, Un, Br	Sa, Un, Br, Hy	Sa, Un, Br, Hy	ND
Cell-wall murein	Type B1δ, Ala, Glu, Thr, Lys contained	ND	ND	ND	ND	Type A, *meso*-Dpm contained
Products from glucose fermentation	a, b, f, h, l	b, l	b, f, l	b, f, l	a, b, l, p	b, e, l
Substrate utilization						
Maltose	+	−	−	−	−	+
Sucrose	+	+	ND	−	−	+
Mannitol	+	−	−	−	+	−
Sorbitol	+	−	−	−	−	−
Gelatin	−	−	−	+	+	−
Enzyme activity						
Acid phosphatase	+	+	−	+	+	+
Alkaline phosphatase	+	+	−	−	+	+
Esterase (C4)	−	+	−	−	−	+
Esterase lipase (C8)	−	+	−	−	−	+
α-Glucosidase	−	+	−	−	−	−
Alanine arylamidase	−	−	−	+	−	−
Arginine arylamidase	−	−	−	+	−	+
Glycine arylamidase	−	−	−	+	−	−
Histidine arylamidase	−	−	−	+	−	−
Leucine arylamidase	−	−	−	+	−	−
Leucyl glycine arylamidase	−	−	−	+	−	−
Pyroglutamic acid arylamidase	−	−	−	+	−	−

Strains: 1, GK12 (data from this study); 2, *Faecalitalea cylindroides* DSM 3983^T (Cato *et al*., 1974; Maesschalck *et al*., 2014); 3, *Faecalicoccus acidiformans* DSM 26963^T (Maesschalck *et al*., 2014); 4, *Faecalicoccus pleomorphus* DSM 20574^T (Barnes *et al*., 1977; Maesschalck *et al*., 2014); 5, *Holdemanella biformis* DSM 3989^T (Eggerth, 1935; Maesschalck *et al*., 2014); 6, *Allobaculum stercoricanis* DSM 13633^T (Greetham *et al*., 2004). +, Positive; −, negative; ND, no data available; Sa, saturated fatty acids; Un, unsaturated fatty acids; Br, branched-chain fatty acids; Hy, hydroxyl fatty acids; DMA, 1,1-dimethoxy alkane; Dpm, diaminopimelic acid; a, acetate; b, butyrate; f, formate; l, lactate; p, propionate; e, ethanol; h, hydrogen.

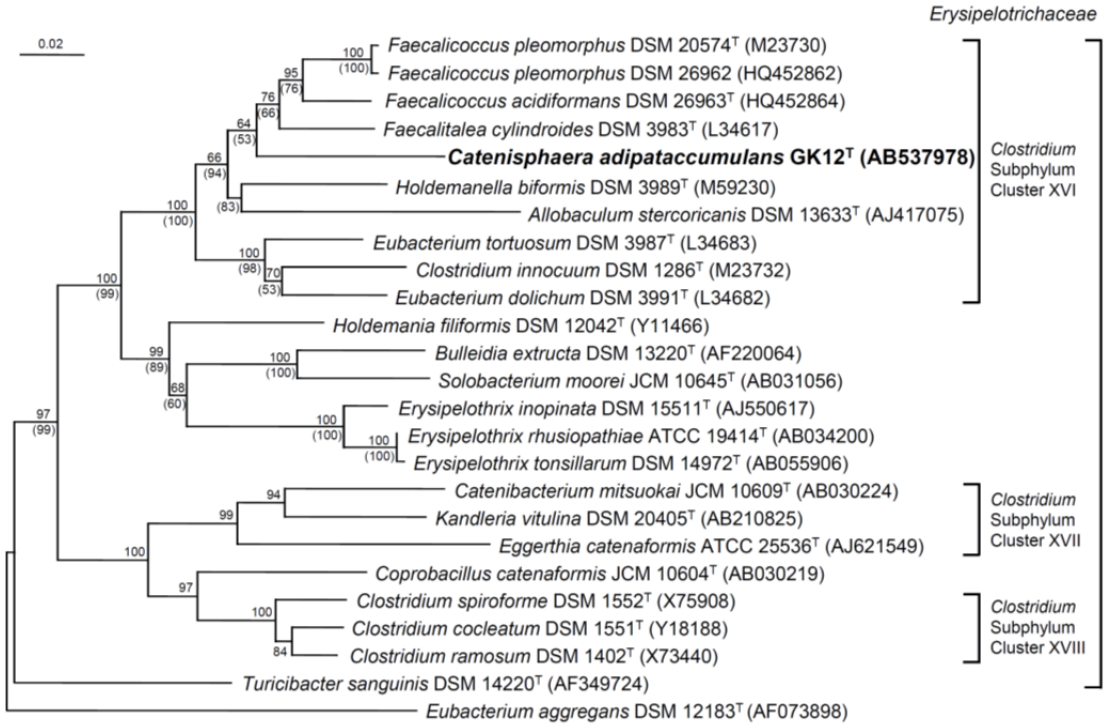

Figure 3.1. The neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences (1355 bp) showing the relationship between strain GK12 and other related species in the family *Erysipelotrichaceae*. The bootstrap values (>50 %) based on 1000 replications are shown at the nodes. The bootstrap values for the maximum-likelihood are shown in the parentheses. The sequence of *Eubacterium aggregans*^{T} (AF073898) was used as an outgroup. Bar: 0.02 substitutions per nucleotide position.

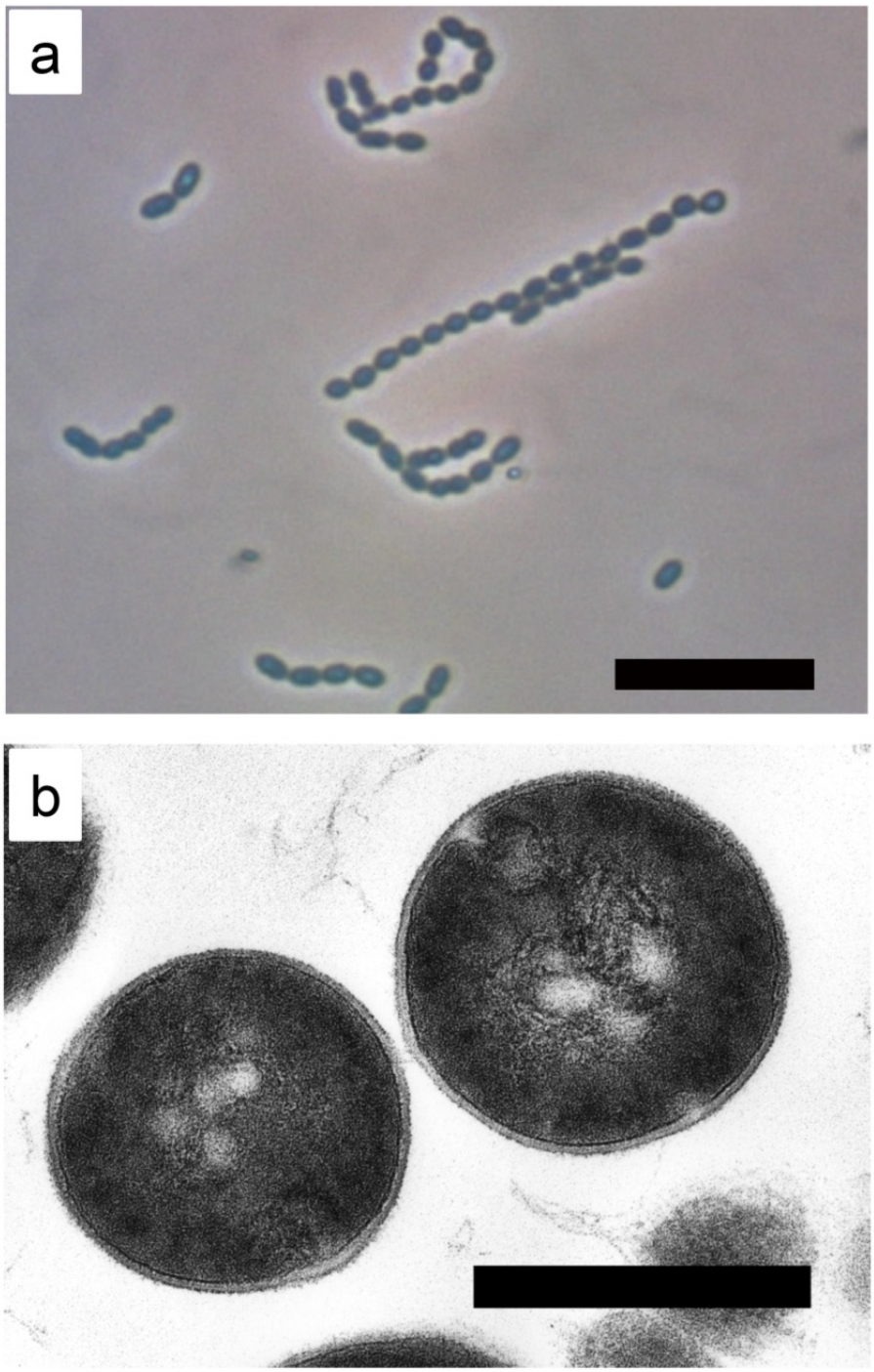

Figure 3.2. Phase-contrast micrograph (a) and transmission electron micrograph (b) of the cells of strain GK12 grown in the late exponential growth phase. Bars: 5 μm (a); 0.5 μm (b).

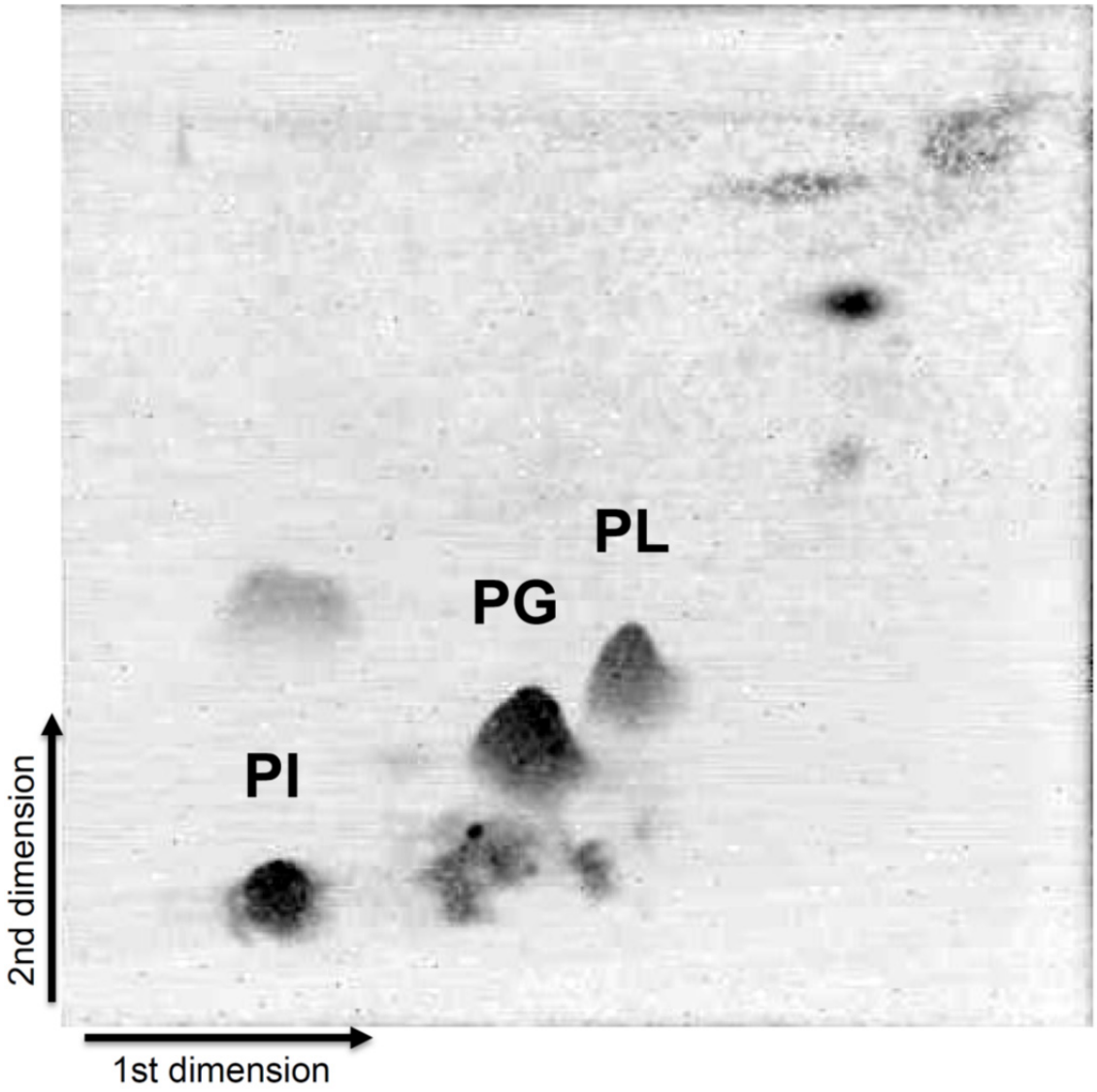

Figure 3.3. The total lipid profile of strain GK12 after separation by two-dimensional TLC on a silica gel plate. The solvent systems composed of chloroform/methanol/7 M ammonium hydroxide (65:25:4, by vol.) and chloroform/methanol/acetic acid/water (170:25:25:2, by vol.) were used for the first and second direction of development, respectively. PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, unidentified phospholipid.

References

Barnes EM, Impey CS, Stevens BJH, Peel JL. 1977. *Streptococcus pleomorphus* sp. nov.: An anaerobic *Streptococcus* isolated mainly from the caeca of birds. J. Gen. Microbiol. 102:45-53.

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911–917.

Bosshard PP, Zbinden R, Altwegg M. 2002. *Turicibacter sanguinis* gen. nov., sp. nov., a novel anaerobic, Gram-positive bacterium. Int. J. Syst. Evol. Microbiol. 52:1263-1266.

Cato EP, Salmon CW, Holdeman LV. 1974. *Eubacterium cylindroides* (Rocchi) Holdeman and Moore: Emended description and designation of neotype strain. Int. J. Syst. Bacteriol. 24:256-259.

Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandez-Garayzabal J, Garcia P, Cai J, Hippe H, Farrow JAE. 1994. The phylogeny of the genus *Clostridium*: Proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. 44:812-826.

Diltmer JC, Lester RL. 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. J. Lipid. Res. 5:126-127.

Downes J, Olsvik B, Hiom SJ, Spratt DA, Cheeseman SL, Olsen I, Weightman AJ, Wade WG. 2000. *Bulleidia extructa* gen. nov., sp. nov., a new genus of gram-positive anaerobic rod isolated from human oral cavities. Int. J. Syst. Evol. Microbiol. 50:979-983.

DSMZ. 1983. *Catalogue of Strains*, 3rd edn. Braunschweig, Germany: Gesellschaft für Biotechnologische Forschung.

Eggerth AE. 1935. The Gram-positive non-spore-bearing anaerobic Bacilli of human feces. J. Bacteriol. 30:277-299.

Felsenstein J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. 17:368–376.

Greetham HL, Gibson GR, Giffard C, Hippe H, Merkhoffer B, Steiner U, Falsen E, Collins MD. 2004. *Allobaculum stercoricanis* gen. nov., sp. nov., isolated from canine feces. Anaerobe. 10:301-307.

Jacin H, Mishkin AR. 1965. Separation of carbohydrates on borate-impregnated silica gel G plates. J. Chromatogr. 18:170-173.

Kageyama A, Benno Y. 2000a. *Catenibacterium mitsuokai* gen. nov., sp. nov., a Gram-positive anaerobic bacterium isolated from human faeces. Int. J. Syst. Evol. Microbiol. 50:1595-1599.

Kageyama A, Benno Y. 2000b. *Coprobacillus catenaformis* gen. nov., sp. nov., a new genus and species isolated from human feces. Microbiol. Immunol. 44:23–28.

Kageyama A, Benno Y. 2000c. Phylogenic and phenotypic characterization of some *Eubacterium*-like isolates from human feces : Description of *Solobacterium moorei* gen. nov., sp. nov. Microbiol. Immunol. 44:223–227.

Kamagata Y, Mikami E. 1991. Isolation and characterization of a novel thermophilic *Methanosaeta* strain. Int. J. Syst. Bacteriol. 41:191-196.

Kanno M, Katayama T, Tamaki H, Mitani Y, Meng X-Y, Hori T, Narihiro T, Morita N, Hoshino T, Yumoto I, Kimura N, Hanada S, Kamagata Y. 2013. Isolation of butanol- and isobutanol-tolerant bacteria and physiological characterization of their butanol tolerance. Appl. Environ. Microbiol. **79:**6998-7005.

Katayama T, Kanno M, Morita N, Hori T, Narihiro T, Mitani Y, Kamagata Y. 2014. An

oleaginous bacterium that intrinsically accumulates long-chain free fatty acids in its cytoplasm. Appl. Environ. Microbiol. 80:1126-1131.

Komagata K, Suzuki K. 1987. Lipid and cell wall analysis in bacterial systematics. Methods. Microbiol. 19:161–207.

Maesschalck CD, Immerseel FV, Eeckhaut V, Baere SD, Cnockaert M, Croubels S, Haesebrouck F, Ducatelle R, Vandamme P. 2014. *Faecalicoccus acidiformans* gen. nov., sp. nov. isolated from the chicken caecum and reclassification of *Streptococcus pleomorphus* (Barnes *et al*., 1977), *Eubacterium biforme* (Eggerth 1935) and *Eubacterium cylindroides* (Cato *et al*., 1974) as *Faecalicoccus pleomorphus* comb. nov., *Holdemanella biformis* gen. nov., comb. nov. and *Faecalitalea cylindroides* gen. nov., comb. nov., respectively, within the family *Erysipelotrichaceae*. Int. J. Syst. Evol. Microbiol. 64:3877-3884.

Migula W. 1990. *System der Bakterien*, vol. 2. Jena: Gustav Fischer.

Nichols BW, James AT. 1964. The lipids of plant storage tissue. Fette. Seifen. Anstrichum. 66:1003-1006.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glockner FO. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35:7188–7196.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.

Salvetti E, Felis GE, Dellaglio F, Castioni A, Torriani S, Lawson PA. 2011. Reclassification of *Lactobacillus catenaformis* (Eggerth 1935) Moore and Holdeman 1970 and *Lactobacillus vitulinus* Sharpe *et al*. 1973 as *Eggerthia catenaformis* gen. nov., comb. nov. and *Kandleria vitulina* gen. nov., comb. nov., respectively. Int. J. Syst.

Evol. Microbiol. 61:2520-2524.

Schleifer KH, Kandler O. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. 36:407–477.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van-Horn DJ, Weber CF. 2009. Introducing mother: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75:7537- 7541.

Schubert K, Fiedler F. 2001. Structural investigations on the cell surface of *Erysipelothrix rhusiopathiae*. System. Appl. Microbiol. 24:26-30.

Skerman VBD, McGowan V, Sneath PHA. 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30:225-420.

Stackebrandt E. 2009. Family 1. *Erysipelotrichaceae.* In Bergey's Manual of Systematic Bacteriology, 2nd edn, 3:1299-1317. Edited by Vos PD, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH, Whitman WB. New York: Springer.

Takahashi T, Fujisawa T, Benno Y, Tamura Y, Sawada T, Suzuki S, Muramatsu M, Mitsuoka T. 1987. *Erysipelothrix tonsillarum* sp. nov., isolated from tonsils of apparently healthy pigs. Int. J. Syst. Bacteriol. 37:166-168.

Takeuchi M, Katayama T, Yamagishi T, Hanada S, Tamaki H, Kamagata Y, Oshima K, Hattori M, Marumo K, Nedachi M, Maeda H, Suwa Y, Sakata S. 2014. *Methyloceanibacter caenitepidi* gen. nov., sp. nov., a facultatively methylotrophic bacterium isolated from marine sediments near a hydrothermal vent. Int. J. Syst. Evol. Microbiol. 64:462-468.

Tamaki H, Hanada S, Kamagata Y, Nakamura K, Nomura N, Nakano K, Matsumura M. 2003.

Flavobacterium limicola sp. nov., a psychrophilic, organic-polymer-degrading bacterium isolated from freshwater sediments. Int. J. Syst. Evol. Microbiol. 53:519-526.

Verbarg S, Rheims H, Emus S, Frühling A, Kroppenstedt RM, Stackebrandt E, Schumann P. 2004. *Erysipelothrix inopinata* sp. nov., isolated in the course of sterile filtration of vegetable peptone broth, and description of *Erysipelotrichaceae* fam. nov. Int. J. Syst. Evol. Microbiol. 54:221-225.

Willems A, Moore WEC, Weiss N, Collins MD. 1997. Phenotypic and phylogenetic characterization of some *Eubacterium*-like isolates containing a novel type B wall murein from human feces: Description of *Holdemania fuliformis* gen. nov., sp. nov. Int. J. Syst. Bacteriol. 47:1201-1204.

Chapter 4

Physiological Characterization of Butanol-Tolerant Strains CM4A and GK12

Abstract

The relatively highly butanol-tolerant strains CM4A (aerobe) and GK12 (obligate anaerobe) were characterized for their butanol tolerance properties. Both strains changed their membrane fatty acid composition in response to 2% butanol exposure, i.e., CM4A and GK12 increased saturated and cyclopropane fatty acids (CFAs), and long-chain fatty acids, respectively, which can serve for enhancing its butanol tolerance. Indeed, the mutants of *Escherichia coli*, overexpressing the cyclopropane fatty acid synthase gene derived from the strain CM4A enhanced the solvent tolerance. Strain CM4A changed the cell surface hydrophobicity by increasing extracellular capsule thickness in the presence of butanol. The exposure of strain GK12 to butanol by consecutive passages even enhanced the growth rate, indicating that yet-unknown mechanisms may also contribute to solvent tolerance. These results demonstrated that microbes have various strategies (e.g. changes in membrane fatty acid composition and cell morphology) to maintain structural integrity against high concentrations of butanol.

Introduction

Microorganisms are known to be sensitive to butanol, which intercalate into the cell membrane and break the lipid hydrogen bonds, resulting in a loss of membrane potential and cell death (Huffer *et al*., 2011; Ingram & Buttke, 1984; Vollherbst-Schneck *et al*., 1984). To date, microbial tolerance mechanism to butanol has been extensively studied on clostridia and *E. coli* through physiological analysis, omics analysis, and genomic library screening of genes related to butanol tolerance (Borden & Papoutsakis, 2007; MacDonald & Goldfine, 1991; Rutherford *et al*., 2010). However, these studies were conducted with low concentrations of butanol (around 1.0% (vol/vol)) due to their sensitivity, and therefore, the mechanism conferring tolerance to high concentrations of the solvents has not been elucidated yet.

Recently, I successfully isolated a wide variety of butanol-tolerant bacteria that could tolerate greater than 2.0% butanol and isobutanol. Among those isolates, aerobic strain CM4A and obligate anaerobic strain GK12 were able to tolerate up to 3.5% and 3.0% butanol, which was not observed for the previously reported bacteria (Li *et al*., 2010; Ting *et al*., 2012). Therefore, I selected strains CM4A and GK12 in order to elucidate the underlying mechanism of such superior tolerance properties. In the present study, two strains were characterized by examining cell surface structures, fatty acid compositions, and genes associated with butanol tolerance.

Materials and Methods

Cell adaptation to butanol. To examine cellular adaptation to butanol, all the isolates were grown with 2.0% butanol and inoculated into the subsequent medium. After 2-15 successive transfers, butanol tolerance of the isolates was evaluated by the same methods as described in the Chapter 2.

Transmission electron microscopy (TEM). Changes in the cell surface morphology of strains CM4A and GK12 in the late exponential growth phase grown with or without butanol were observed by TEM (Hitachi H-7600) (Toju *et al*., 2010). The thickness of the extracellular capsule of strain CM4A was measured from the transmission electron micrographs of 10 randomly selected cells (two spots per single cell, n=20).

Cell surface hydrophobicity measurement. The cell surface hydrophobicity of strain CM4A grown with or without butanol was assessed by the bacterial adhesion to hydrocarbon (BATH) test (Yamashita *et al*., 2007). In brief, 0.2 ml of organic solvent was added to 5 ml of cell suspension in a late exponential-phase of growth, which was adjusted to 1.0 of OD_{600}. The solution was then vortex-agitated for 30 s and left to stand for 15 min to allow separation. Butanol, *n*-hexane, *n*-tetradecane, toluene, and xylene were used as the solvents in the assay. The percentage of cells that adhered to the solvent was calculated by the following formula: [1-(OD_{600} of aqueous phase after mixing)/(OD_{600} of initial suspension)]×100 (Reid *et al*., 1992).

Fatty acid analysis. An analysis of whole-cell lipid extracts from strains CM4A and GK12

grown with or without butanol was performed using previously described methods (Hanada *et al*., 2002). Briefly, cells in the late exponential phase of growth were directly methanolyzed. The products containing fatty acid methyl esters were then extracted with *n*-hexane and analyzed by gas chromatography-mass spectrometry (M7200A GC/3DQMS system; Hitachi, Japan).

Cloning and expression of the cyclopropane fatty acid (CFA) synthase gene (*cfa*) in *E. coli.* The *cfa* gene of strain CM4A was amplified by using the primers cfa-F and cfa-R, designed from the *cfa* gene sequence in the *Enterococcus faecalis* V583 genome (NC004668), and then sequenced by using the primers cfa362R and cfa871F. The primers cfa-5EF and cfa-5XR, containing *Eco*RI and *Xho*I sites, were used to amplify the *cfa* gene for cloning into the pET-28b expression vector (Novagen) to produce an N-terminal His 6-tagged fusion protein. The primers used for the PCR amplification, direct sequencing, and cloning of the *cfa* gene are listed in Table 4.1. *E. coli* DH5α cells were transformed and subsequently plated onto LB medium supplemented with 30 μg/ml kanamycin. The expression and activity of the recombinant protein in the positive clone, designated as *E. coli*/pCFA, were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by the above-described fatty acid analysis, respectively. *E. coli*/pCFA and the negative-control clone *E. coli*/pET28, which was transformed with an empty pET-28b vector, were subjected to the solvent tolerance assay, as described above. Briefly, the cells were grown on LB medium containing 0.7-1.0 % butanol or isobutanol, 0.01 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), and 30 μg/ml kanamycin. The cell growth was monitored using a spectrophotometer.

Nucleotide sequence accession numbers. The GenBank/EMBL/DDBJ accession number of the *cfa* gene of strain CM4A is AB669597.

Results and Discussion

Morphological characterization of strains CM4A and GK12. The cell morphologies were investigated by TEM. As shown in Fig. 4.1, changes in the cell surface properties in the presence of a high butanol concentration (2.0%) were evident in strain CM4A. The cells exhibited a diplococcus shape with a hyaluronic acid capsular polysaccharide (Fig. 4.1A, 4.1C), and the thickness of the extracellular capsule increased twofold from 17±3.7 nm to 32±6.2 nm when the cells were grown with butanol ($p<0.01$ by t-test) (Fig. 4.1B, 4.1D). These changes in cell surface properties in response to butanol exposure have not been reported to date. The BATH test (Yamashita *et al*., 2007) was performed to clarify whether the cell surface hydrophobicity of strain CM4A was altered by the increased extracellular capsule thickness. Cells grown with 2% butanol exhibited a significantly lower affinity for butanol and other organic solvents than did the cells grown without butanol ($p<0.01$ by t-test) (Table 4.2). This result suggests that the changes in the capsule structure of strain CM4A decrease the cell surface hydrophobicity and thus may function as a physical defense against butanol. Previous studies have reported that the solvent-tolerant microorganisms *Rhodococcus rhodochrous* and *Staphylococcus* sp. produce extracellular hydrophilic compounds that may prevent hydrophobic solvents, such as *n*-hexadecane and toluene, from intercalating into the membrane (Iwabuchi *et al*., 2000; Zahir *et al*., 2006). In contrast to CM4A, such change in morphology was not observed in strain GK12.

Changes in the fatty acid compositions of strains CM4A and GK12 in the presence of butanol. To elucidate the underlying mechanism of solvent tolerance, I further investigated the physiological response of strains CM4A and GK12 to butanol. Exposure to solvents impairs the integrity and stability of the cytoplasmic membrane, and several solvent-tolerant

microorganisms are known to reestablish membrane fluidity and decrease solvent permeability by altering their membrane lipid compositions (Huffer *et al*., 2011; Torres *et al*., 2011; Weber & De Bont, 1996). Thus, to monitor the modification of the cell membrane to high concentrations of butanol, changes in the fatty acid compositions of the highly solvent-tolerant strains CM4A and GK12 were examined at a concentration of up to 2.5% butanol.

In the absence of butanol, strain CM4A contained saturated fatty acids (44% of total), together with unsaturated fatty acids (56%), such as palmitic acid (C16:0) and *cis*-vaccenic acid (C18:1ω7c). When the cells were grown with butanol, the proportion of C16:0 and CFAs (*cis*-11,12-methylene octadecanoic acid [cyclo-C19:0]) increased, whereas the proportion of C18:1ω7c decreased, resulting in an increase in the proportion of total saturated fatty acids and CFAs by up to 56% (Table 4.3). Although the *cis*-hexadecenoic acid (C16:1ω7c) level relatively increased with 2.0% butanol but decreased with 2.5%, the total unsaturated fatty acid proportion tended to decrease in a butanol dose-dependent manner (Table 4.3). In contrast, strain GK12 did not contain unsaturated fatty acids but rather saturated fatty acids and saturated 1,1-dimethoxy alkanes (DMAs) (Rainey *et al*., 2000). In the presence of butanol, the proportions of longer-chain fatty acids (stearic acid [C18:0] and arachidic acid [C20:0]) significantly increased (Table 4.4). Although the proportion of C18:0 DMAs relatively increased with 2.0% butanol but decreased with 2.5%, the total proportion of alkyl groups of a length of 18 increased in a butanol dose-dependent manner (Table 4.4). Because increases in the acyl chain length and proportion of saturated fatty acids and the cyclization of unsaturated fatty acids promote more rigid membrane structures (Grogan & Cronan, 1997; Mykytczuk *et al*., 2007), these changes in response to butanol exposure in both strains may compensate for the increased membrane fluidity imposed by butanol. Other microorganisms, such as

Clostridium acetobutylicum and *Pseudomonas putida*, have also been reported to maintain membrane rigidity by increasing both the mean length of the acyl chain and the proportion of saturated fatty acids and by synthesizing CFAs in the presence of solvents (Heipieper & De Bont, 1994; Heipieper *et al*., 2007; Lepage *et al*., 1987; Pini *et al*., 2009), which is in accordance with the findings in this study.

Overall, butanol dose-dependent changes in the saturated and CFA levels and acyl chain length in strains CM4A and GK12, respectively, were clearly observed (Tables 4.3 and 4.4). In particular, strain GK12 exhibited a marked alteration in membrane composition than strain CM4A. In general, an increase in the length of the acyl chain has a smaller effect on the fluidity of the lipid bilayer than does the saturation of fatty acids (Mykytczuk *et al*., 2007). Because strain GK12 lacked unsaturated fatty acids, this strain would have to markedly alter the length of the acyl chain of its saturated fatty acids to maintain membrane fluidity.

Heterologous expression of the *cfa* gene derived from strain CM4A improved solvent tolerance in *E. coli*. The fatty acid analysis indicated that CFAs may contribute to the butanol tolerance of strain CM4A. To verify this phenomenon, I examined whether the cyclization of unsaturated fatty acids among the membrane lipids could enhance the tolerance of *E. coli* to butanol and isobutanol. Recombinant strain *E. coli*/pCFA was generated by the introduction of the *cfa* gene derived from strain CM4A. The deduced 388 amino acid sequence resulting from the *cfa* gene (AB669597) of strain CM4A is 36% identical to the *E. coli cfa* gene (AM946981) product. Expression of the recombinant protein and the increase in the proportion of CFAs (cyclo-C19:0) were confirmed by SDS-PAGE and fatty acid analysis, respectively (Fig. 4.2 and Table 4.5). A solvent tolerance assay showed that the relative growth rate of recombinant *E. coli*/pCFA in the presence of butanol or isobutanol was significantly higher than the growth rate of the negative-control strain *E. coli*/pET28 ($p<0.01$

by t-test) (Fig. 4.3 and 4.4). These findings strongly demonstrate that CFA synthesis due to *cfa* gene transformation enhanced the tolerance of *E. coli* to butanol and isobutanol. CFA synthase is known to directly modify unsaturated fatty acids in the membrane and therefore does not require energy or carbon for *de novo* fatty acid synthesis (Grogan & Cronan, 1997). The enhancement of butanol tolerance by reinforcing CFA synthesis thus did not compete with butanol production, at least in terms of energy and carbon consumption. Introduction of the *cfa* gene into a candidate microbial platform may serve as a new strategy to improve butanol production efficiency.

Mechanisms involved in the butanol adaptation of strain GK12. Microorganisms are known to adapt to organic solvents during the exposure to non-lethal levels (Ruhl *et al*., 2009; Weber *et al*., 1993). To verify whether the isolates could adapt to butanol, the 16 isolates were subjected to successive subculturing with non-lethal concentrations of butanol. Only strain GK12 exhibited a significant adaptation after 15 consecutive passages in the presence of 2.0% butanol (Fig. 4.5). Interestingly, the butanol-treated GK12 cells grew even faster in the presence of 2.0% butanol than in the absence of butanol (Fig. 4.6, comparison between Fig. 2.3 and 4.5). In addition, when the butanol-treated cells were grown in 2.5% or 3.0% butanol, the cell population density in the stationary phase was higher than the untreated cells, clearly demonstrating the ability of the cells to adapt to butanol. Conversely, the enhanced tolerance was completely abolished by repeatedly culturing the butanol-treated cells in the absence of butanol, suggesting that the butanol adaption of strain GK12 was due to physiological responses rather than genetic mutations. To our knowledge, no other microorganism can grow faster in the presence of such toxic and non-metabolizable organic solvents than in the absence of those solvents.

To elucidate the adaptation mechanisms, I analyzed the fatty acid and DMA compositions of the adapted cells in comparison to the non-adapted cells. Similar to the non-adapted cells, the adapted cells increased their acyl chain lengths in a butanol dose-dependent manner (Table 4.4). However, considerable differences in the compositions were observed between the non-adapted and adapted cells grown with 2.0% butanol. In particular, the proportion of short chains (myristic acid [C14:0]) in the butanol-adapted cells was much higher than in the non-adapted cells, whereas the proportion of long chains (C18:0 and C20:0) in the butanol-adapted cells was lower than in the non-adapted cells. It should also be noted that, when comparing the adapted cells grown with or without 2.0% butanol, there were no significant differences in the proportions of short and long chains; rather, such proportions were similar to those of the non-adapted cells grown without butanol (Table 4.4). These results strongly imply that 2.0% butanol-adapted cells may use alternative strategies to acquire butanol tolerance instead of altering the chain length of fatty acids and DMAs, at least at the concentration (2.0%) to which the cells had adapted. Interestingly, the proportions of long chains and short chains markedly increased and decreased, respectively, when the adapted cells were grown with 2.5% butanol (Table 4.4), showing that a 0.5% increase was critical for the collapse of the homeostatic state of the fatty acid composition.

A similar finding was reported for the adaptation of *Pseudomonas putida* to toluene (Neumann *et al*., 2005). Changes in the membrane fatty acid compositions via the isomerization of *cis*- to *trans*-unsaturated fatty acids in response to solvent exposure were observed in non-adapted *P. putida* cells but not in adapted cells, suggesting that alternative mechanisms in the adapted cells, such as a solvent efflux system, allowed for improved solvent tolerance (Isken & De Bont, 1996).

Taken together, strain CM4A responded to butanol exposure by adjusting membrane

fatty acid compositions and by increasing the production of capsule structure. Another strain GK12 improved butanol tolerance by alternative mechanisms, which was indicated by comparison of the non-adapted with butanol-adapted cells, as well as by change in membrane fatty acid and DMA compositions. These findings shed light into the largely unknown mechanisms of bacteria to tolerate high concentrations of butanol.

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Figures and Tables

Table 4.1. Primers used for the amplification, direct sequencing, and cloning of the *cfa* gene of strain CM4A.

Primer name	Use	Primer sequence (5' to 3')
cfa-F [a]	Amplification	GAGGGAATGCAATGTTAG
cfa-R [a]	Amplification	TCTATTAACCAATCCGG
cfa362R [a]	Sequencing	CCTAGATCGTAATGGCTGTG
cfa871F [a]	Sequencing	GGTGGCTATATTCCTGGTG
cfa-5EF [b]	Cloning	CCGAATTCGAATGCAATGTTAG
cfa-5XR [b]	Cloning	CCCTCGAGATTAACCAATCCG

[a]The primers were designed from the *cfa* gene sequence in the *Enterococcus faecalis* V583 genome (NC004668). [b]Primers containing *Eco*RI and *Xho*I sites, as underlined, were used to amplify the *cfa* gene for cloning into the pET-28b expression vector (Novagen) to produce an N-terminal His 6-tagged fusion protein.

Table 4.2. Cell surface hydrophobicity of strain CM4A grown with or without 2.0% butanol.

CM4A cells	BATH (%) [a]				
	Butanol	*n*-Hexane	*n*-Tetradecane	Toluene	Xylene
without butanol	11.6 ± 2.94	22.3 ± 0.26	15.9 ± 0.45	30.5 ± 0.45	21.9 ± 1.58
with 2.0% butanol	0.8 ± 4.11	4.3 ± 1.62	6.5 ± 1.39	5.3 ± 1.39	2.6 ± 1.87

[a]The value represents the percentage of cells adhering to a given solvent in three independent measurements. [1-(OD_{600} of aqueous phase after mixing)/(OD_{600} of initial suspension)]×100.

Table 4.3. Changes in the fatty acid composition of strain CM4A in the presence of 2.0% and 2.5% butanol.

	No solvents	2.0% butanol	2.5% butanol
C9:1	ND	ND	1.1 ± 0.3
C14:0	5.7 ± 0.2	6.9 ± 0.6	6.6 ± 0.2
C14:1ω7c	0.6 ± 0.1	0.8 ± 0.1	ND
C15:0	0.1 ± 0.0	0.1 ± 0.0	ND
C16:0	35.7 ± 1.3	41.0 ± 0.8	45.8 ± 0.7
C16:1	ND	ND	0.9 ± 0.0
C16:1ω7c	8.9 ± 0.2	9.7 ± 0.4	7.9 ± 0.3
C18:0	1.9 ± 0.1	1.2 ± 0.0	1.7 ± 0.2
C18:1ω7c	46.4 ± 1.5	38.5 ± 1.6	33.6 ± 0.4
cyclo-C19:0	0.6 ± 0.1	1.8 ± 0.2	2.4 ± 0.1
Total saturated fatty acids	43.4 ± 1.4	49.2 ± 1.3	54.1 ± 0.5

Each fatty acid composition is described as a percentage of the whole-cell lipids. The values are the means ± standard deviations of three independent measurements. The double-bond positions of C9:1 and C16:1 were not identified. “Total saturated fatty acids” is the sum of myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), and stearic acid (C18:0). Abbreviations: X:YωZc, a fatty acid containing X carbon atoms with Y double bonds at position Z, counted from the methyl terminus in the *cis* configuration; cyclo-C19:0, *cis*-11,12-methylene octadecanoic acid; ND, not detected.

Table 4.4. Changes in the fatty acid and DMA compositions of non-adapted and butanol-adapted cells of strain GK12 in the presence of butanol.

	non-adapted cells			butanol-adapted cells			
	No solvents	2.0% butanol	2.5% butanol	No solvents	2.0% butanol	2.5% butanol	3.0% butanol
C14:0	24.5 ± 1.1	6.6 ± 0.5	1.5 ± 0.8	27.0 ± 1.6	26.1 ± 0.4	5.2 ± 0.7	1.1 ± 0.4
C16:0	23.5 ± 1.4	23.7 ± 2.2	21.6 ± 1.1	25.1 ± 0.3	27.8 ± 0.6	31.2 ± 0.9	19.2 ± 0.4
C18:0	10.8 ± 0.6	30.4 ± 0.7	42.1 ± 1.3	3.8 ± 0.5	5.2 ± 0.5	35.2 ± 0.2	48.1 ± 0.8
C20:0	2.8 ± 0.6	10.1 ± 1.3	14.1 ± 1.3	2.6 ± 0.5	4.7 ± 0.5	6.6 ± 0.2	19.4 ± 0.6
C14:0 DMA	4.9 ± 1.3	0.6 ± 0.2	ND	5.5 ± 0.5	2.7 ± 1.2	ND	ND
C16:0 DMA	18.0 ± 1.7	1.8 ± 0.5	1.6 ± 0.4	10.3 ± 1.3	4.2 ± 0.3	2.1 ± 0.3	1.1 ± 0.4
C18:0 DMA	15.5 ± 0.8	26.8 ± 2.2	19.1 ± 1.9	25.7 ± 0.6	29.3 ± 0.5	19.6 ± 0.4	11.2 ± 0.9
Total C14	29.4 ± 1.4	7.2 ± 0.4	1.5 ± 0.7	32.5 ± 1.4	28.8 ± 1.1	5.2 ± 0.7	1.1 ± 0.4
Total C16	41.5 ± 1.9	25.5 ± 2.0	23.3 ± 1.0	35.5 ± 1.2	32.0 ± 0.6	33.3 ± 0.8	20.3 ± 0.5
Total C18	26.3 ± 0.9	57.3 ± 2.0	61.2 ± 2.0	29.4 ± 0.7	34.5 ± 0.6	54.8 ± 0.4	59.3 ± 1.0

Each fatty acid composition is described as a percentage of whole-cell lipids. Cell adaptation was achieved by 15 consecutive passages with 2.0% butanol. The values are the means ± standard deviations of three independent measurements. Abbreviations: X:Y, fatty acid containing X carbon atoms with Y double bonds; total X, the sum of fatty acids and DMAs with an acyl chain length of X; ND, not detected.

Table 4.5. Changes in the membrane fatty acid compositions of the strain *E. coli*/pCFA and the control strain *E. coli*/pET28 in response to the presence of 0.1 mM IPTG[a].

	E. coli/pET28	*E. coli*/pCFA
C12:0	4.1 ± 0.1	4.3 ± 0.3
C14:0	8.8 ± 0.5	11.1 ± 0.1
C14:0-3OH	3.4 ± 0.3	3.4 ± 1.4
C14:1ω7c	ND	0.1± 0.1
C15:0	0.2 ± 0.0	0.1 ± 0.1
C16:0	43.2 ± 0.2	45.2 ± 0.6
C16:1ω7c	16.3 ± 0.1	17.6 ± 0.6
cyclo-C17:0	3.9 ± 0.1	5.6 ± 0.7
C18:0	0.5 ± 0.1	0.2 ± 0.2
C18:1ω7c	19.4 ± 0.7	6.1 ± 0.6
cyclo-C19:0	0.3 ± 0.0	6.5 ± 1.3

[a]Each fatty acid composition is described as a percentage of the total fatty acids. The values are the means ± standard deviations of three independent measurements. Abbreviations: X:YωZc, fatty acid containing X carbon atoms with Y double bonds at position Z, counted from the methyl terminus in the *cis* configuration; C14:0-3OH, 3-hydroxy tetradecanoic acid; cyclo-C17:0, *cis*-9,10-methylene hexadecanoic acid; cyclo-C19:0, *cis*-11,12-methylene octadecanoic acid; ND, not detected.

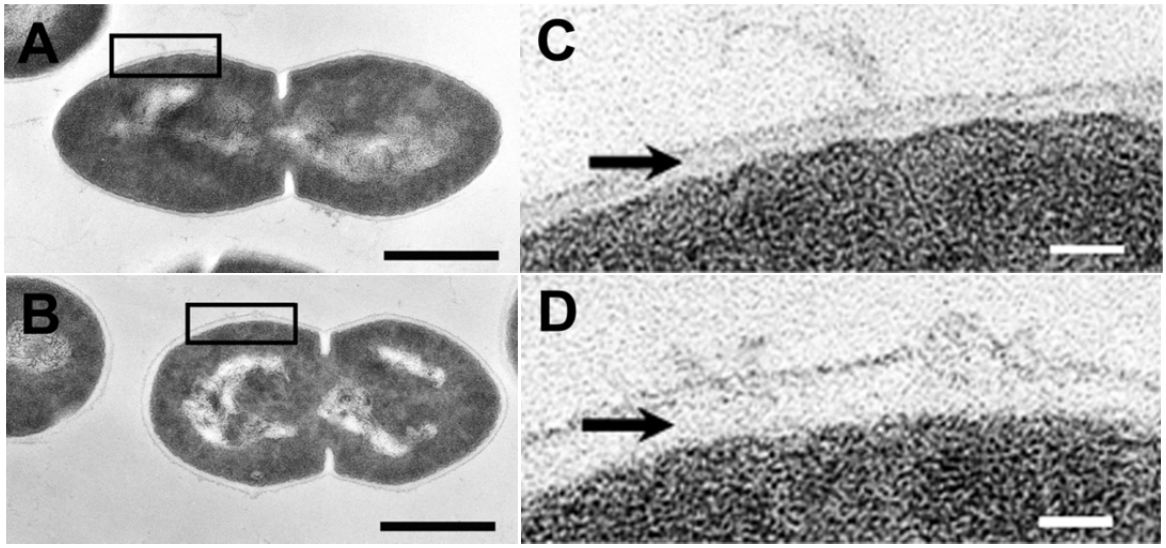

Figure 4.1. Transmission electron micrographs (A-D) of strain CM4A in the absence (A, C) and presence (B, D) of 2.0% butanol. The transmission electron micrographs in C and D are magnified views of the boxes in A and B, respectively. Scale bars, 0.5 μm (A, B) and 50 nm (C, D). The arrows point to the positions of the capsule. The average capsule thicknesses were 17 ± 3.7 nm and 32 ± 3.7 nm in the absence and presence, respectively, of 2.0% butanol ($p<0.01$ by t-test).

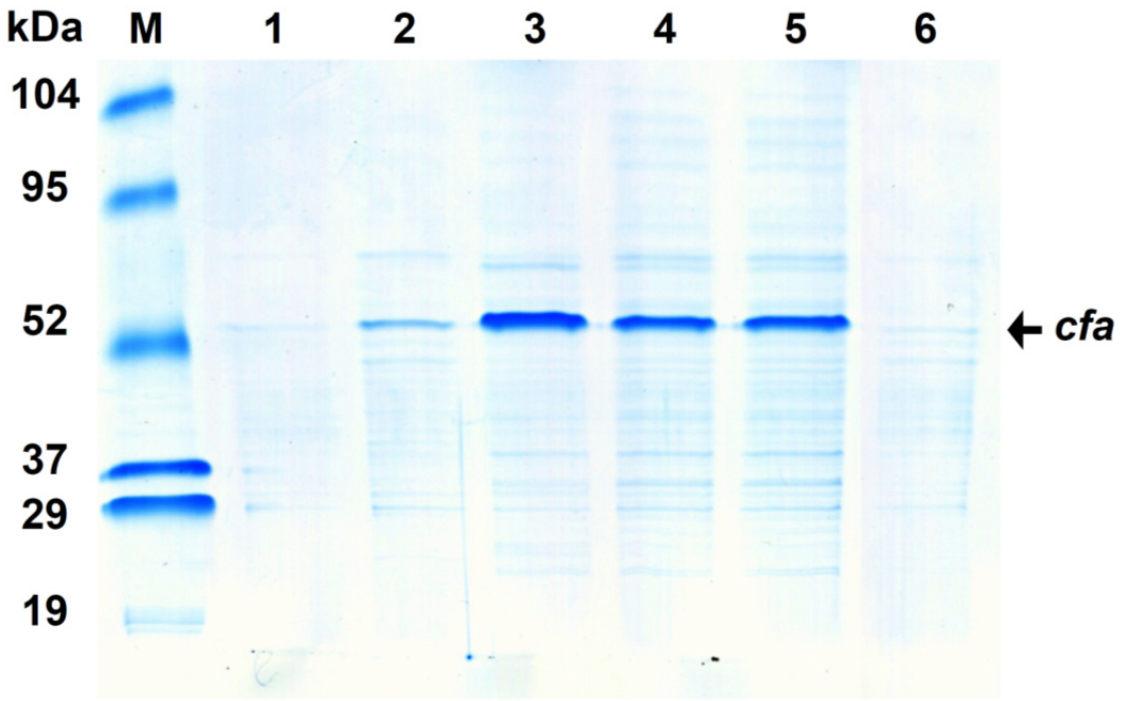

Figure 4.2. SDS-PAGE analysis of *cfa* gene expression in *E. coli*/pCFA (lanes 1-5) and *E. coli*/pET28 (lane 6) at different concentration of IPTG. In total, 60 μg of protein in the sonicated supernatant was purified by His-selective nickel affinity gel chromatography and analyzed by SDS-PAGE. Lane M, molecular weight marker; lane 1, 10 mM glucose without IPTG (negative control); lane 2, no IPTG; lane 3, 0.01 mM IPTG; lane 4, 0.1 mM IPTG; lane 5, 1.0 mM IPTG; lane 6, *E. coli*/pET28 grown without IPTG (negative control).

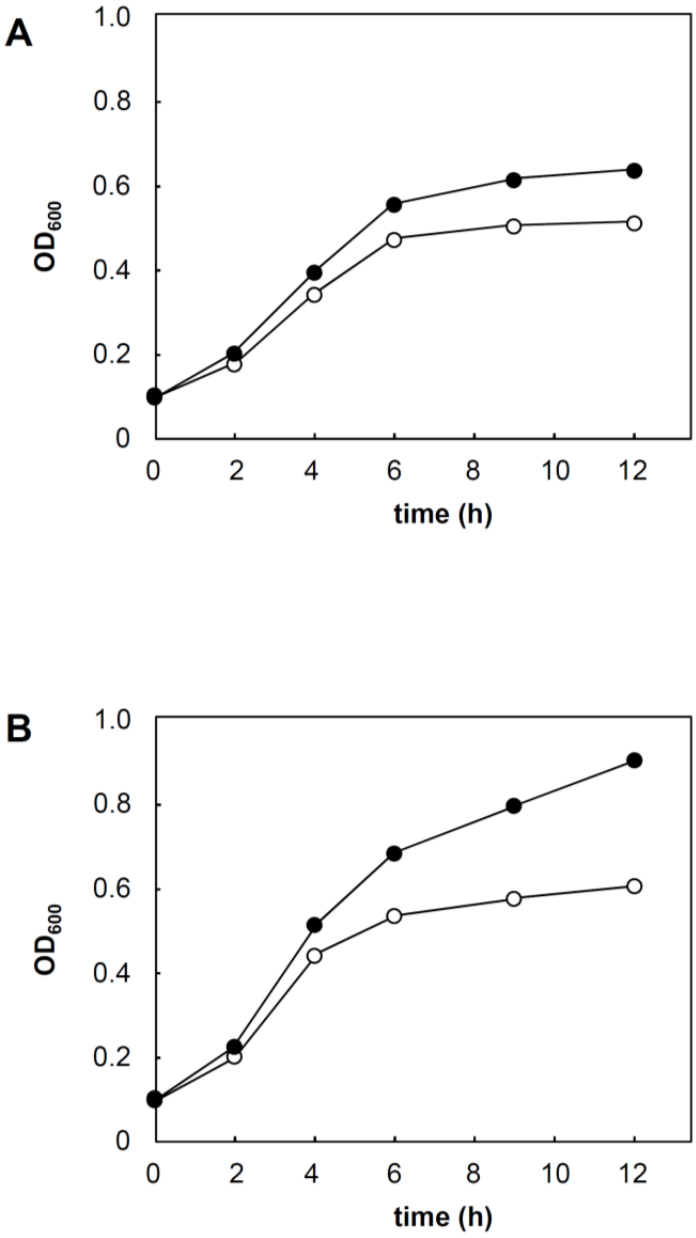

Figure 4.3. Growth of *E. coli*/pCFA (●) and the control strain *E. coli*/pET28 (○) in the presence of 0.8% butanol (A) or 0.8% isobutanol (B). The values represent the mean of triplicate experiments.

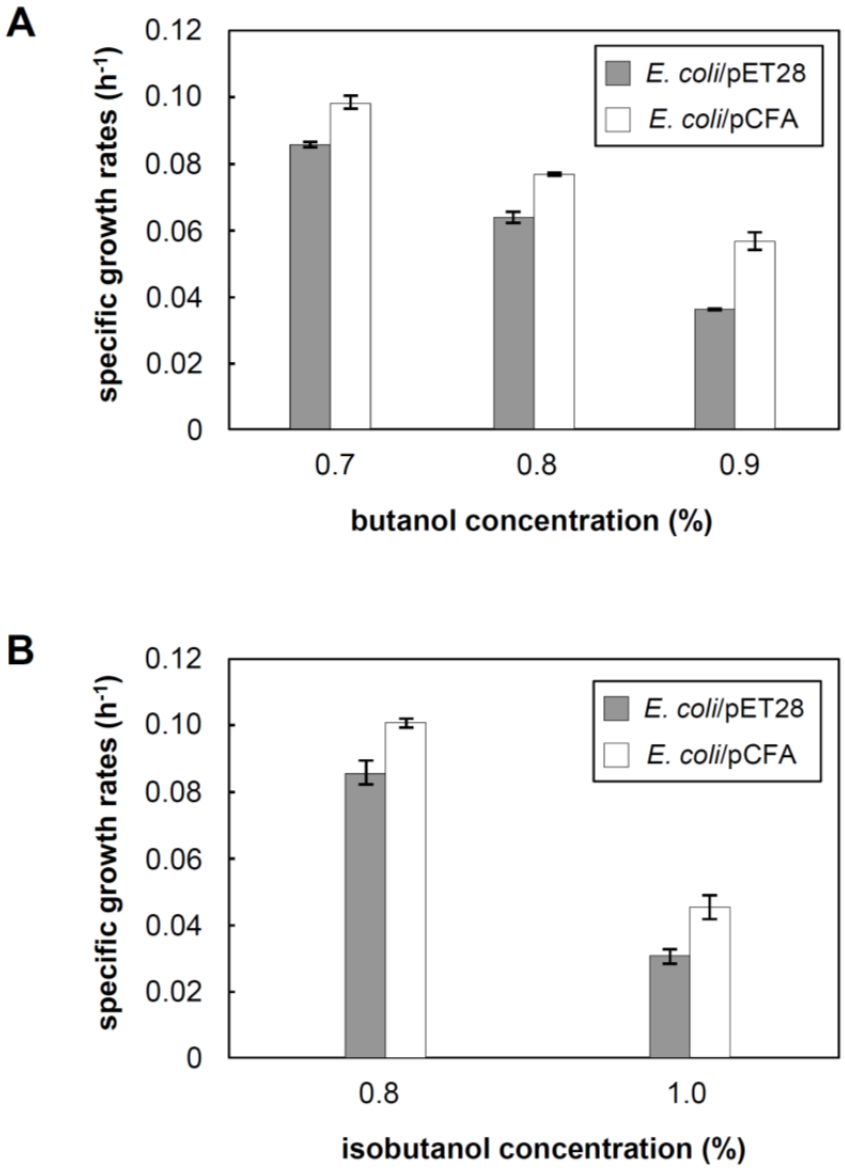

Figure 4.4. Butanol (A) and isobutanol (B) tolerance of *E. coli*/pCFA and the control strain *E. coli*/pET28 showing the differences in growth rates in the presence of butanol and isobutanol. To induce heterologous gene expression, 0.01 mM IPTG was added to each strain. The values and error bars represent the mean and SD of triplicate experiments. The growth rates of the *E. coli*/pCFA and *E. coli*/pET28 strains without solvent were equal.

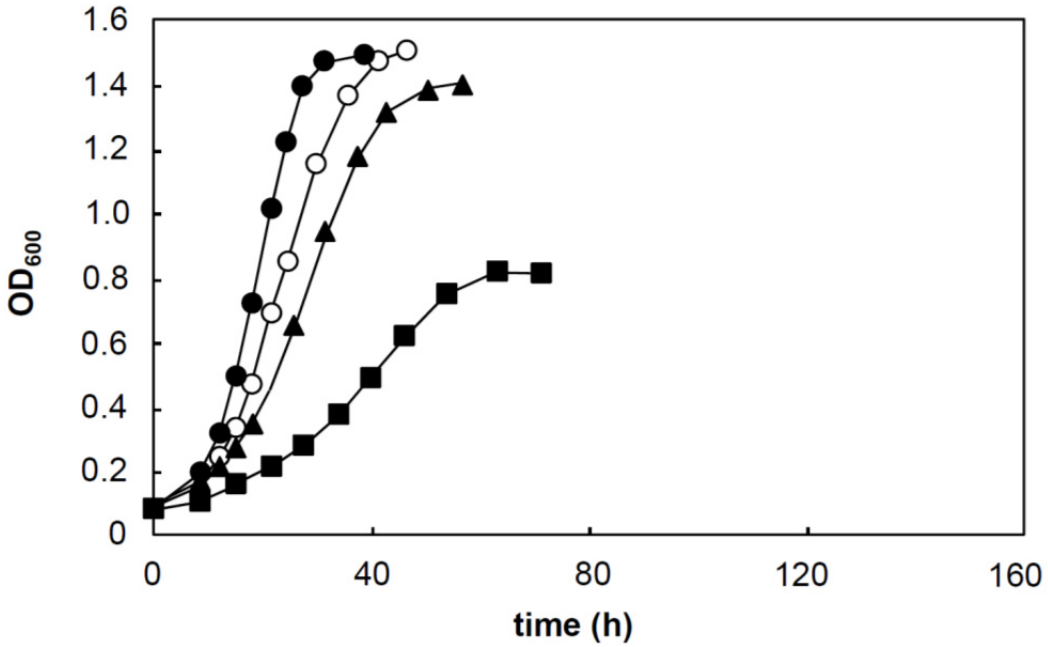

Figure 4.5. Effect of butanol on the growth of butanol-adapted cells of strain GK12. The values represent the mean of triplicate experiments. Butanol concentrations: (○) 0%, (●) 2%, (▲) 2.5%, and (■) 3.0%.

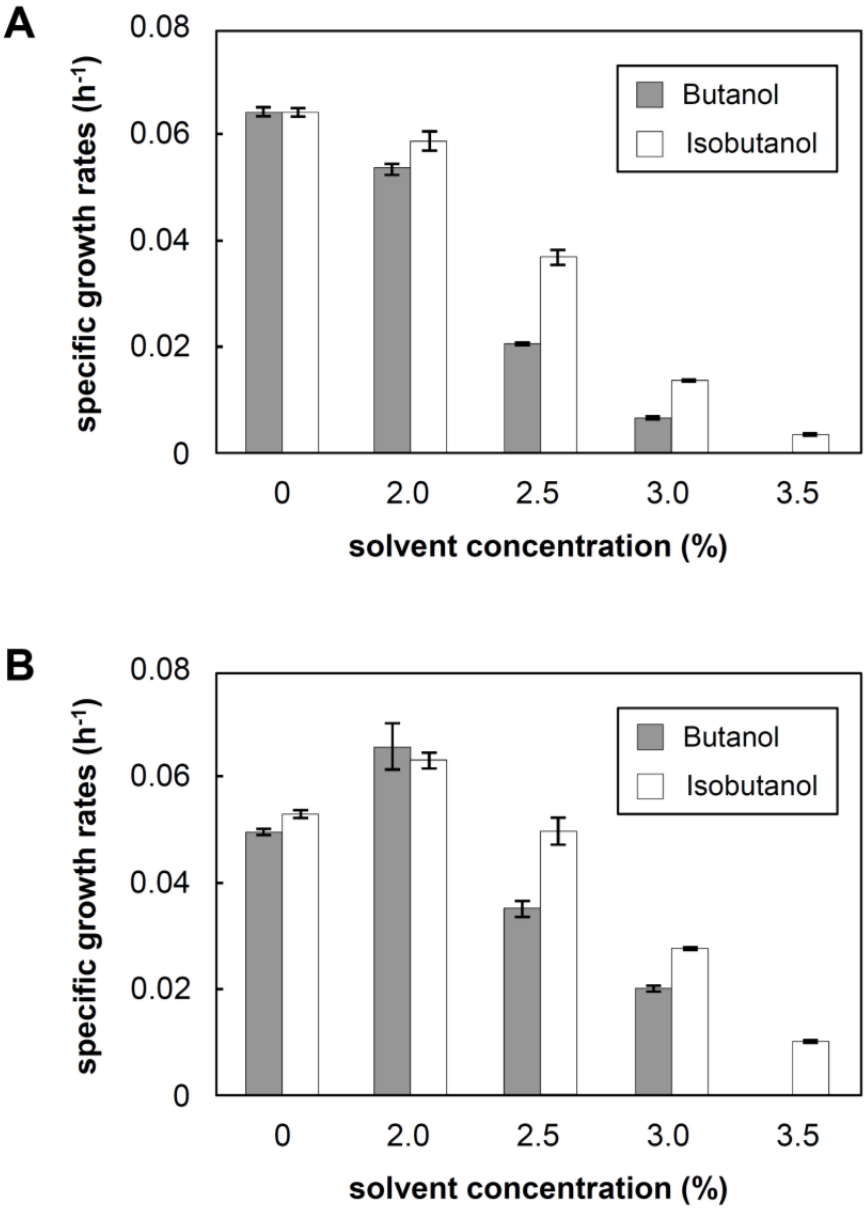

Figure 4.6. Butanol and isobutanol tolerance of strain GK12. The specific growth rates of non-adapted (A) and butanol-adapted (B) cells following different butanol or isobutanol challenges. Cell adaptation was previously achieved by 15 consecutive passages with 2.0% butanol. The values and error bars represent the mean and SD of triplicate experiments.

References

Borden JR, Papoutsakis ET. 2007. Dynamics of genomic-library enrichment and identification of solvent tolerance genes for *Clostridium acetobutylicum*. Appl. Environ. Microbiol. 73:3061-3068.

Grogan DW, Cronan JE Jr. 1997. Cyclopropane ring formation in membrane lipids of bacteria. Microbiol. Mol. Biol. Rev. 61:429-441.

Hanada S, Takaichi S, Matsuura K, Nakamura K. 2002. *Roseiflexus castenholzii* gen. nov., sp. nov., a thermophilic, filamentous, photosynthetic bacterium that lacks chlorosomes. Int. J. Syst. Evol. Microbiol. 52:187-193.

Heipieper HJ, De Bont JA. 1994. Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of fatty acid composition of membranes. Appl. Environ. Microbiol. 60:4440-4444.

Heipieper HJ, Neumann G, Cornelissen S, Meinhardt F. 2007. Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. Appl. Microbiol. Biotechnol. 74:961-973.

Huffer S, Clark ME, Ning JC, Blanch HW, Clark DS. 2011. The role of alcohols in growth, lipid composition, and membrane fluidity of yeast, bacteria, and archaea. Appl. Environ. Microbiol. 77:6400-6408.

Ingram LO, Buttke TM. 1984. Effects of alcohols on micro-organisms. Adv. Microb. Physiol. 25:253-300.

Isken S, de Bont JAM. 1996. Active efflux of toluene in a solvent-resistant bacterium. J. Bacteriol. 178:6056-6058.

Iwabuchi N, Sunairi M, Anzai H, Nakajima M, Harayama S. 2000. Relationships between

colony morphotypes and oil tolerance in *Rhodococcus rhodochrous*. Appl. Environ. Microbiol. 66:5073-5077.

Lepage C, Fayolle F, Hermann M, Vandecasteele JP. 1987. Changes in membrane lipid composition of *Clostridium acetobutylicum* during acetone-butanol fermentation: effects of solvents, growth temperature and pH. Microbiology 133:103-110.

Li J, Zhao JB, Zhao M, Yang YL, Jiang WH, Yang S. 2010. Screening and characterization of butanol-tolerant micro-organisms. Lett. Appl. Microbiol. 50:373-379.

MacDonald DL, Goldfine H. 1991. Effects of solvents and alcohols on the polar lipid composition of *Clostridium butyricum* under conditions of controlled lipid chain composition. Appl. Environ. Microbiol. 57:3517-3521.

Mykytczuk NCS, Trevors JT, Leduc LG, Ferroni GD. 2007. Fluorescence polarization in studies of bacterial cytoplasmic membrane fluidity under environmental stress. Prog. Biophys. Mol. Biol. 95:60-82.

Neumann G, Kabelitz N, Zehnsdorf A, Miltner A, Lippold H, Meyer D, Schmid A, Heipieper HJ. 2005. Prediction of the adaptability of *Pseudomonas putida* DOT-T1E to a second phase of a solvent for economically sound two-phase biotransformations. Appl. Environ. Microbiol. 71:6606-6612.

Pini CV, Bernal P, Godoy P, Ramos JL, Segura A. 2009. Cyclopropane fatty acids are involved in organic solvent tolerance but not in acid stress resistance in *Pseudomonas putida* DOT-T1E. Microb. Biotechnol. 2:253-261.

Rainey F, Andersson M, Lassila ELN, Ulrych U, Busse H, Weiss N, Mikkola R, Salkinoja-Salonen M. 2000. *Frigoribacterium faeni* gen. nov., sp. nov., a novel psychrophilic genus of the family *Microbacteriaceae*. Int. J. Syst. Evol. Microbiol. 50:355-363.

Reid G, Cuperus PL, Bruce AW, van der Mei HC, Tomeczek L, Khoury AH, Busscher HJ. 1992. Comparison of contact angles and adhesion to hexadecane of urogenital, dairy, and poultry Lactobacilli: Effect of serial culture passages. Appl. Environ. Microbiol. 58:1549-1553.

Ruhl J, Schmid A, Blank LM. 2009. Selected *Pseudomonas putida* strains can grow in the presence of high butanol concentrations. Appl. Environ. Microbiol. 75:4653-4656.

Rutherford BJ, Dahl RH, Price RE, Szmidt HL, Benke PI, Mukhopadhyay A, Keasling JD. 2010. Functional genomic study of exogenous *n*-butanol stress in *Escherichia coli*. Appl. Environ. Microbiol. 76:1935-1945.

Ting CNW, Wu J, Takahashi K, Endo A, Zhao H. 2012. Screened butanol-tolerant *Enterococcus faecium* capable of butanol production. Appl. Biochem. Biotech. 168: 1672-1680.

Toju H, Hosokawa T, Koga R, Nikoh N, Meng XY, Kimura N, Fukatsu T. 2010. "*Candidatus* Curculioniphilus buchneri," a Novel clade of bacterial endocellular symbionts from Weevils of the genus *Curculio*. Appl. Environ. Microbiol. 76:275-282.

Torres S, Pandey A, Castro GR. 2011. Organic solvent adaptation of Gram positive bacteria: Applications and biotechnological potentials. Biotechnol. Adv. 29:442-452.

Vollherbst-Schneck K, Sands JA, Montenecourt BS. 1984. Effect of butanol on lipid composition and fluidity of *Clostridium acetobutylicum* ATCC 824. Appl. Environ. Microbiol. 47:193-194.

Weber FJ, De Bont JA. 1996. Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. Biochim. Biophys. Acta 1286:225-245.

Weber FJ, Ooijkaas LP, Schemen RM, Hartmans S, De Bont JA. 1993. Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic

solvents. Appl. Environ. Microbiol. 59:3502-3504.

Yamashita S, Satoi M, Iwasa Y, Honda K, Sameshima Y, Omasa T, Kato J, Ohtake H. 2007. Utilization of hydrophobic bacterium *Rhodococcus opacus* B-4 as whole-cell catalyst in anhydrous organic solvents. Appl. Microbiol. Biotechnol. 74:761-767.

Zahir Z, Seed KD, Dennis JJ. 2006. Isolation and characterization of novel organic solvent-tolerant bacteria. Extremophiles 10:129-138.

Chapter 5

Effect of pH on butanol tolerance of an aerobic butanol-tolerant strain CM4A

Abstract

I investigated pH effect on butanol tolerance of a high butanol-tolerant bacterium, *Enterococcus faecalis* strain CM4A. The strain grew over a broad pH range (pH 4.0-12.0) and preferred alkaline pH (pH 8.0 and 10.0) in the absence of butanol. However, in the presence of butanol, strain CM4A grew better under acidic and neutral pH conditions (pH 6.0 and 6.8). Membrane fatty acid analysis revealed that the cells exposed to butanol exhibited increased cyclopropane and saturated fatty acids, which contribute to butanol tolerance of the strain by decreasing membrane fluidity, more evidently at acidic and neutral pH than at alkaline pH. Meanwhile, the strain grown under alkaline pH without butanol increased short chain fatty acids, which is involved in increasing membrane fluidity for alkaline adaptation. Such a change was not observed in the cells grown under alkaline pH with butanol. These results suggested that strain CM4A simultaneously exposed to butanol and alkali stresses was not likely able to properly adjust membrane fluidity due to the opposite response to each stress and thereby showed low butanol tolerance under alkaline pH. Our findings indicate the non-negligible impact of pH on microbial butanol tolerance, providing a new insight into efficient butanol production.

Introduction

Microbial fuel production from renewable resources has gained increased attention in view of energy security and environmental concerns. Recently, heterologous butanol production has become realized by the development of genetic and metabolic engineering (Atsumi *et al*., 2008; Nielsen *et al*., 2009), and thus butanol-tolerant bacteria have been regarded as alternative hosts overcoming growth interruption problem during butanol production. To date, a number of butanol-tolerant bacteria able to grow in the presence of greater than 2.0% (vol/vol) butanol have been reported (Knoshaug & Zhang, 2009; Ruhl *et al*., 2009; Li *et al*., 2010; Kataoka *et al*., 2011; Ting *et al*., 2012; Kanno *et al*., 2013). I isolated an aerobic butanol-tolerant bacterium *Enterococcus faecalis* strain CM4A from grease-contaminated soil (Kanno *et al*., 2013). Strain CM4A exhibited superior butanol tolerance with the ability to grow up to 3.5% butanol without assimilation and degradation.

Microbial butanol tolerance is possibly influenced by physicochemical properties such as temperature and pH for cultivation and fermentation. However, very little is known about the influence of those culture conditions to butanol tolerance, since the well-studied bacterial species grew under the limited temperature and pH conditions. Indeed, the effect of culture pH on the butanol tolerance has not been investigated, although only a few studies on the effect of temperature were reported (Knoshaug & Zhang, 2009; Baer *et al*., 1987).

In the present study, I aimed to investigate how culture pH affects microbial butanol tolerance using *E. faecalis* strain CM4A, which can grow over a broad pH range (pH 4.0-12.0). This study further characterized the response of cell membrane to both butanol and pH stresses.

Materials and Methods

Strains and culture conditions. Previously isolated butanol-tolerant strains able to grow in greater than 2.0% (vol/vol) butanol at pH 6.8, *E. faecalis* strain CM4A, *Bacillus amyloliquefaciens* strain FW5A, and *Lysinibacillus xylanilyticus* strain SK7A in the phylum *Firmicutes* (Kanno *et al*., 2013), were used in this study. Cultivation was performed at 30 °C with shaking in TGY medium consisting of (per liter) 20 g tryptone, 5 g glucose, 5 g yeast extract, 7 ml basal salt solution, 1 ml vitamin solution (Hanada *et al*., 1997), and 25 mM buffer as described below.

Growth test under different pH conditions. To assess the effect of pH, strains were cultured under various pH conditions (pH 3.0-12.5). Growth was monitored by measuring the optical density (OD_{600}) as described in the Chapter 2. The pH value of culture medium was adjusted by using the following appropriate buffer: sodium citrate buffer (pH 3.0), sodium acetate buffer (pH 4.0-5.0), potassium phosphate buffer (pH 6.0-9.0), sodium carbonate buffer (pH 10.0), and disodium hydrogen phosphate buffer plus NaOH (pH 11.0-12.5). After autoclaving, the pH value was measured by a pH meter and readjusted by adding NaOH or HCl.

Butanol tolerance assay. Growth in the presence of butanol was evaluated in the same way as mentioned above. Specific growth rate was calculated from the linear range of exponential growth. Relative growth rate was defined as the specific growth rate in the presence of

butanol relative to that without butanol.

Physiological and morphological characterization. Membrane fatty acid composition and cell morphology of strain CM4A in the late exponential growth phase were investigated in the same methods as described in the Chapter 4. Briefly, fatty acid methyl esters were extracted from whole-cell methanolyzed products into *n*-hexane and then analyzed by gas chromatography-mass spectrometry system. Cell morphology was observed by using transmission electron microscopy.

Results and Discussion

Alkali tolerance of *E. faecalis* strain CM4A. *E. faecalis* strain CM4A was cultured aerobically in a glucose-rich medium. The strain grew over a broad pH range (pH 4.0-12.0), with the highest growth rate at pH 8.0, but the highest maximum OD_{600} was obtained at pH 10.0 (Fig. 5.1). The alkali tolerance of this strain was comparable to that of obligate alkaliphiles, such as *Alkaliphilus transvaalensis* and *Bacillus marmarensis* (Takai *et al*., 2001; Denizci *et al*., 2010). Strain CM4A is thus considered as an alkali-tolerant microorganism. The ability to withstand alkaline pH up to 11.9 has been reported in the type strain of *E. faecalis* (Flahaut *et al*., 1997), which is consistent with the results of strain CM4A.

pH effect on butanol tolerance of *E. faecalis* strain CM4A. To elucidate the effect of pH

on butanol tolerance, strain CM4A was tested for butanol tolerance under various pHs. Since cells did not grow sufficiently at pH 5.0 or pH 11.0 in the presence of 2.0% butanol, the relative growth rates were compared under four different pH conditions (pH 6.0, 6.8, 8.0, 10.0) in the presence or the absence of butanol. Interestingly, the strain exhibited the highest growth rate at neutral pH (pH 6.8) in the presence of 2.0% butanol, although its optimum growth was observed at alkaline pH (pH 8.0) in the absence of butanol (Fig. 5.2). Note that this alkali-tolerant strain also showed significantly higher growth rate at pH 6.0 than at pH 10.0 in the presence of butanol ($p<0.01$ by t-test). Likewise, other butanol-tolerant strains, *B. amyloliquefaciens* strain FW5A and *L. xylanilyticus* strain SK7A in the phylum *Firmicutes* (Kanno *et al*., 2013), also showed relatively high butanol tolerance at acidic pH conditions (Fig. 5.3 and 5.4), suggesting that this phenomenon might be a common trait in butanol-tolerant strains within the *Firmicutes*.

Changes in the fatty acid compositions of *E. faecalis* strain CM4A in the absence of butanol at alkaline pH. To elucidate the mechanism of alkali tolerance, the response of the cell membrane to alkali challenge was further investigated. In particular, changes in the fatty acid composition of strain CM4A were determined under different pH conditions (pH 6.0, 6.8, 8.0, 10.0) in the absence of butanol. The cellular fatty acids of strain CM4A were mainly composed of cyclopropane, and normal saturated or unsaturated chains in the range from C14 to C20, with a high abundance of palmitic acid (C16:0) and *cis*-vaccenic acid (C18:1ω7c). No significant change was observed between acidic and neutral pH ($p>0.01$ by t-test). In contrast, the proportion of C14 chain length fatty acids (myristic acid [C14:0] and *cis*-tetradecenoic acid [C14:1ω7c]) tended to increase with increasing pH (Table 5.1). Such increase in the

proportion of short chain fatty acids are known to increase membrane fluidity (Mykytczuk *et al*., 2007) that is a key to microbial alkaline adaptation. It has been reported, indeed, that *E. coli*, *Listeria monocytogenes,* and alkaliphilic *Bacillus* spp. also enhance membrane fluidity under alkaline conditions by increasing unsaturated or branched-chain fatty acids (Clejan *et al*., 1986; Yeo *et al*., 1998; Yuk & Marshall, 2004; Giotis *et al*., 2007). Membrane fluidity is known to affect the configuration and activity of membrane proteins such as ATP synthase and various transporters (e.g., cation/proton antiporters, etc), which are regulating proton entry and cytoplasmic retention that serve to prevent intracellular alkalinization (Borochov & Shinitzky, 1976; Shinitzky *et al*., 1980; Padan *et al*., 2005). Taken together, our findings revealed that strain CM4A altered membrane fatty acid compositions for increasing its membrane fluidity and subsequently adapted to alkaline conditions.

Changes in the fatty acid compositions of *E. faecalis* strain CM4A in the presence of butanol at different pHs. The fatty acid composition of strain CM4A was also investigated at different pHs in the presence of 2% butanol. The increase in the proportion of both cyclopropane fatty acid (*cis*-11,12-methylene octadecanoic acid [cyclo-C19:0]) and total saturated fatty acids in response to butanol was found regardless of the culture pH (Table 5.1). These alterations causing a decrease in membrane fluidity likely contribute to maintain cellular integrity upon exposure to butanol as suggested in the previous studies (Huffer *et al*., 2011; Kanno *et al*., 2013; Pini *et al*., 2009). It is noteworthy that the responses of the cell membrane to butanol exposure were more evident at acidic pH (Table 5.1). For example, the cells grown at pH 6.0 contained higher proportions of cyclopropane fatty acid (3.5 ± 1.1%) and total saturated fatty acids (57.9 ± 4.7%) than the cells grown at other pH conditions. In

contrast, these changes were little observed in the cells grown at pH 8.0 and 10.0, although the proportion of saturated fatty acids was slightly high at pH 10.0 (53.2 ± 1.8%). The results suggested that the cells failed to decrease membrane fluidity at alkaline pHs in the presence of butanol, which appeared to be a main cause of lowered butanol tolerance under alkaline conditions.

Furthermore, the increase in the proportion of C14 chain length fatty acids with increasing pH, which occurred in the absence of butanol, was not observed in the cells grown with butanol (Table 5.1). This indicated that the adjustment of membrane fluidity in response to alkali challenge did not work in the presence of butanol. Thus, whereas strain CM4A would tolerate butanol by decreasing membrane fluidity, it would adapt to alkaline pH by increasing membrane fluidity (Fig. 5.5). Due to these two mechanisms working oppositely, the strain might lose the ability to adjust membrane fluidity under simultaneous exposure to both butanol and alkali stresses, hence the strain might increase cell susceptibility to butanol under alkaline conditions. Indeed, by transmission electron microscopy, the cells grown with 2.0% butanol at pH 10.0 showed irregular shape with disrupted membrane structure (Fig. 5.6). This is contrary to the cells grown in other conditions, which showed well-preserved cell structure (Fig. 5.6).

Acknowledgements

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Figures and Tables

Table 5.1. Changes in the fatty acid composition of strain CM4A*

	No solvents				2.0% butanol			
	pH 6.0	pH 6.8	pH 8.0	pH 10.0	pH 6.0	pH 6.8	pH 8.0	pH 10.0
C14:0	4.2 ± 0.5	4.6 ± 1.3	6.5 ± 1.9	6.9 ± 0.7	5.8 ± 0.3	5.0 ± 1.9	5.8 ± 0.1	4.6 ± 1.9
C14:1ω7c	0.1 ± 0.2	0.2 ± 0.2	0.5 ± 0.2	0.6 ± 0.1	0.3 ± 0.3	0.3 ± 0.3	0.6 ± 0.1	0.2 ± 0.2
C16:0	41.5 ± 1.1	41.6 ± 1.0	39.6 ± 3.3	39.2 ± 2.9	49.4 ± 4.1	44.8 ± 1.8	41.0 ± 1.9	43.5 ± 1.7
C16:1	0.8 ± 0.7	1.2 ± 1.1	0.9 ± 0.9	0.9 ± 0.9	1.0 ± 1.0	1.9 ± 2.0	1.3 ± 1.5	0.8 ± 0.7
C16:1ω7c	4.4 ± 1.3	6.1 ± 2.0	6.5 ± 0.8	7.5 ± 1.1	6.4 ± 1.2	7.2 ± 2.6	7.5 ± 1.8	6.9 ± 4.4
C18:0	2.2 ± 0.6	1.5 ± 0.2	1.3 ± 0.5	0.9 ± 0.2	2.7 ± 1.2	1.6 ± 0.6	1.2 ± 0.6	5.1 ± 3.6
C18:1ω7c	46.3 ± 2.4	44.9 ± 4.8	44.3 ± 3.0	43.8 ± 1.7	30.4 ± 0.9	37.6 ± 4.9	42.0 ± 4.1	38.6 ± 2.8
cyclo-C19:0	0.6 ± 0.8	ND	0.4 ± 0.7	ND	**3.5 ± 1.1**	**1.3 ± 0.6**	**0.7 ± 0.2**	**0.3 ± 0.3**
C20:1	0.0 ± 0.1	ND	0.1 ± 0.1	ND	0.5 ± 0.9	0.1 ± 0.2	ND	ND
Total C14	**4.3 ± 0.5**	**4.8 ± 1.5**	**6.9 ± 2.1**	**7.5 ± 0.8**	**6.1 ± 0.6**	**5.4 ± 2.3**	**6.4 ± 0.1**	**4.8 ± 1.8**
Total saturated fatty acids	47.9 ± 0.6	47.6 ± 1.6	47.4 ± 4.7	47.1 ± 3.3	**57.9 ± 4.7**	**51.5 ± 1.6**	**47.9 ± 1.4**	**53.2 ± 1.8**

* Each fatty acid composition is described as a percentage of the whole-cell lipids. The values are the means ± standard deviations of three independent measurements. The double-bond positions of C16:1 and C20:1 were not identified. “Total saturated fatty acids” is the sum of myristic acid (C14:0), palmitic acid (C16:0), and stearic acid (C18:0). Abbreviations: X:YωZc, a fatty acid containing X carbon atoms with Y double bonds at position Z, counted from the methyl terminus in the *cis* configuration; cyclo-C19:0, *cis*-11,12-methylene octadecanoic acid; ND, not detected. The bold values are discussed in the text.

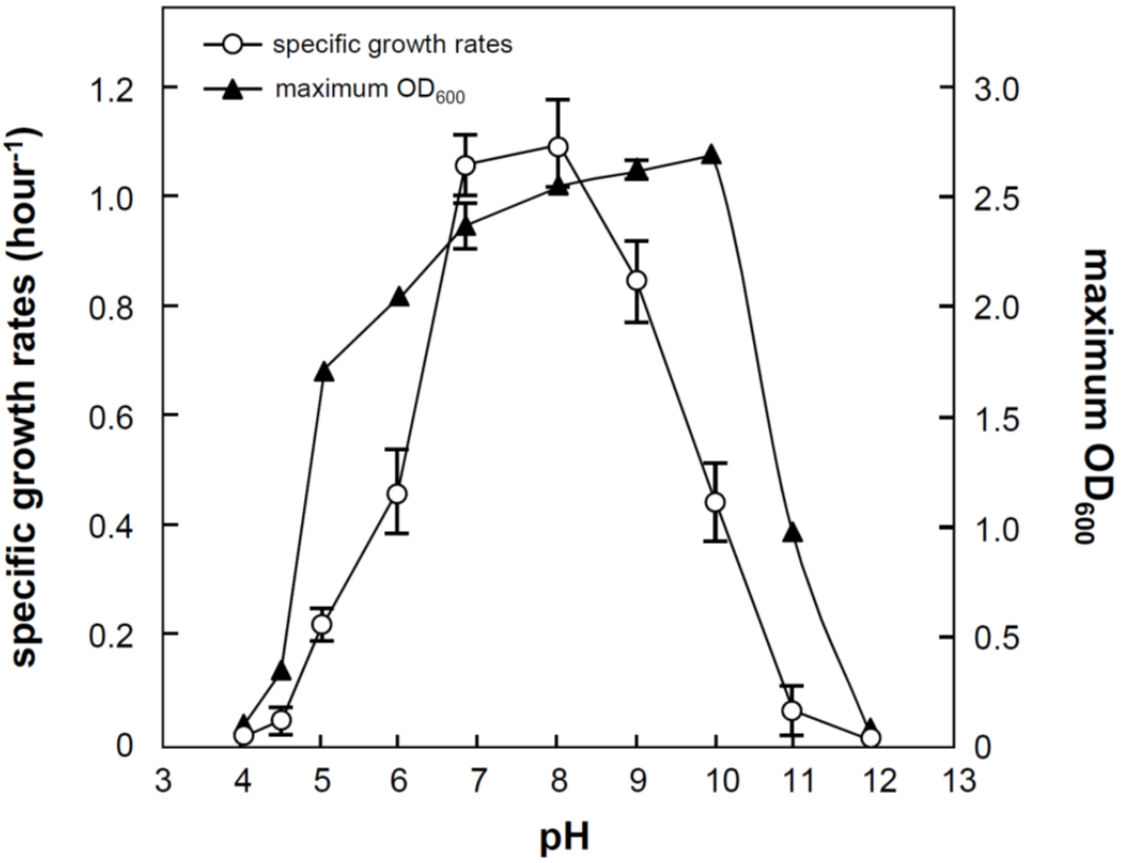

Figure 5.1. Growth characteristics of *E. faecalis* strain CM4A at various pHs. Effect of pH on growth as determined by specific growth rates (○) and maximum OD_{600} (▲) in the absence of butanol. The values and error bars represent the mean and SD of triplicate experiments.

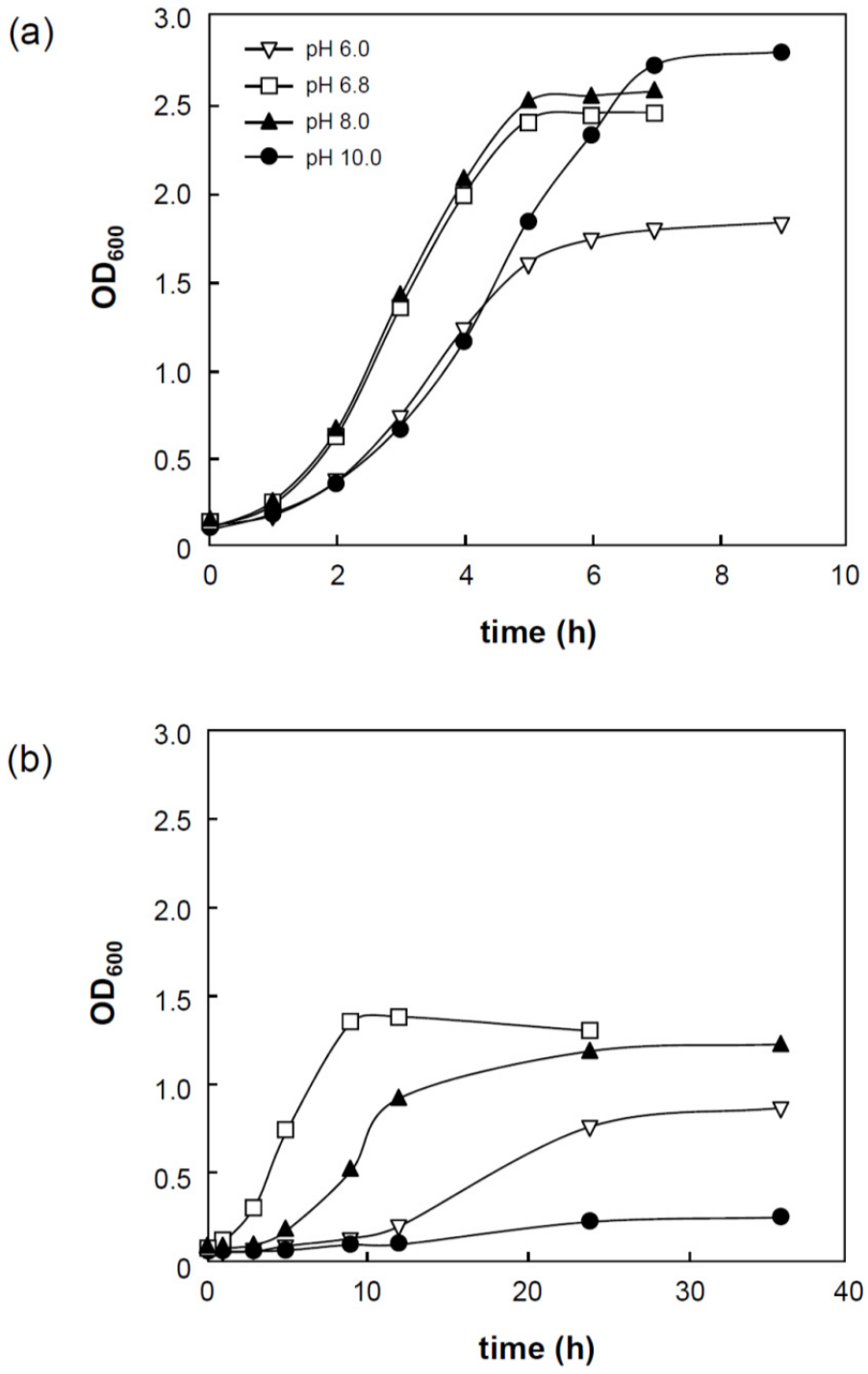

Figure 5.2. Butanol tolerance assay of *E. faecalis* strain CM4A. Growth curves of strain CM4A in the absence (a) and presence (b) of 2.0% butanol under different pH conditions. The values represent the mean of triplicate experiments.

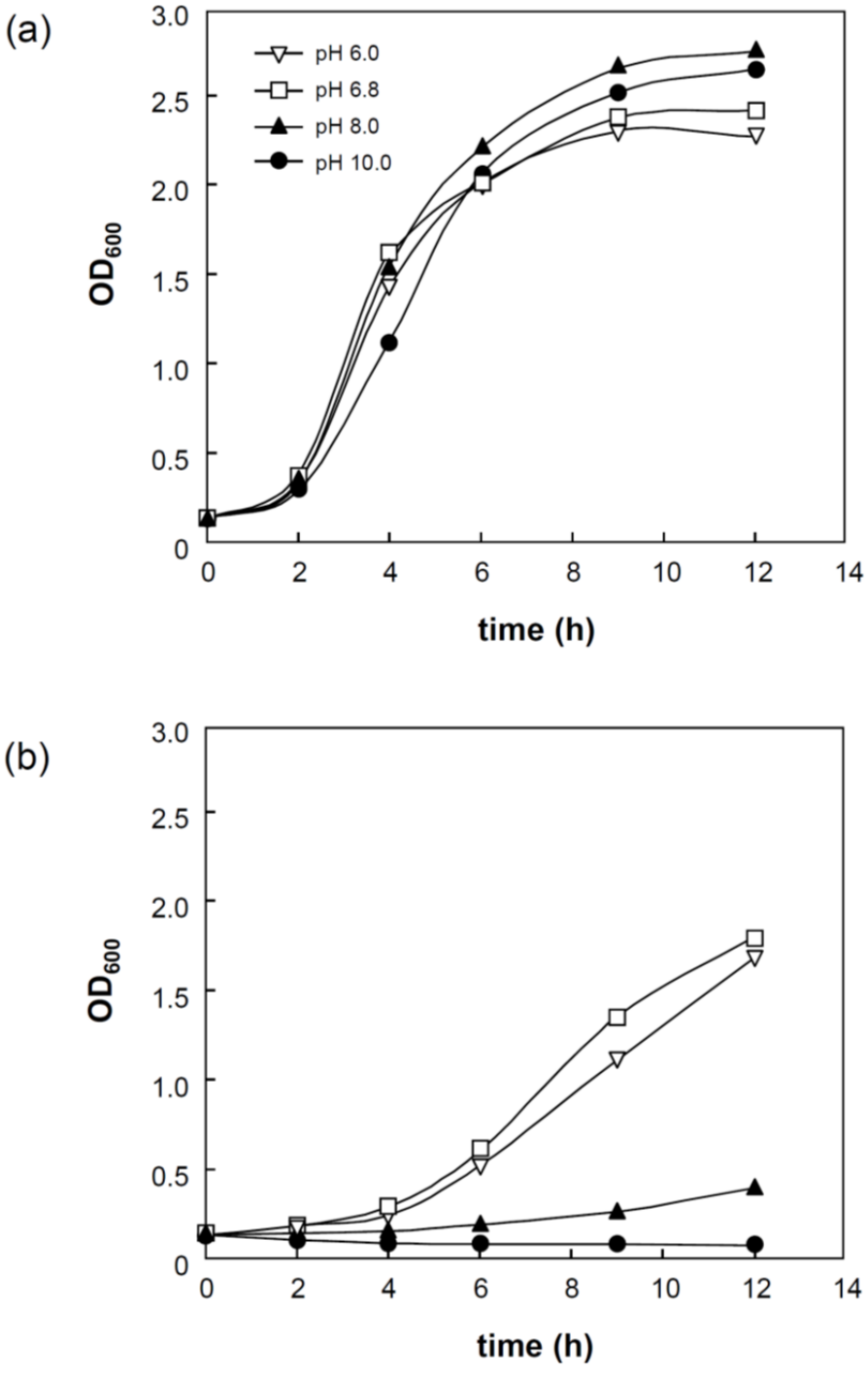

Figure 5.3. Butanol tolerance assay of *Bacillus amyloliquefaciens* strain FW5A. Growth curves of strain FW5A in the absence **(a)** and presence **(b)** of 2.0% butanol under different pH conditions. The values represent the mean of triplicate experiments.

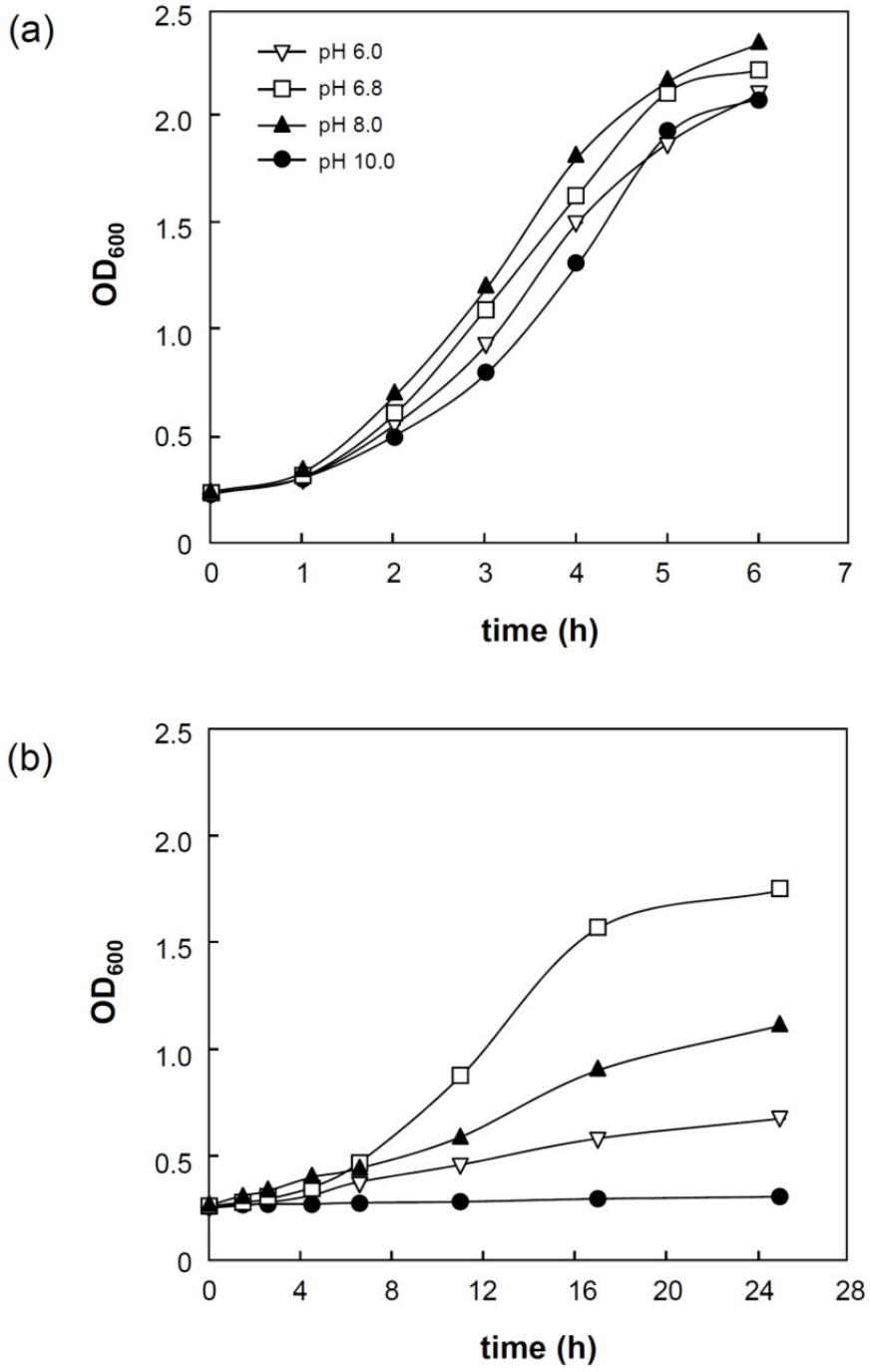

Figure 5.4. Butanol tolerance assay of *Lysinibacillus xylanilyticus* strain SK7A. Growth curves of strain SK7A in the absence **(a)** and presence **(b)** of 2.0% butanol under different pH conditions. The values represent the mean of triplicate experiments.

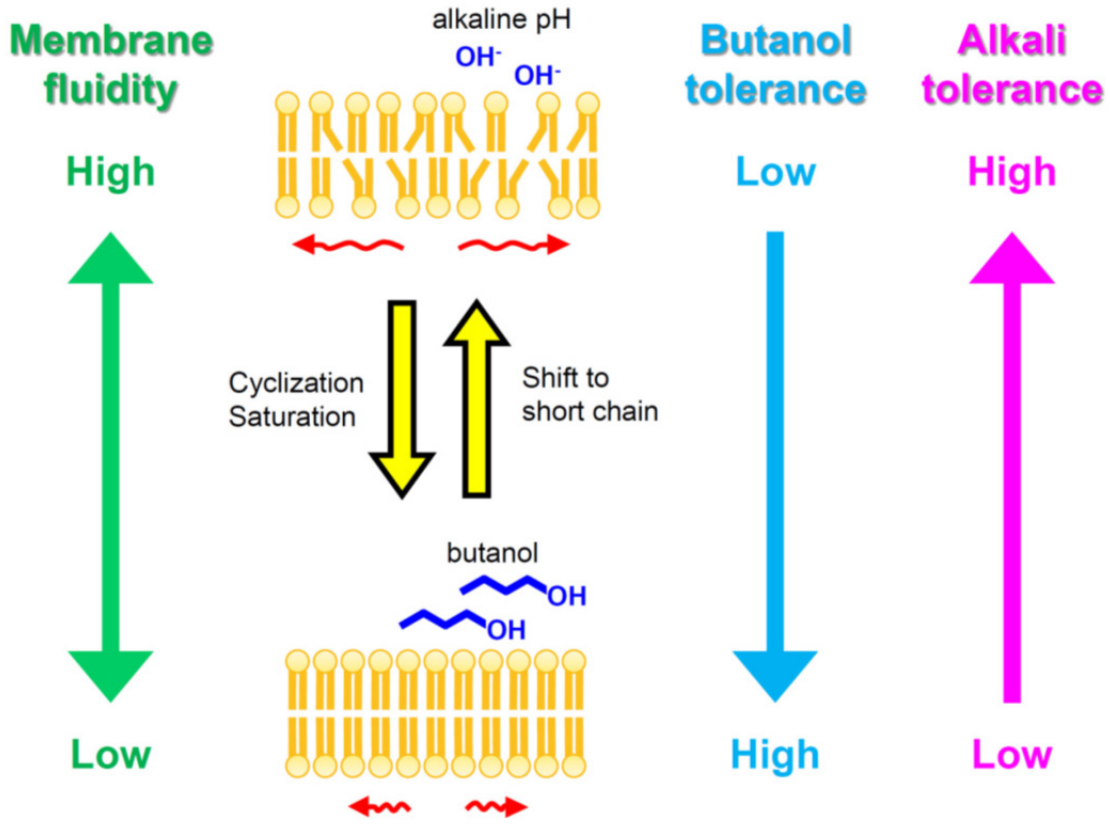

Figure 5.5. Schematic representation of membrane fluidity adjustment in *E. faecalis* strain CM4A. The opposite mechanisms involved in membrane fluidity adjustment behind butanol and alkali tolerance. The strain alters the proportion of membrane fatty acid components (i.e. cyclization, saturation, or shift to short chain) in response to butanol exposure or alkaline pH.

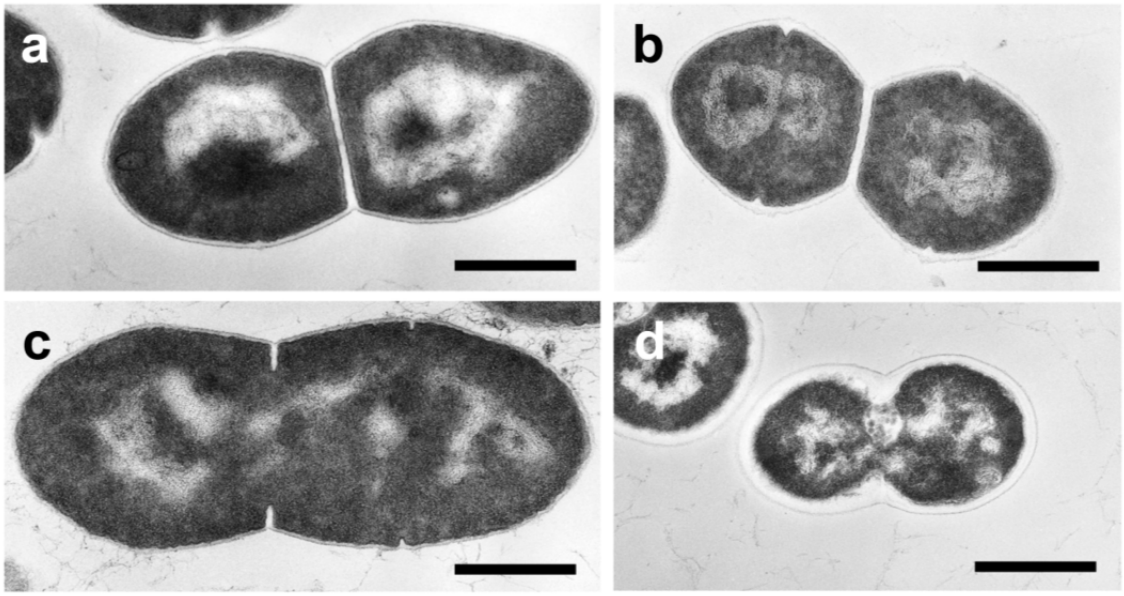

Figure 5.6. Cell morphology of *E. faecalis* strain CM4A. Transmission electron micrographs of the cell grown at pH 6.8 (a, b) or pH 10.0 (c, d) in the absence (a, c) and presence (b, d) of 2.0% butanol. Bars, 0.5 μm.

References

Atsumi S, Hanai T, Liao JC. 2008. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature. 451: 86-89.

Baer SH, Blaschek HP, Smith TL. 1987. Effect of butanol challenge and temperature on lipid composition and membrane fluidity of butanol-tolerant *Clostridium acetobutylicum*. Appl. Environ. Microbiol. 53:2854-2861.

Borochov H, Shinitzky M. 1976. Vertical displacement of membrane proteins mediated by changes in microviscosity. Proc. Natl. Acad. Sci. U S A. 73:4526-4530.

Clejan S, Krulwich TA, Mondrus KR, Setoyoung D. 1986. Membrane lipid composition of obligately and facultatively alkalophilic strains of *Bacillus* spp. J. Bacteriol. 168:334-340.

Denizci AA, Kazan D, Erarslan A. 2010. *Bacillus marmarensis* sp. nov., an alkaliphilic, protease-producing bacterium isolated from mushroom compost. Int. J. Syst. Evol. Microbiol. 60:1590-1594.

Flahaut S, Hartke A, Giard JC, Auffray Y. 1997. Alkaline stress response in *Enterococcus faecalis*: adaptation, cross-protection, and changes in protein synthesis. Appl. Environ. Microbiol. 63:812-814.

Giotis ES, McDowell DA, Blair IS, Wilkinson BJ. 2007. Role of branched-chain fatty acids in pH stress tolerance in Listeria monocytogenes. Appl. Environ. Microbiol. 73:997-1001.

Hanada S, Kawase Y, Hiraishi A, Takaichi S, Matsuura K, Shimada K, Nagashima KVP. 1997. *Porphyrobacter tepidarius* sp. nov., a moderately thermophilic aerobic photosynthetic bacterium isolated from a hot spring. Int. J. Syst. Evol. Microbiol.

47:408-413.

Huffer S, Clark ME, Ning JC, Blanch HW, Clark DS. 2011. The role of alcohols in growth, lipid composition, and membrane fluidity of yeast, bacteria, and archaea. Appl. Environ. Microbiol. 77:6400-6408.

Kanno M, Katayama T, Tamaki H, Mitani Y, Meng XY, Hori T, Narihiro T, Morita N, Hoshino T, Yumoto I, Kimura N, Hanada S, Kamagata Y. 2013. Isolation of butanol- and isobutanol-tolerant bacteria and physiological characterization of their butanol tolerance. Appl. Environ. Microbiol. 79:6998-7005.

Kataoka N, Tajima T, Kato J, Rachadech W, Vangnai A. 2011. Development of butanol-tolerant *Bacillus subtilis* strain GRSW2-B1 as a potential bioproduction host. AMB Express. 1:1-11.

Knoshaug EP, Zhang M. 2009. Butanol tolerance in a selection of microorganisms. Appl. Biochem. Biotechnol. 153:13-20.

Li J, Zhao JB, Zhao M, Yang YL, Jiang WH, Yang S. 2010. Screening and characterization of butanol-tolerant micro-organisms. Lett. Appl. Microbiol. 50:373-379.

Mykytczuk NCS, Trevors JT, Leduc LG, Ferroni GD. 2007. Fluorescence polarization in studies of bacterial cytoplasmic membrane fluidity under environmental stress. Prog. Biophys. Mol. Biol. 95:60-82.

Nielsen DR, Leonard E, Yoon SH, Tseng HC, Yuan C, Prather KLJ. 2009. Engineering alternative butanol production platforms in heterologous bacteria. Metab. Eng. 11:262-273.

Padan E, Bibi E, Ito M, Krulwich TA. 2005. Alkaline pH homeostasis in bacteria: new insights. Biochim. Biophys. Acta. 1717:67-88.

Pini CV, Bernal P, Godoy P, Ramos JL, Segura A. 2009. Cyclopropane fatty acids are

involved in organic solvent tolerance but not in acid stress resistance in *Pseudomonas putida* DOT-T1E. Microbial. Biotechnol. 2:253-261.

Ruhl J, Schmid A, Blank LM. 2009. Selected *Pseudomonas putida* strains can grow in the presence of high butanol concentrations. Appl. Environ. Microbiol. 75:4653-4656.

Shinitzky M, Borochov H, Wilbrandt W. 1980. Lipid fluidity as a physiological regulator of membrane transport and enzyme activities. In Membrane Transport in Erythrocytes. Edited by Lassen UV, With HH, Wiesing IO. Copenhagen: Munksgaard; 91-102.

Takai K, Moser DP, Onstott TC, Spoelstra N, Pfiffner SM, Dohnalkova A, Fredrickson JK. 2001. *Alkaliphilus transvaalensis* gen. nov., sp. nov., an extremely alkaliphilic bacterium isolated from a deep south african gold mine. Int. J. Syst. Evol. Microbiol. 51:1245-1256.

Ting CNW, Wu J, Takahashi K, Endo A, Zhao H. 2012. Screened butanol-tolerant *Enterococcus faecium* capable of butanol production. Appl. Biochem. Biotechnol. 168:1672-1680.

Yeo IH, Han SK, Yu JH, Bai DH. 1998. Isolation of novel alkalophilic *Bacillus alcalophilus* subsp. YB380 and the characteristics of its yeast cell wall hydrolase. J. Microbiol. Biotechnol. 8:501-508.

Yuk HG, Marshall DL. 2004. Adaptation of *Escherichia coli* O157:H7 to pH alters membrane lipid composition, verotoxin secretion and resistance to simulated gastric fluid acid. Appl. Environ. Microbiol. 70:3500-3505.

Chapter 6

Free Fatty Acids Accumulation of an Anaerobic Butanol-Tolerant Strain GK12 Caused by Butanol Exposure

Abstract

Medium- and long-chain fatty acids are present in organisms in esterified forms that serve as cell membrane constituents and storage compounds. A large number of organisms are known to accumulate lipophilic materials as a source of energy and carbon. I found a bacterium, designated strain GK12, intrinsically accumulates free fatty acids (FFAs) as intracellular droplets without exhibiting cytotoxicity. Strain GK12 is an obligatory anaerobic, taxonomically-novel lactic acid bacterium that was screened as butanol-tolerant bacterium. Saturated fatty acids with carbon chain lengths of 14, 16, 18 and 20 were produced from glucose under stress conditions, including higher-than-optimum temperatures and the presence of organic solvents that affect cell membrane integrity. FFAs were produced at levels corresponding to up to 25% (w/w) of the dry cell mass. On the basis of the results, one possible explanation for FFA accumulation is the imbalance between excess membrane fatty acid biosynthesis due to homeoviscous adaptation and limited β-oxidation activity due to anaerobic growth involving lactic acid fermentation, at which FFAs might prove a sink for reducing equivalents. FFA droplets were not further utilized as an energy and carbon source, even under conditions of starvation. A naturally occurring bacterium that accumulates significant amounts of long-chain FFAs with non-cytotoxicity would provide useful strategies for microbial biodiesel production.

Introduction

Fatty acids with medium and long aliphatic tails are ubiquitously found in living organisms as cell membrane components in the form of ester- or ether-linked lipids. In response to environmental changes, such as variations in temperature, pH and salinity, microorganisms alter the physicochemical properties of their membrane lipids to maintain membrane fluidity and integrity in a response referred to as homeoviscous adaptation (Sinensky, 1974). Strategies for adjusting membrane fluidity usually entail alterations of the membrane fatty acid composition, including saturation, *cis* and *trans* isomerization, chain length modification, iso- and anteiso-branching and cyclization, as the biophysical properties of the cell membrane are determined mainly by fatty acid structures (Zhang & Rock, 2008; Mykytczuk *et al*., 2007).

Storage lipid compounds are another class of vital fatty acid-derived compounds. Many eukaryotic and prokaryotic organisms store large amounts of lipophilic compounds in the form of intracellular droplets and use them as energy and carbon sources (Zweytick *et al*., 2000; Wältermann & Steinbüchel, 2005). The major lipophilic storage compounds that occur naturally in eukaryotes are fatty acyl lipids such as triacylglycerols (TAGs) and wax esters (WEs) (Zweytick *et al*., 2000). Although a few bacterial species accumulate these neutral lipids, liner polyesters, specifically polyhydroxyalkanoates (PHAs), are the most common in prokaryotic storage compounds (Wältermann & Steinbüchel, 2005; Alvarez & Steinbüchel, 2002). These lipophilic materials are ideal for energy storage because of their minimal space requirements, higher caloric values compared to proteins or carbohydrates and lack of cellular toxicity (Manilla-Pérez *et al*., 2010). Besides a store of carbon and energy, these lipophilic compounds serve as a sink for reducing equivalents in microorganisms (Alvarez &

Steinbüchel, 2002; Dawes & Senior, 1973). In contrast, nonesterified fatty acids (i.e., free fatty acids (FFAs)) are toxic due to their amphiphilic nature (Desbois & Smith, 2010). Indeed, endogenously produced FFAs dramatically reduce cell viability in metabolically engineered *Escherichia coli* (Lennen *et al*., 2011). FFAs have been found to constitute a minor fraction of lipid droplets (Leman, 1997; Alvarez *et al*., 1996), but no living organisms that naturally store only large amounts of intracellular FFAs have been encountered thus far.

Previously, obligate anaerobic bacterium strain GK12 was isolated as a butanol-tolerant bacterium, and characterized further to elucidate mechanism behind high butanol tolerance (Kanno *et al*., 2013). Based on the phenotypic, phylogenetic and chemotaxonomic properties, the strain was also described as a new genus and species in the family *Erysipelotrichaceae* as a type strain, with the name *Catenisphaera adipataccumulans* gen. nov., sp. nov (Kanno *et al*., 2015).

Here, I report the further characterization of strain GK12 that accumulates FFAs by exposure to butanol or heat stresses. I also investigated its unique nature with regard to homeoviscous adaptation.

Materials and Methods

Cultivation of GK12 under stress conditions. Cultivation was conducted anaerobically at 37 °C under an atmosphere of N_2/CO_2 (80:20, v/v). The medium was prepared based on Widdel medium (Pfennig *et al*., 1981) with the following composition (per liter): 10 g glucose, 5 g yeast extract, 0.53 g NH_4Cl, 0.14 g KH_2PO_4, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.15 g $CaCl_2 \cdot 2H_2O$, and 2.52 g $NaHCO_3$ supplemented with 0.5 g $Na_2S \cdot 9H_2O$, 0.5 g cysteine·HCl, 1 ml trace-elements (Sekiguchi *et al*., 2000) and 2 ml vitamin solution (Sekiguchi *et al*., 2000).

The effects of environmental stresses on accumulated free fatty acid (FFA) levels and compositions were determined under the following culture conditions: solvent stress (medium amended with 1.0%, 2.0%, 2.5% butanol, 2.0% isobutanol, 5.0% ethanol, 0.6% hexane or 0.2% toluene (v/v)); osmotic stress (medium supplemented with 0.1 M NaCl); heat stress (culturing at 45 °C). Cells grown at 37 °C in the absence of organic solvents and NaCl were used as a control. Under all of these stress conditions, the cell population densities reached levels comparable to those observed in 2% butanol cultures. Cells in the mid-exponential phase of growth were harvested and subjected to lipid analysis.

To examine the utilization of accumulated FFAs and cell survival, cells growing in late exponential phase at 45 °C were harvested, washed and subsequently cultured at 37 °C in fresh medium containing yeast extract (1 g l^{-1}), but not glucose. No increase in turbidity was observed under these conditions. The numbers of viable cells were counted in triplicate in the starting cultures and after 20 days in culture based on the most probable number technique. Additionally, the contents of FFAs were measured in the starting cultures and after 7 days in culture as described above. Cells grown at 37 °C were used as controls.

DNA extraction and draft genome sequence analysis. Genomic DNA was extracted using ISOPLANT II (NIPPON GENE, Tokyo, Japan). A draft genome sequence was obtained via pyrosequencing using the 454 Life Sciences GS FLX Titanium platform (Roche, Basel,

Switzerland), followed by Sanger sequencing for gap closure at the Hokkaido System Science Co., Ltd. (Sapporo, Japan).

Transmission electron microscopy. Cells in the late exponential phase of growth were treated as described in the Chapter 4, and viewed with a Hitachi H-7600 electron microscope operated at 80 kV.

Metabolite and lipid analysis. The levels of glucose, organic acids and butanol in the culture supernatant were quantified via high-performance liquid chromatography (HPLC) using an apparatus equipped with a cation exchange column and a refractive index detector. The cells were stained with Sudan Black (Thakur *et al*., 1988) to determine whether intracellular droplets were lipophilic.

Cellular lipids were extracted following the method of Bligh and Dyer (Bligh & Dyer, 1959). To avoid the degradation of lipids by lipase during the experiments, the cells were treated with boiled water prior to extraction. This heat treatment had no effect on the content of FFAs. The total lipids were separated into fatty acids, polar lipids and neutral lipids via thin layer chromatography (TLC) using silica gel plates with a solvent system composed of *n*-hexane/diethyl ether/acetic acid (70:30:2, by vol.). In the TLC analyses, 1,2-dipalmitoyl-*sn*-glycerol, palmitic acid, stearic acid, trimyristin, and phosphatidylglycerol were used as reference standards. All chemicals were purchased from Sigma-Aldrich. Note that no wax ester was applied to TLC plate as a reference standard because wax esters show the highest R*f* value among all reference standards employed in this solvent system (Hernandez *et al*., 2008). Each spot was visualized using 0.01% (w/v) primeline in 80% (v/v) acetone in water under UV irradiation. Spots corresponding to fatty acids and polar lipids were scraped off the plate and subsequently methanolyzed with 10% (v/v) acetyl chloride in methanol at 100 °C for 3 hours.

Fatty acid methyl esters (FAMEs) were identified and quantified via gas chromatography-mass spectrometry (GC-MS) and GC, respectively, by the same methods as described in the Chapter 4. Standard curves for fatty acids quantification were generated based on serial dilutions of the FAME reference standard (GL Sciences, Tokyo, Japan). PHAs were detected following extraction from cells via GC-MS analysis as described by Brandl *et al* (Brandl *et al*., 1988).

The extent of the relative increase in the chain length of cell membrane phospholipid-derived fatty acids (PLFAs) in a given culture condition was expressed as the weighted average of the alkyl chain lengths and calculated using the following equation: $\Sigma\, n\, P_{n:0} / \Sigma\, n\, PC_{n:0}$, where n is the number of carbons in PLFAs, P is the proportion of PLFAs in the cells grown under stress conditions, and PC is the proportion of the PLFAs under the no-stress condition (i.e., cultured at 37 °C in the absence of organic solvents and NaCl).

Purification of FFA droplets. Droplets were purified via density gradient centrifugation according to Preusting *et al* (Preusting *et al*., 1993). Cells in the late exponential phase of growth were washed twice and then physically disrupted through bead beating (Yasui Kikai, Osaka, Japan). Cell-free extracts were fractionated using a discontinuous density gradient composed of 0.2, 0.4, 0.6, 1.0 and 1.5 M sucrose in 10 mM Tri-HCl (pH 7.5). The collected droplets were washed twice with 10 mM Tri-HCl and subjected to lipid extraction.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The molecular mass of the purified sample was determined via MALDI-TOF MS using the Autoflex II TOF/TOF system with Flex Control software (Bruker Daltonics) (Matsuo, 2011). The sample was dissolved in chloroform-methanol (2:1, v/v) at a concentration of 1.0 μg μl^{-1}, and 2,5-dihydroxybenzoic acid or α-cyano-4-hydroxy-cinnamic acid dissolved in 0.1% TFA in H_2O/CH_3CN (1:1, v/v) at a concentration of 10 μg μl^{-1} was

used as a matrix. One microliter of the matrix was spotted on a sample plate (MTP 384 target plate ground steel TF, Bruker Daltonics) and subsequently mixed with an equal amount of the sample. Following co-crystallization, MALDI-TOF MS spectra were acquired in positive ion and reflectron mode.

Quantitative reverse transcription PCR (qRT-PCR). SYBR Green-based real-time PCR was performed for the quantification of gene expression. Draft sequence data for the GK12 genome were searched for target genes encoding the enzymes responsible for the primary or essential reactions involved in fatty acid metabolism (Table 6.1). Putative ORFs were determined using BLASTX (Altschul *et al*., 1990). Total RNA was extracted from cells in the mid-exponential phase of growth using ISOGEN-LS (NIPPON GENE). Complementary DNA was generated using the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa). cDNA amplification was carried out using Power SYBR Green PCR master mix and the indicated primers (Table 6.2) with the ABI PRISM 7900 sequence detection system (Applied Biosystems). Standard curves for quantification were determined based on serial dilutions of total cDNA at known concentrations. All reactions including the non-template control were performed in triplicate in three independent experiments. The presence of a single PCR product without any nonspecific amplicons was confirmed via agarose gel and melting curve analyses. The level of gene expression across samples was normalized based on the expression of the GAPDH housekeeping gene (*gapA*). Glucose consumption was constant regardless of the presence of butanol in the cultures.

Nucleotide sequence accession numbers. The sequence information for the GK12 strain has been deposited at GenBank under accession numbers AB537978 and AB745670 through AB745675.

Results and Discussion

Identification and characterization of intracellular droplets. An obligate anaerobic, butanol-tolerant bacterium, designated GK12, was isolated from a methanogenic reactor (Kanno *et al*., 2013). GK12 is a mesophilic heterotroph that ferments glucose, mainly producing lactate. GK12 neither assimilates nor degrades butanol. Transmission electron microscopy revealed that strain GK12 accumulated electron transparent droplets (later identified as FFAs) within the cytoplasm (Fig. 6.1). These droplets became more evident in the cells grown with 2% butanol (Fig. 6.1b).

The droplets that accumulated within the cells were sudanophilic, suggesting that they consisted of lipophilic materials. TLC and GC-MS analyses of the crude cellular lipid extracts revealed the absence of previously reported lipophilic storage compounds, such as TAGs, WEs and PHAs, but the presence of FFAs (Fig. 6.2). The size of the TLC spots corresponding to FFAs increased significantly after culturing the cells with 2% butanol (Fig. 6.2, lanes 1 and 2), in agreement with the electron microscopy observations. Following density gradient centrifugation of cell-free extracts, a layer of white fluid appeared in cells grown with butanol, which was nearly invisible without butanol (Fig. 6.3). GC-MS and MALDI-TOF MS revealed that this fraction was comprised of myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) and arachidic acid (C20:0), demonstrating that GK12 cells accumulate droplets consisting of straight-chain saturated fatty acids. FFAs were not detected in the culture supernatants.

The FFA levels and carbon chain lengths increased in the presence of butanol in a dose-dependent manner (Fig. 6.4 and Fig. 6.5a, respectively). When grown in the presence of 2.5% butanol, GK12 cells produced an approximately 13-fold higher FFA content associated with a relative increase in C18:0 and C20:0 and a decrease in C14:0 compared to the bacterium cultured without butanol. Significant accumulation of FFAs was also induced by culturing the bacterium with 2-methyl-1-propanol (isobutanol) and toluene and by heat stress

(cultivation at 45 °C), whereas this phenomenon was not observed in the cultures subjected to ethanol, hexane or osmotic stress (Fig. 6.6). GK12 cells accumulated FFAs consisting of C18:0 (55% of total FFAs), C16:0 (23%) and C20:0 (22%) at levels up to 25% of dry cell weight (w/w), showing a yield of 9.8% (w/w) of the total glucose consumed when grown in stationary phase in batch cultures at 45 °C. There has been, so far, no anaerobic bacterium that produces significant amounts of fatty acid-derived lipids such as TAGs or WEs. Fatty acid biosynthesis is an energy intensive process and the excess production over the demand for membrane fatty acid seems to be unusual for cells growing under obligatory anaerobic conditions.

The influence of FFA accumulation on cell viability was examined in cultures performed under non-growth conditions. The numbers of viable FFA-accumulating and control cells, grown at 45 °C and 37 °C, respectively, were compared using the most probable number method. The cell numbers in the two groups (cells grown at 45 °C and 37 °C) was not differ initially or after 20 days in culture (10^9 and 10^7 cells ml^{-1} at zero time and after 20 days, respectively), demonstrating that intracellular FFAs do not exhibit cytotoxicity in GK12 cells. Transmission electron microscopy clearly showed that FFAs formed multiple droplets that were segregated from the cytoplasm (Fig. 6.1). This segregation of FFAs may be due to an amphiphilic layer consisting of phospholipids and proteins preventing their integration into membrane lipid bilayers and undesired chemical reactions with other cellular constituents (Zweytick *et al*., 2000; Athenstaedt & Daum, 2006), resulting in the non-toxicity of these droplets in GK12 cells.

Relationship between FFA accumulation and environmental stress. The fatty acid components of the accumulated FFAs were also found in PLFAs and consisted of saturated fatty acids and 1,1-dimethoxy alkanes with carbon chain lengths of 14, 16 and 18. Similar to the droplet FFAs, the PLFAs also exhibited butanol dose-dependent increases in alkyl chain length (Fig. 6.5b). Because the cytoplasmic membrane is the main target of organic solvents,

bacteria exhibit homeoviscous adaptation in response to solvent exposure (Ingram, 1976; Sikkema *et al*., 1995). In the case of GK12 cells, we hypothesized that the relative increase in PLFA chain length occurred to maintain the integrity of the membrane in response to butanol, thus revealing the extent of environmental stress affecting the cell membrane. When the degree of the relative increase in PLFA chain length in a given culture condition relative to the no-stress condition was expressed as the weighted average (see Methods), a significant correlation was found between FFA accumulation and the weighted average (r=0.86, $p<0.001$; Fig. 6.6). The consistency of the increases in both PLFA chain length and FFA concentrations under the stress conditions suggested that FFA accumulation is promoted by environmental stress that affects the cell membrane.

Mechanisms of FFA accumulation. Given that the composition of the FFAs was identical to that of the PLFAs and that the chain lengths of both FFAs and PLFAs increased in a butanol dose-dependent manner (Fig. 6.5), I postulated that FFA accumulation was an excess product of fatty acid synthesis and/or phospholipid degradation initiated by the environmental stresses. To verify the mechanism underlying FFA biosynthesis, the expression of genes involved in fatty acid metabolism was quantified by qRT-PCR. As the enzyme responsible for the primary or essential reactions of fatty acid biosynthesis, β-oxidation, phospholipid synthesis or degradation, β-ketoacyl-acyl carrier protein (ACP) synthase III (FabH), acetyl-CoA carboxylase (ACC), 1-acyl-sn-glycerol-3-phosphate acyltransferase, phosphatidate phosphatase and acyl-CoA synthetase (FadD) were selected from the draft sequence data of the GK12 genome (Table 6.1). The relative expression of the genes encoding FabH and ACC, both of which are involved in fatty acid biosynthesis, were significantly upregulated in response to butanol exposure compared to the other examined genes (Table 6.3). It has been reported that an increase in alkyl chain length in PLFAs has a smaller effect on the rigidification of the membrane than the saturation of unsaturated alkyl chains, which is a process usually observed in bacteria for homeoviscous adaptation (Mykytczuk *et al*., 2007).

Because GK12 cells intrinsically lacks unsaturated or methyl-branched fatty acids, this bacterium must dramatically alter the length of saturated PLFAs via *de novo* fatty acid biosynthesis in response to stresses (Fig. 6.5b). Because C20:0 was present in the detected FFAs, but rarely found in membrane PLFAs (Fig. 6.5), it is very likely that the FFAs are generated via the hydrolysis of acyl-ACPs, resulting in the release of feedback inhibition of enzymes such as ACC and FabH (Jiang & Cronan, 1994; Heath & Rock, 1996; Davis *et al*., 2000), and, in turn, the activation of fatty acid biosynthesis (Fig. 6.7b). However, the β-oxidation of a large amount of fatty acids may be restricted as GK12 is a strictly anaerobic lactate-fermenting bacterium. β-Oxidation generates large amounts of reducing equivalents and, thus, requires the regeneration of NAD^+, which explains why all of the TAG- and WE-accumulating bacteria described to date are aerobic (Wältermann & Steinbüchel, 2005; Alvarez & Steinbüchel, 2002). In addition to the limited regeneration of NAD^+ via anaerobic fermentation relative to aerobic respiration, acetyl-CoA generated through β-oxidation is not available for the oxidation of NADH to NAD^+ in lactic acid fermentation (Fig. 6.7). Under stress conditions, the β-oxidation of fatty acids with longer alkyl chains requires more NAD^+. The limited availability of NAD^+, which leads to high concentrations of reduced pyridine nucleotides, may also drive a fatty acid synthesis to provide a sink for reducing equivalents in which it reoxidizes NADPH. The resulting imbalance between the anabolic and catabolic processes yields surplus fatty acids. In this scenario, FFAs generated through phospholipid degradation would also accumulate, consistent with the upregulation of *ppap* encoding phosphatidate phosphatase in the presence of butanol (Table 6.3).

As described previously, FFA droplets could serve as a sink for excess reducing equivalents to maintain intracellular redox balance under anaerobic growth by lactic acid fermentation. On the other hand, the accumulated FFAs did not decrease during the cultivation under starvation conditions (Fig. 6.8), indicating that the stain GK12 did not utilize the FFAs for growth under starvation conditions, possibly due to limited β-oxidation activity. Another potential function of the droplets in the cells is to serve as a donor or reservoir for phospholipids, as previously postulated for intracellular TAG (Alvarez & Steinbüchel, 2002;

Athenstaedt & Daum, 2006). The primarily localization of C20:0 in FFAs and not in PLFAs seems to be inconsistent with its production for homeoviscous adaptation and for the storage of membrane lipids. It has been recognized that biosynthesis of fatty acid available for membrane or storage lipids is directly correlated with the substrate specificity of the involved enzymes (Magnuson *et al*., 1993; Serrano-Vega *et al*., 2005). I speculated that strain GK12 produces C20:0 as homeoviscous adaptation but cannot incorporate it into membrane lipids significantly due to the substrate specificity of the enzymes such as acyl-ACP thioesterase (Frentzen *et al*., 1983) and glycerophosphate acyltransferase (Lu *et al*., 2008).

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I thank Xian-Ying Meng and Mizuho Muramatsu for their technical assistance with transmission electron microscopy and GC-MS analysis, respectively.

Figures and Tables

Table 6.1. Enzymatic description of the genes examined via qRT-PCR.

Gene	Accession no.	Enzyme (EC number)	Pathway	Reaction	Protein sequence identity against a database (Strain, Accession no.)
fabH	AB745670	β-ketoacyl-acyl carrier protein (ACP) synthase III (EC 2.3.1.180)	fatty acid biosynthesis	acetyl-CoA with malonyl-ACP to acetoacetyl-ACP	49% (*Erysipelotrichaceae* bacterium 5_2_54FAA, ZP_06646275)
acc	AB745671	acetyl-CoA carboxylase (EC 6.4.1.2)	fatty acid biosynthesis	acetyl-CoA plus bicarbonate to malonyl-CoA	66% (*Erysipelotrichaceae* bacterium 21_3, ZP_06646266)
pls	AB745672	1-acyl-sn-glycerol-3-phosphate acyltransferase (EC 2.3.1.51)	phospholipid biosynthesis	acylation of 1-acyl-sn-glycerol-3 phosphate, yielding phosphatidic acid	34% (*Erysipelotrichaceae* bacterium 5_2_54FAA, ZP_09538775)
ppap	AB745673	phosphatidate phosphatase (EC 3.1.3.4)	phospholipid degradation	dephosphorylation of 1, 2-diacylglycerol-3-phosphate, yielding 1, 2-diacyl-sn-glycerol	44% (*Faecalibacterium prausnitzii* L2-6, CBK99553)
fadD	AB745674	acyl-CoA synthetase (EC 6.2.1.3)	β-oxidation	long-chain fatty acid to CoA thioester	69% (*Coprococcus* sp. ART55/1, CBK83344)
gapA	AB745675	glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	glycolysis	oxidative phosphorylation of glyceraldehyde-3-phosphate, yielding glycerate 3-phosphate	82% (*Erysipelotrichaceae* bacterium 5_2_54FAA, ZP_06644559)

*The *gapA* gene sequence was used as an internal standard.

Table 6.2. The primers and characteristics of the standard curves used for qRT-PCR analysis.

Gene	Primers (5' to 3')*	Amplicon length (bp)	Intercept point	Efficiency (%)	R^2	Non template control
fabH	F: TTGCGAAGATCCTGGATACC	235	36.8	101.1	0.988	Not detected
	R: GGCAGTTCTAAACGGTCCTTC					
acc	F: GACCCTGGATGAAAACATGG	249	38.8	109.0	0.947	Not detected
	R: CTGCCGCCTTCTGATAAAAC					
pls	F: GTGCCGCTGAGCTTTATTTC	272	38.2	71.8	0.982	Not detected
	R: AGTGTCACCGGTACAATGGTC					
ppap	F: ACATCTGATCGTGTCGCTTG	263	45.4	140.2	0.956	Not detected
	R: CGCCGACAAGTACATCTGAC					
fadD	F: TCGGAGAACTCTGTGTCAAGG	214	39.4	86.7	0.996	Not detected
	R: GCCAGGAAATCTTCGATCTG					
gapA	F: GAACTGCTTAGCTCCGATGG	214	36.6	77.9	0.991	Not detected
	R: CAATTCCGGAACAACCAAAC					

*F, forward primer; R, reverse primer

Table 6.3. Relative increments of the expression of genes involved in fatty acid and phospholipid metabolism following 2% (v/v) butanol exposure.

Gene	Pathway	Fold difference
fabH	fatty acid biosynthesis	7.3 ± 0.3
acc	fatty acid biosynthesis	13.2 ± 3.1
pls	phospholipid biosynthesis	3.3 ± 0.8
ppap	phospholipid degradation	16.5 ± 12.3
fadD	β-oxidation	2.0 ± 0.4

The values were calculated relative to control cells grown without butanol following normalization to the *gapA* gene and expressed as the mean and SD of three independent experiments.

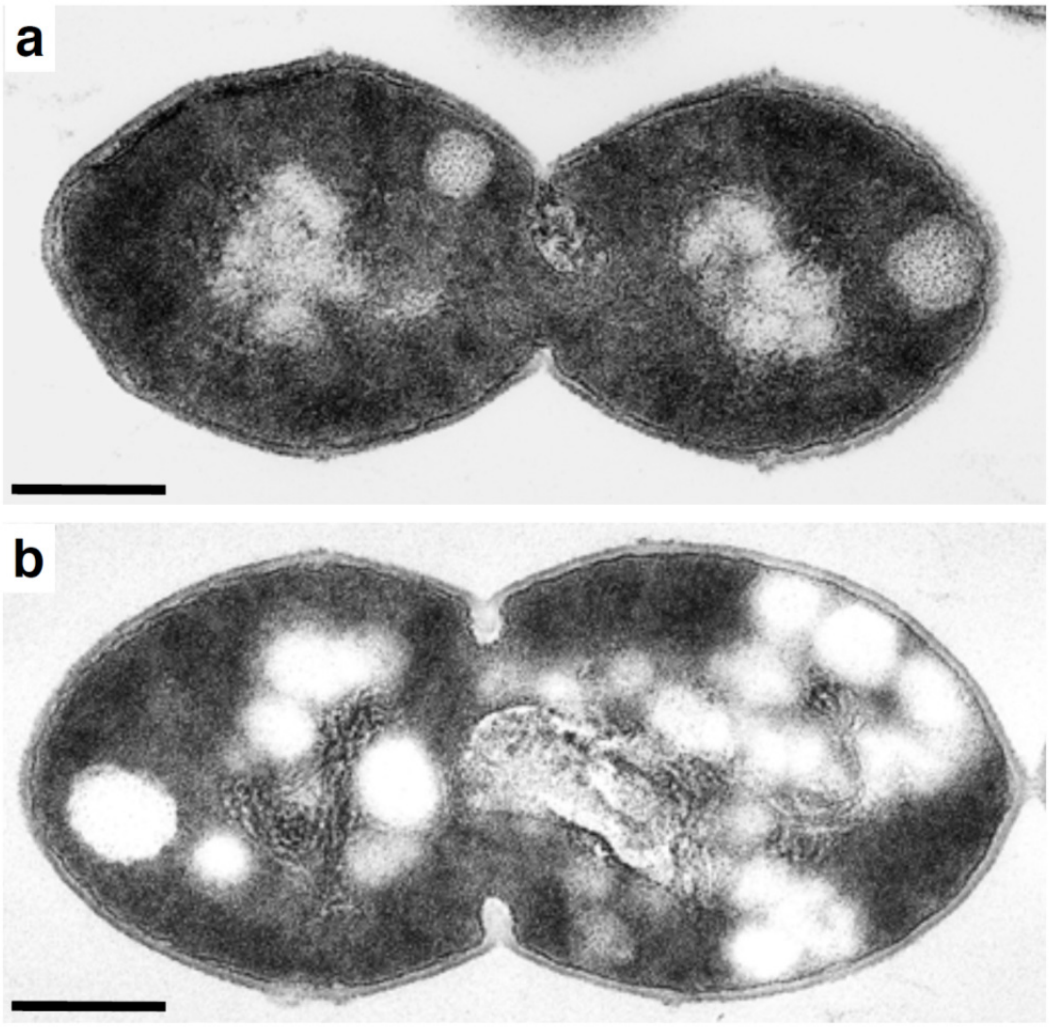

Figure 6.1. Electron photomicrographs of GK12 cells. Transmission electron micrographs showing electron-transparent droplets in cells cultured in the absence (a) or presence of 2% butanol (b) (scale bar, 1 μm).

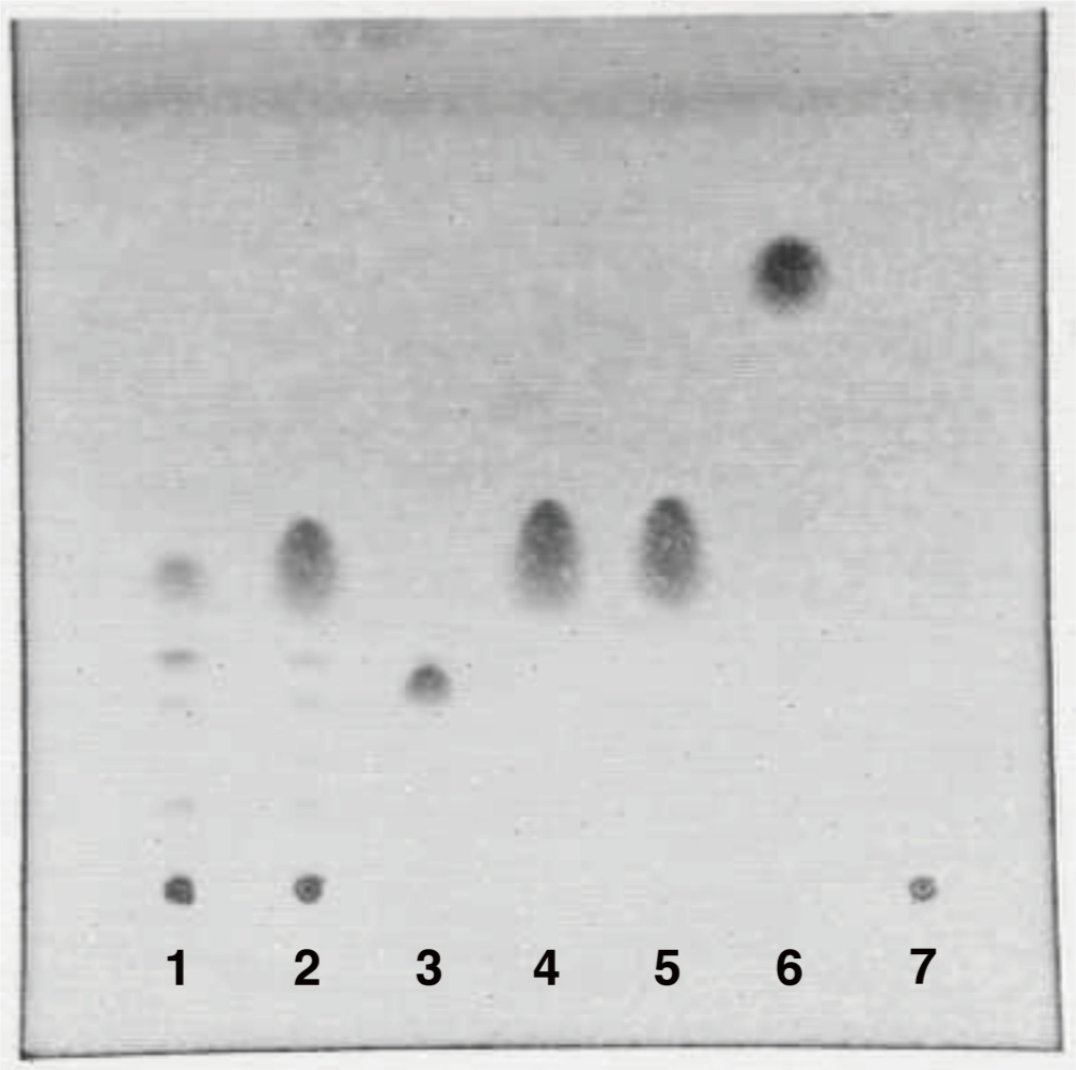

Figure 6.2. Thin-layer chromatography of crude lipid extracts of GK12 cells. This photograph is representative of three replicate experiments. Lanes: 1, crude lipid extracts of cells grown without butanol; 2, cells grown with 2% butanol; 3, 1,2-dipalmitoyl-*sn*-glycerol; 4, palmitic acid; 5, stearic acid; 6, trimyristin; 7, phosphatidyl glycerol.

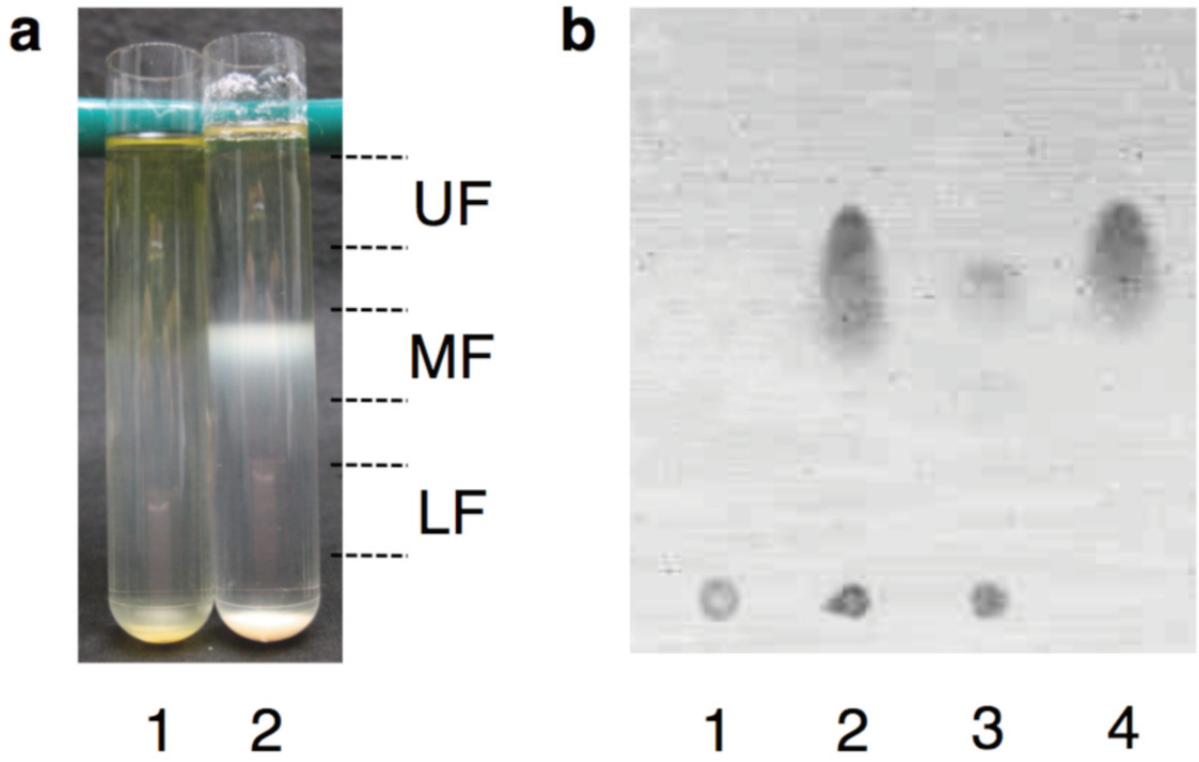

Figure 6.3. Purification of lipid droplets. Sucrose gradient centrifugation of cell-free extracts and fractionation of layers (a). 1, normal cells; 2, butanol-grown cells; UF, upper fraction; MF, middle fraction; LF, lower fraction. Thin-layer chromatography of lipid extracts from each fraction of butanol-grown cells (b). This image is representative of three replicate experiments. Lanes: 1, UF; 2, MF; 3, LF; 4, palmitic acid.

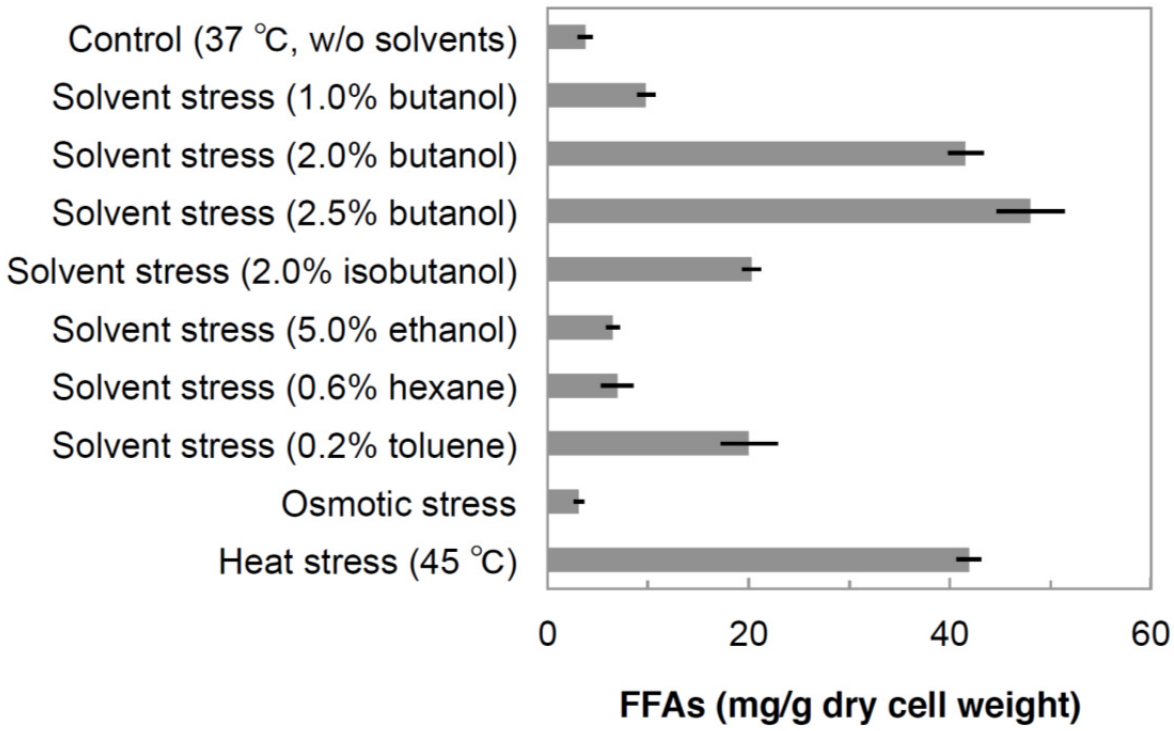

Figure 6.4. The effect of various stresses on FFA accumulation. Cells in the mid-exponential phase of growth were analyzed. Values and error bars represent the mean and SDs of triplicate experiments.

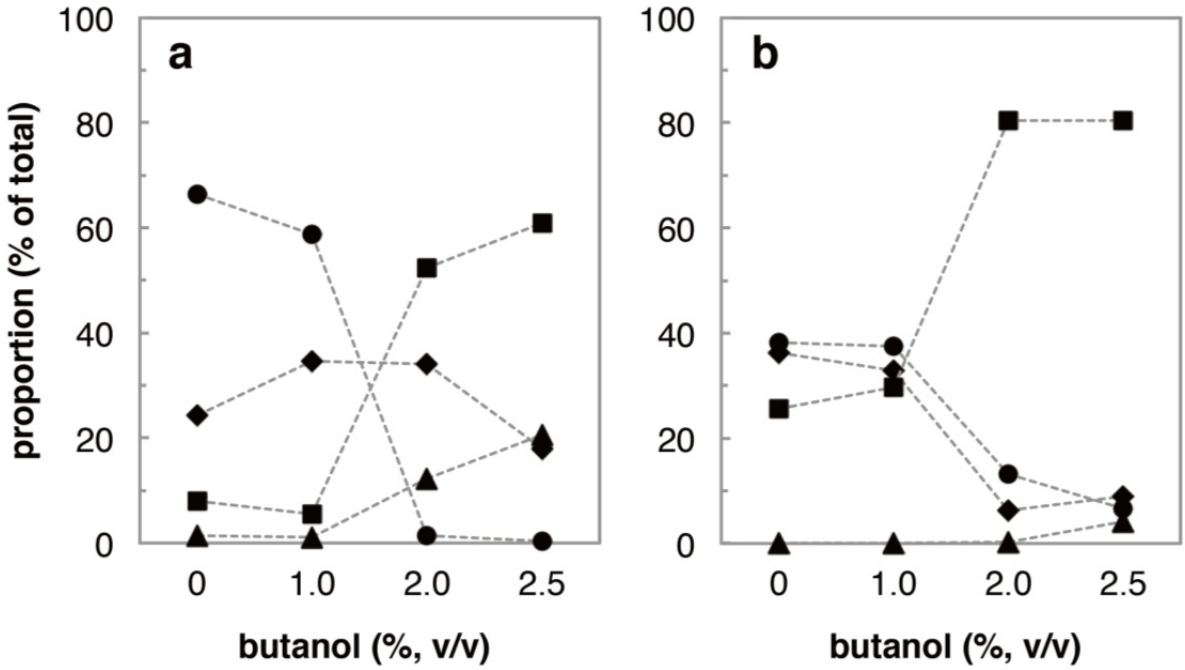

Figure 6.5. The effect of butanol concentrations on the alkyl chain lengths of FFAs (a) and PLFAs (b) (●, C14:0; ◆, C16:0; ■, C18:0; ▲, C20:0). Values represent the mean of three replicates. The level of standard deviation in each value was less than 1.5% of total fatty acids.

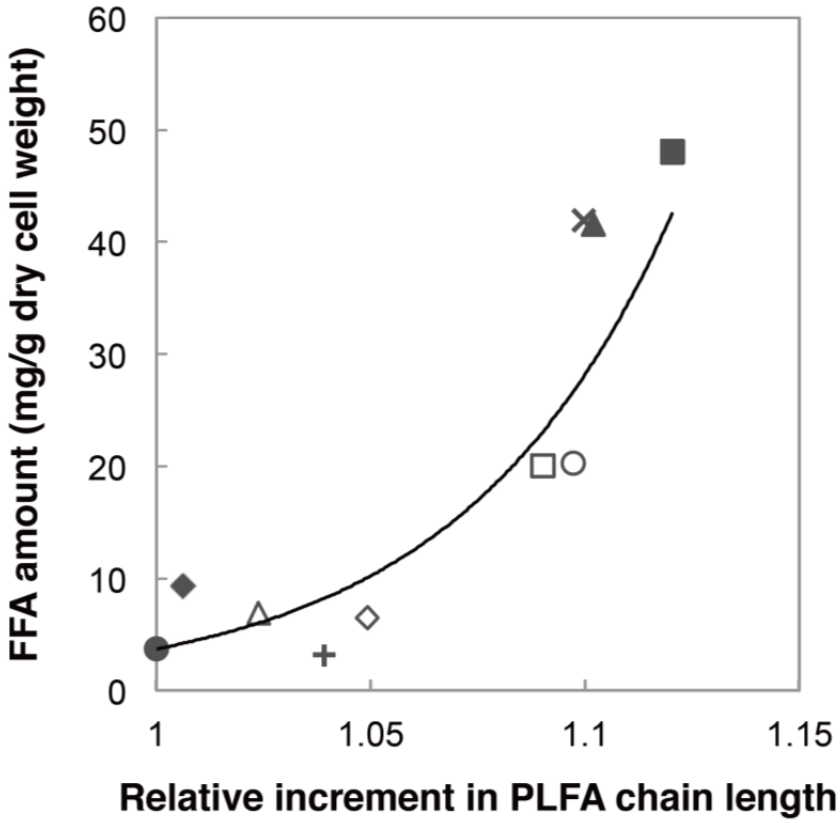

Figure 6.6. The correlation between FFA accumulation and the relative increment in PLFA chain length. The values on the x-axis represent the weighted average showing the relative increase in PLFA chain length under stress condition relative to the no-stress condition (control). For the details of the calculation, see the Materials and Methods section. Symbols represent the differences in culture conditions as follows: ●, control; ◆, 1.0% butanol; ▲, 2.0% butanol; ■, 2.5% butanol; ○, 2.0% isobutanol; ◇, 5.0% ethanol; ∆, 0.6% hexane; □, 0.2% toluene; +, osmotic stress; ×, heat stress. The trend line fitting the data points is also displayed.

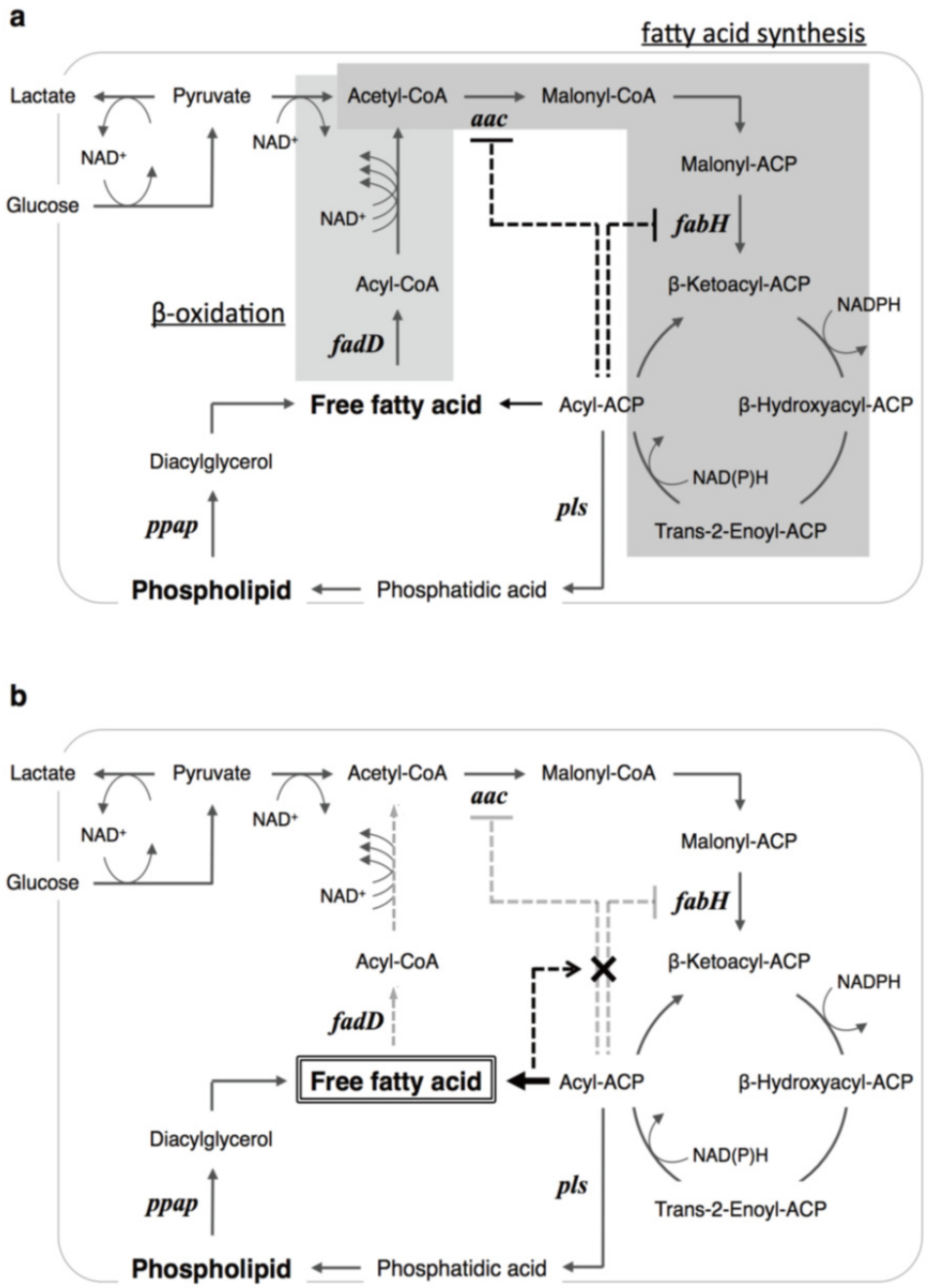

Figure 6.7. Putative fatty acid metabolism pathways (**a**) and the changes caused by environmental stress (**b**). The genes examined via qRT-PCR are indicated. Feedback inhibition of ACC and FabH by acyl-ACP (dashed line) is released (dashed arrow) due to the generation of FFAs via the hydrolysis of acyl-ACPs.

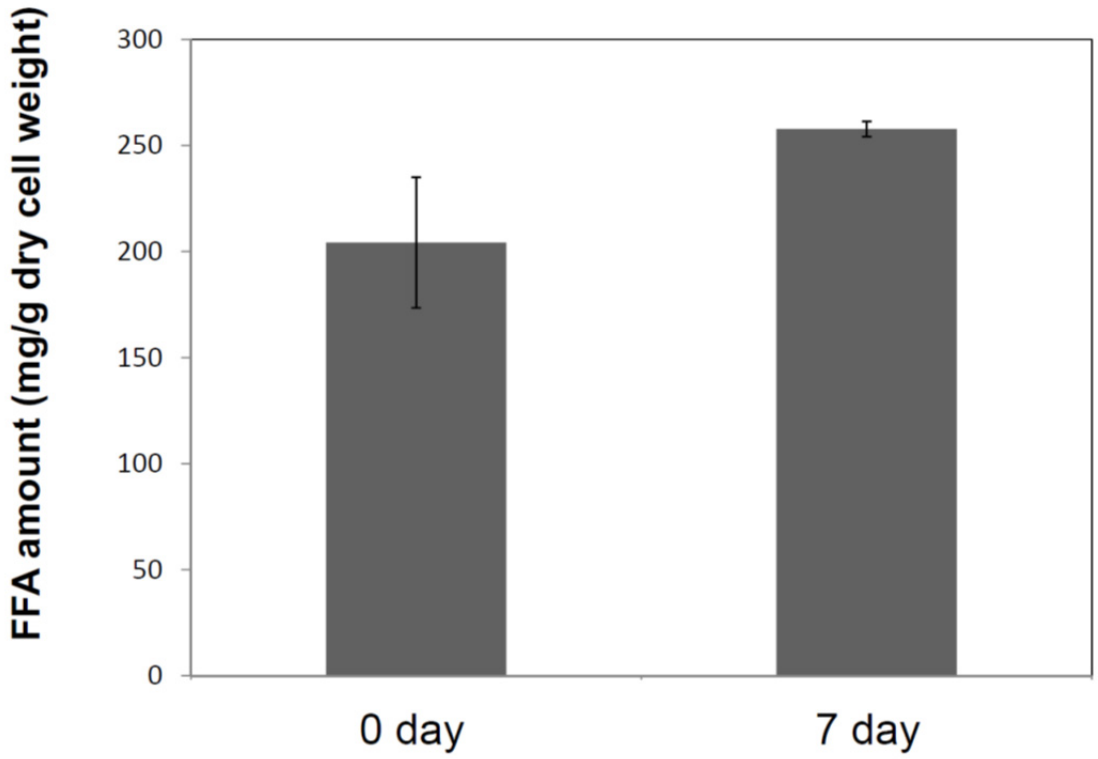

Figure 6.8. FFA amount before and after period of starvation. Dry cell weight measured in the starting cultures and after 7 days in starved culture were 28.2±0.9 mg and 12.9±1.0 mg, respectively.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-10.

Alvarez HM, Steinbüchel A. 2002. Triacylglycerols in prokaryotic microorganisms. Appl. Microbiol. Biotechnol. 60:367-376.

Alvarez HM, Mayer F, Fabritius D, Steinbüchel A. 1996. Formation of intracytoplasmic lipid inclusions by *Rhodococcus opacus* strain PD630. Arch. Microbiol. 165:377-386.

Athenstaedt K, Daum G. 2006. The life cycle of neutral lipids: synthesis, storage and degradation. Cell. Mol. Life Sci. 63:1355-1369.

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911-917.

Brandl H, Gross RA, Lenz RW, Fuller RC. 1988. *Pseudomonas oleovorans* as a Source of Poly(beta-Hydroxyalkanoates) for Potential Applications as Biodegradable Polyesters. Appl. Environ. Microbiol. 54:1977-1982.

Davis MS, Solbiati J, Cronan Jr JE. 2000. Overproduction of acetyl-CoA carboxylase activity increases the rate of fatty acid biosynthesis in *Escherichia coli*. J. Biol. Chem. 275:28593-28598.

Dawes, EA, Senior, PJ. 1973. The role of regulation of energy reserve polymers in micro-organisms. Adv. Microbiol. Physiol. 10:135-266.

Desbois AP, Smith VJ. 2010. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Appl. Microbiol. Biotechnol. 85:1629-1642.

Frentzen M, Heinz E, McKeon, TA, Stumpf, PK. 1983. Specificities and selectivities of glycerol-3-phosphate acyltransferase and monoacylglycerol-3-phosphate acyltransferase from pea and spinach chloroplasts. Eur J. Biochem. 129:629-636.

Heath RJ, Rock CO. 1996. Inhibition of beta-ketoacyl-acyl carrier protein synthase III (FabH) by acyl-acyl carrier protein in *Escherichia coli*. J. Biol. Chem. 271:10996-11000.

Hernandez MA, Mohn WW, Martinez E, Rost E, Alvarez AF, Alvarez HM. 2008. Biosynthesis of storage compounds by *Rhodococcus jostii* RHA1 and global identification of genes involved in their metabolism. BMC Genomics 9:600.

Ingram LO. 1976. Adaptation of membrane lipids to alcohols. J. Bacteriol. 125:670-678.

Jiang P, Cronan Jr JE. 1994. Inhibition of fatty acid synthesis in *Escherichia coli* in the absence of phospholipid synthesis and release of inhibition by thioesterase action. J. Bacteriol. 176:2814-2821.

Kanno M, Katayama T, Tamaki H, Mitani Y, Meng X-Y, Hori T, Narihiro T, Morita N, Hoshino T, Yumoto I, Kimura N, Hanada S, Kamagata Y. 2013. Isolation of butanol- and isobutanol-tolerant bacteria and physiological characterization of their butanol tolerance. Appl. Environ. Microbiol. 79:6998-7005.

Kanno M, Katayama T, Morita N, Tamaki H, Hanada S, Kamagata Y. 2015. *Catenisphaera adipataccumulans* gen. nov., sp. nov., a member of the family *Erysipelotrichaceae* isolated from an anaerobic digester. Int. J. Syst. Evol. Microbiol. 65:805-810.

Leman J. 1997. Oleaginous microorganims: an assessment of the potential. Adv. Appl. Microbiol. 43:195-243.

Lennen RM, Kruziki MA, Kumar K, Zinkel RA, Burnum KE, Lipton MS, Hoover SW, Ranatunga DR, Wittkopp TM, Marner WD II, Pfleger BF. 2011. Membrane stresses induced by overproduction of free fatty acids in *Escherichia coli*. Appl. Environ. Microbiol. 77:8114-8128.

Lu X, Vora H, Khosla C. 2008. Overproduction of free fatty acids in *E. coli*: Implications for biodiesel production. Metab. Eng. 10:333-339.

Magnuson K, Jackowski S, Rock CO, Cronan JE Jr. 1993. Regulation of fatty acid biosynthesis in *Escherichia coli*. Mcirobiol. Rev. 57:522-542.

Manilla-Pérez E, Lange AB, Hetzler S, Steinbüchel A. 2010. Occurrence, production, and export of lipophilic compounds by hydrocarbonoclastic marine bacteria and their potential use to produce bulk chemicals from hydrocarbons. Appl. Microbiol. Biotechnol. 86:1693-1706.

Matsuo K. 2011. Small-scale, high-throughput method for plant N-glycan preparation for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis. Anal. Biochem. 413:200-202.

Mykytczuk NCS, Trevors JT, Leduc LG, Ferroni GD. 2007. Fluorescence polarization in studies of bacterial cytoplasmic membrane fluidity under environmental stress. Prog. Biophys. Mol. Bio. 95:60-82.

Preusting H, van Houten R, Hoefs A, van Langenberghe EK, Favre-Bulle O, Witholt B. 1993. High cell density cultivation of Pseudomonas oleovorans: growth and production of poly (3-hydroxyalkanoates) in two-liquid phase batch and fed-batch systems. Biotechnol. Bioeng. 41:550-556.

Sekiguchi Y, Kamagata Y, Nakamura K, Ohashi A, Harada H. 2000. *Syntrophothermus lipocalidus* gen. nov., sp. nov., a novel thermophilic, syntrophic,

fatty-acid-oxidizing anaerobe which utilizes isobutyrate. Int. J. Syst. Evol. Microbiol. 50:771-779.

Serrano-Vega MJ, Garcés R, Martinez-Force E. 2005. Cloning, characterization and structural model of a FatA-type thioesterase from sunflower seeds (*Helianthus annuus* L.). Planta. 221:868-880.

Sikkema J, de Bont JA, Poolman B. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. 59:201-222.

Sinensky M. 1974. Homeoviscous adaptation—a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 71:522-525.

Thakur MS, Prapulla SG, Karanth NG. 1988. Microscopic observation of Sudan Black B staining to monitor lipid production by microbes. J. Chem. Technol. Biotechnol. 42:129-134.

Wältermann M, Steinbüchel A. 2005. Neutral lipid bodies in prokaryotes: recent insights into structure, formation, and relationship to eukaryotic lipid depots. J. Bacteriol. 187:3607-3619.

Widdel F, Pfennig N. 1981. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov., sp. nov. Arch. Microbiol. 129:395-400.

Zhang YM, Rock CO. 2008. Membrane lipid homeostasis in bacteria. Nat. Rev. Microbiol. 6:222-233.

Zweytick D, Athenstaedt K, Daum G. 2000. Intracellular lipid particles of eukaryotic cells. Biochim. Biophys. Acta 1469:101-120.

Chapter 7

Summary and Concluding Remarks

As I mentioned in the **Chapter 1 (General Introduction)**, butanol is expected as a good alternative to the traditional biofuel ethanol due to the advantages of its high energy content, miscibility with existing fuels, octane rating, and low volatility (Lee *et al*., 2008; Schwarz & Gapes, 2006). However, mainly owing to the high toxicity of *n*-butanol (referred to as butanol) and isobutanol to microbial cells, their biological production is very limited, and thus it gets economically unfavorable. In fact, the previously known butanol producing microbes including the clostridia, the genetically engineered *E. coli* and *Saccharomyces cerevisiae* are highly sensitive to butanol (Knoshaug & Zhang, 2009; Qureshi *et al*., 2007; Woods, 1995). Such toxicity of butanol to microbial cell growth has long been a critical issue for practical biobutanol production.

To date, only a few microbial species have been reported to grow in the presence of greater than 2.0% butanol (Li *et al*., 2010; Knoshaug & Zhang, 2009; Kataoka *et al*., 2011; Ruhl *et al*., 2009; Ting *et al*., 2012), which were mainly screened from deposited species in the culture collections. Meanwhile, few attempts for isolating butanol-tolerant bacteria from natural environments have been made so far, thus very little is known about butanol-tolerant bacteria that are potentially present in nature.

I extensively attempted to screen from various environmental samples as described in the **Chapter 2**. Eventually, 16 bacteria that could tolerate more than 2.0% butanol and isobutanol were successfully isolated. It is noteworthy that the ten isolates were phylogenetically distinct from previously identified butanol-tolerant bacteria. In particular, three of them were phylogenetically novel microbes, showing less than 94% sequence similarity of 16S rRNA gene to the most closely related species. In the next Chapter (**Chapter 3**), phenotypic, phylogenetic and chemotaxonomic methods were

used to characterize an obligate anaerobic isolate, designated strain GK12, and based on these results I propose a novel species and genus in the family *Erysipelotrichaceae* for this isolate (Kanno *et al*., 2015a). These results clearly indicate that there is a wider variety of butanol- and isobutanol-tolerant bacteria that can grow in the presence of greater than 2.0% solvent than previously recognized.

As described in the following chapters (**Chapters 4-6**), aerobic strain CM4A and anaerobic strain GK12 were selected and characterized further. Although microbial mechanisms of butanol tolerance has been extensively studied on clostridia and *E. coli* through physiological analysis, omics analysis, and genomic library screening of genes related to butanol tolerance, these studies were conducted with low concentrations of butanol (around 1.0%) due to their high sensitivity to butanol (Borden & Papoutsakis, 2007; MacDonald & Goldfine, 1991; Rutherford *et al*., 2010). Furthermore, the mechanism conferring tolerance to high concentrations of butanol has not been elucidated yet. In the present study, strains CM4A and GK12 could tolerate up to 3.5% and 3.0% butanol, respectively. Because of their high butanol tolerance, we could test them in the presence of greater than 2.0% butanol.

In the **Chapter 4**, I characterized strains CM4A and GK12 to elucidate the underlying mechanism of high butanol tolerance. The results suggested that the organisms perhaps maintain their structural integrity by increasing the extracellular capsule thickness, adjusting the membrane fluidity, and adaptation via unknown mechanisms (Kanno *et al*., 2013). Note that the tolerance mechanism seems to be different between strain CM4A and GK12. Strain GK12 improved butanol tolerance by the alternative unknown mechanism, whereas the strain did not change cell morphology, which was observed in strain CM4A, indicating the presence of various mechanisms to

adapt cell upon butanol exposure.

Although high butanol-tolerant bacteria and their tolerance mechanisms have been searched in the present study (**Chapters 2 & 4**), one remaining key issue to be addressed is how physicochemical properties such as pH and temperature affect microbial butanol tolerance. In the **Chapter 5**, I present a conceptual study of combined effects of butanol and pH stresses on bacterial cells. It is convincingly concluded that strain CM4A shows lower ability to tolerate butanol under alkaline conditions due to the interactive effects of butanol and alkali stresses (Kanno *et al*., 2015b). To our knowledge, this is the first report describing the effect of pH on microbial butanol tolerance. Our findings indicate the non-negligible impact of pH on butanol tolerance of bacterial species, which provide a new insight into an efficient heterologous butanol production using alternative bacterial host.

Furthermore, as described in the **Chapter 6**, I found lipid droplets within the cytoplasm when strain GK12 cells grown with 2% butanol were observed by transmission electron microscopy. The strain intrinsically accumulated long-chain fatty acids, which may serve as a sink for excess reducing equivalents but not energy and carbon source. The deduced intrinsic FFA accumulation machinery in this bacterium is similar to the mechanism of FFA overproduction in *E. coli* metabolically engineered to develop sustainable biofuels, i.e. involving the release of fatty acid biosynthesis feedback inhibition and the knocking out of the genes involved in β-oxidation (Lu *et al*., 2008; Steen *et al*., 2010; San & Li, 2012). The FFA accumulation observed in the GK12 cells is non-cytotoxic, does not appear to degrade and results in an increase in carbon chain length, and hence, potentially improves the practicality and yield of biodiesel production.

It could be concluded that the findings presented in the thesis provide further strategies for developing potential solvent-tolerant platforms for microbial fuel production. Recently, butanol-tolerant bacteria have been proposed as alternative butanol production hosts that are able to overcome the end product inhibition. The two isolates, strains CM4A and GK12, could be expected as promising host candidates since their tolerance levels were comparable or even greater than the tolerance of previously-identified butanol-tolerant bacteria. The screening from environmental samples would be continuously useful because the presence of a wide variety of butanol-tolerant bacteria could be demonstrated in this thesis.

Previously, the study of microbial solvent tolerance has been mainly focused on aromatic organic solvents (e.g. toluene, benzene, etc) (Inoue & Horikoshi, 1989; Zahir *et al*., 2006). However, due to the difference of solvent hydrophobicity, some strains exhibiting superior tolerance to aromatic solvents did not necessarily tolerate to other organic solvents such as alcohols (Kongpol *et al*., 2008). Indeed, toluene- and benzene-tolerant *Rhodococcos* strain cannot grow in the presence of 2.0% butanol (Li *et al*., 2010). This indicates that the mechanism of butanol tolerance might be inconsistent with the tolerance mechanism of aromatic solvents. Compared to the previously identified microbial mechanism of aromatic solvent tolerance, the current understanding of butanol tolerance is very limited.

Although it is generally hard to elucidate complex unknown mechanisms of butanol tolerance, the combined approach presented in this thesis was effective. I successfully identified the novel properties of butanol-tolerant isolates such as changes in the cell surface properties, fatty acid compositions, and lipid accumulation by conducting morphological and physiological analyses, gene expression profiling and

heterogeneous expression. The results contribute to better understanding of the mechanism related to high butanol tolerance. These findings represent a significant breakthrough in this research field.

References

Borden JR, Papoutsakis ET. 2007. Dynamics of genomic-library enrichment and identification of solvent tolerance genes for *Clostridium acetobutylicum*. Appl. Environ. Microbiol. 73:3061-3068.

Inoue A, Horikoshi K. 1989. A Pseudomonas thrives in high concentrations of toluene. Nature. 338:264-266.

Kanno M, Katayama T, Tamaki H, Mitani Y, Meng X-Y, Hori T, Narihiro T, Morita N, Hoshino T, Yumoto I, Kimura N, Hanada S, Kamagata Y. 2013. Isolation of butanol- and isobutanol-tolerant bacteria and physiological characterization of their butanol tolerance. Appl. Environ. Microbiol. **79:**6998-7005.

Kanno M, Katayama T, Morita N, Tamaki H, Hanada S, Kamagata Y. 2015a. *Catenisphaera adipataccumulans* gen. nov., sp. nov., a member of the family *Erysipelotrichaceae* isolated from an anaerobic digester. Int. J. Syst. Evol. Microbiol. 65:805-810.

Kanno M, Tamaki H, Mitani Y, Kimura N, Hanada S, Kamagata Y. 2015b. pH-induced change in cell susceptibility to butanol in a high butanol-tolerant bacterium, *Enterococcus faecalis* strain CM4A. Biotechnology for biofuels. 8:69.

Kataoka N, Tajima T, Kato J, Rachadech W, Vangnai A. 2011. Development of butanol-tolerant *Bacillus subtilis* strain GRSW2-B1 as a potential bioproduction host. AMB Express 1:10-20.

Katayama T, Kanno M, Morita N, Hori T, Narihiro T, Mitani Y, Kamagata Y. 2014. An oleaginous bacterium that intrinsically accumulates long-chain free fatty acids in its cytoplasm. Appl. Environ. Microbiol. 80:1126-1131.

Knoshaug EP, Zhang M. 2009. Butanol tolerance in a selection of microorganisms. Appl. Biochem. Biotechnol. 153:13-20.

Kongpol A, Kato J, Vangnai AS. 2008. Isolation and characterization of *Deinococcus geothermalis* T27, a slightly thermophilic and organic solvent-tolerant bacterium able to survive in the presence of high concentrations of ethyl acetate. FEMS microbial. lett. 286:227-235.

Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, Jung KS. 2008. Fermentative butanol production by Clostridia. Biotechnol. Bioeng. 101:209-28.

Li J, Zhao JB, Zhao M, Yang YL, Jiang WH, Yang S. 2010. Screening and characterization of butanol-tolerant micro-organisms. Lett. Appl. Microbiol. 50:373-379.

Lu X, Vora H, Khosla C. 2008. Overproduction of free fatty acids in *E. coli*: Implications for biodiesel production. Metab. Eng. 10:333-339.

MacDonald DL, Goldfine H. 1991. Effects of solvents and alcohols on the polar lipid composition of *Clostridium butyricum* under conditions of controlled lipid chain composition. Appl. Environ. Microbiol. 57:3517-3521.

Qureshi N, Saha BC, Cotta MA. 2007. Butanol production from wheat straw hydrolysate using C*lostridium beijerinckii*. Bioprocess. Biosyst. Eng. 30:419-427.

Ruhl J, Schmid A, Blank LM. 2009. Selected *Pseudomonas putida* strains can grow in the presence of high butanol concentrations. Appl. Environ. Microbiol. 75:4653-4656.

Rutherford BJ, Dahl RH, Price RE, Szmidt HL, Benke PI, Mukhopadhyay A, Keasling JD. 2010. Functional genomic study of exogenous n-butanol stress in

Escherichia coli. Appl. Environ. Microbiol. 76:1935-1945.

San KY, Li M. 2012. Bacteria and method for synthesizing fatty acids. U.S. patent. WO2013059218A1.

Schwarz WH, Gapes JR. 2006. Butanol-rediscovering a renewable fuel. BioWorld Europe 1:16-19.

Steen EJ, Kang Y, Bokinsky G, Hu Z, Schirmer A, McClure A, Del Cardayre SB, Keasling JD. 2010. Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature 463:559-562.

Ting CNW, Wu J, Takahashi K, Endo A, Zhao H. 2012. Screened butanol-tolerant *Enterococcus faecium* capable of butanol production. Appl. Biochem. Biotech. 168: 1672-1680.

Woods DR, 1995. The genetic engineering of microbial solvent production. Trends in Biotechnol. 13:259-264.

Zahir Z, Seed KD, Dennis JJ. 2006. Isolation and characterization of novel organic solvent-tolerant bacteria. Extremophiles 10:129-138.