Appl Microbiol Biotechnol (2007) 76:773–781
DOI 10.1007/s00253-007-1046-1

BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Cell envelope fluidity modification for an effective glutamate excretion in *Corynebacterium glutamicum* 2262

Dimitrios Bokas · Davin Uy · Franck Grattepanche · Guy Duportail · Emmanuel Guedon · Stéphane Delaunay · Jean-Louis Goergen

Received: 30 November 2006 / Revised: 3 May 2007 / Accepted: 21 May 2007 / Published online: 7 July 2007

Abstract 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) was used to assess the cell envelope fluidity of *Corynebacterium glutamicum* 2262 during a temperature-triggered glutamate producing process. Because the fluorescence lifetime of TMA-DPH was shown to be constant all over the process, fluorescence anisotropy can be considered as a good index of cell envelope fluidity. When the temperature of the fed-batch culture was increased from 33 to 39°C to induce glutamate excretion, the fluorescence anisotropy values decreased from 0.212±0.002 to 0.186±0.002 (corresponding to an increase in the cell fluidity), while the specific glutamate production rate reached its maximal value. The increase in fluidity of the *C. glutamicum* cell envelope was not due to a physical effect related to the temperature elevation, but rather to an alteration of the composition of the cell envelope. Using a mutant devoid of corynomycolates, significant differences in fluorescence anisotropy values were obtained compared to the wild-type strain, suggesting that TMA-DPH is mainly anchored into the corynomycomembrane. Differences in fluorescence anisotropy were also observed when the bacteria were cultivated at 33, 36, 38, and 39°C in batch cultures, and a linear relationship was obtained between the maximum specific glutamate production rate and the measured fluidity. When using the glutamate non-producing variant of *C. glutamicum* 2262, the fluorescence anisotropy remained constant at 0.207±0.003 whatever the applied temperature shift. This suggests that the fluidity of the Corynebacteria mycomembrane plays an important role in glutamate excretion during the temperature-triggered process.

Keywords Corynebacterium · Glutamate · Fluidity · Mycolic acid · TMA-DPH

D. Bokas · D. Uy · F. Grattepanche · E. Guedon · S. Delaunay · J.-L. Goergen (✉)
Laboratoire des Sciences du Génie Chimique, UPR CNRS 6811,
ENSAIA, Institut National Polytechnique de Lorraine,
2 Avenue de la Forêt de Haye, BP 172,
F-54505 Vandoeuvre-lès-Nancy, France
e-mail: Jean-Louis.Goergen@ensaia.inpl-nancy.fr

G. Duportail
Laboratoire de Pharmacologie et Physicochimie,
UMR CNRS 7175 du CNRS, Faculté de Pharmacie,
Université Louis Pasteur de Strasbourg,
BP 60024, F-67401 Illkirch Cedex, France

Introduction

Since 1957 and the selection of the glutamate producing bacterium, *Corynebacterium glutamicum*, by Kinoshita et al. (1957), production of this amino acid have increased from 100,000 to approximately 1,500,000 t (Hermann 2003). Strains of *C. glutamicum* produce naturally few amounts of glutamate while under specific conditions; maximal production rate can reach up to 100 g l^{-1} (Miescher 1975). Several conditions can induce glutamate overproduction: biotin limitation, addition of antibiotics (penicillin, ethambutol), detergents, or cerulenin (an inhibitor of the condensing enzyme of the fatty acid synthase complex), and temperature upshift (Momose and Takagi 1978; Delaunay et al. 1999; Kimura 2005; Hashimoto et al. 2006). In particular, when the temperature is used, the process is divided in two phases: (1) a first phase with an optimal temperature for the cell growth and (2) a second phase, where a temperature upshift is applied to induce glutamate overproduction. Using such a process, Delaunay et al. (1999) obtained a final glutamate titer of 85 g l^{-1} after 24 h of culture in fed-batch process.

To improve the production of the bulk product glutamate, the identification of the limiting steps and under-

standing of the controlling factors involved in the glutamate excretion are of crucial importance. Recent data suggest that a major kinetic bottleneck is likely located at the level of glutamate excretion. Indeed, a sixfold increase in glutamate excretion flux is measured when the glutamate export system is activated by a temperature shift during the temperature-triggered process (Delaunay et al. 1999; Lapujade et al. 1999); besides, the amplification of the enzyme activity of the last synthesis step, i.e., glutamate dehydrogenase, yielded a higher intracellular glutamate titer, but an identical excretion rate in Corynebacteria (Börmann-El Kholy et al 1993; Lapujade 2000). To identify the parameters modulating the glutamate efflux in *C. glutamicum*, modifications of the composition of the membrane, of the cellular energy and of the membrane potential of producing and non-producing cells have been carried out. Recent findings have shown that the cellular energy is needed for an active glutamate excretion (Gutmann et al. 1992; Sekine et al. 2001), whereas the alteration of the membrane potential does not affect the glutamate production (Lapujade 2000; Gutmann et al. 1992).

Many studies were performed to give clues regarding the mechanisms responsible for glutamate excretion in Corynebacteria. In biotin limitation or surfactant processes, it was proposed that a modification of plasma membrane phospholipids (increase in the saturated to unsaturated fatty acids ratio (Demain and Birnbaum 1968; Shibukawa and Ohsawa 1966; Marquet et al. 1986), a decrease in phospholipids content (Clément et al. 1984; Clément and Lanéelle 1986; Huchenq et al. 1984) or of the total lipids (Hoischen and Krämer 1990) were necessary for glutamate excretion. Besides, during the glutamate production phase of the temperature triggered process, if no reduction of the total lipids content was observed, an increase in the fatty acids unsaturation ratio was measured (Gourdon 1999).

On the other hand, the induction of glutamate excretion by penicillin addition is not related to a modification of the composition of cytoplasmic membrane (Shibukawa et al. 1968). In fact, the modification of the arabinogalactan–peptidoglycan structure upon penicillin addition might explain the glutamate excretion as swelling of the glutamate-producing cells occurred (Shibukawa et al. 1968) as a result of the arabinogalactan–peptidoglycan network weakening (Wijayarathna et al. 2001). In addition, an *alr*-disrupted mutant (gene encoding for D-alanine racemase, which generates D-alanine necessary for the formation of the peptidoglycan network) was able to excrete glutamate in a D-alanine-deficient medium (Eggeling et al. 2001).

Another hypothesis is the glutamate excretion as a result of a modification of the glutamate biosynthesis pathway. Kimura (2002) suggested that the glutamate efflux might be due to a change in cell metabolism at the 2-oxoglutarate branch point; in fact, a mutant deficient in 2-oxoglutarate dehydrogenase (ODHC) activity was capable of excreting high amounts of glutamate without external triggering mean, and presented a comparable fatty acid composition compared to its parental strain. Recently, Niebisch et al. (2006) have shown that ODHC activity was regulated through the phosphorylation status of a protein belonging to this enzymatic complex, OdhI. This protein is phosphorylated via PknG, a serine/threonine protein kinase, that is partially located in cytoplasmic membrane in *Mycobacterium tuberculosis* (Koul et al. 2001; Cowley et al. 2004) and that can act as a sensor of environmental changes (Narayan et al. 2007).

At last, cell envelope fluidity has recently been identified as a key factor of the glutamate excretion process. A temperature sensitive mutant of *C. glutamicum* KY9714, which was generated by a mutation on *ltsA*, a putative gene involved in cell envelope fluidity regulation (Hirasawa et al. 2000), was able to produce high amounts of glutamate at a temperature of 37°C, higher than its optimal growth temperature. However, only a few data on the fluidity of the membrane lipids of glutamate producing *C. glutamicum* have been reported. It was demonstrated that the fluidity of extracted lipids of *C. glutamicum* membrane was not modified during the glutamate excretion induced by local anesthetic addition (Lambert et al. 1995). In addition, modifications of the cell envelope fluidity of *Brevibacterium* sp. ATCC 13869 measured during growth and glutamate production induced by biotin limitation could not be evidenced because of irregular values of fluorescence anisotropy (Neubeck et al. 1993). Nevertheless, recent studies revealed that mycolic acid content of *Mycobacteria* decreased under biotin limitation, addition of ethambutol, Tween 40, penicillin, or cerulenin addition, whereas short corynomycolic acids increased under biotin limitation and cerulenin supplementation (Radmacher et al. 2005; Hashimoto et al. 2006).

The aim of the present paper was to investigate the fluidity of cell envelope during growth and glutamate overproduction of *C. glutamicum* 2262 triggered by an increase in culture temperature from 33 to 39°C. Both the anisotropy and the fluorescence lifetime of the TMA-DPH probe, a molecule widely used to assess the fluidity of biological membranes (Cranney et al. 1983; Neubeck et al. 1993; Denich et al. 2003), were measured to evaluate the fluidity of cell envelope. The effects of culture temperature (33, 36, 38, and 39°C) on the fluidity of cell envelope and on the specific glutamate production rate were assessed. Finally, using a mutant of *C. glutamicum* 2262 defective in corynomycolic acid production, binding site of the fluorescent probe into the cell envelope is discussed.

Materials and methods

Bacterial strain and medium composition

Strains used throughout this study were *Corynebacterium glutamicum* 2262 (provided by Orsan-Amylum S.A.) and

2262NP. This last strain, a non-glutamate-producing variant of *C. glutamicum* 2262, has been isolated upon prolonged continuous fermentation (Uy et al. 2003). The composition of the glutamate production medium used was based on MCGC medium (Von der Osten et al. 1989) in which the citrate was replaced by deferoxamine. This medium consisted of: glucose (60 g l^{-1}), Na_2HPO_4 (3 g l^{-1}), KH_2PO_4 (6 g l^{-1}), NaCl (2 g l^{-1}), $(NH_4)_2SO_4$ (8 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.4 g l^{-1}), $FeSO_4 \cdot 7H_2O$ (40 mg l^{-1}), $FeCl_3$ (4 mg l^{-1}), $ZnSO_4 \cdot 7H_2O$ (1 mg l^{-1}) $CuCl_2 \cdot 2H_2O$ (0.4 mg l^{-1}), $MnSO_4 \cdot H_2O$ (4 mg l^{-1}), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.2 mg l^{-1}), $Na_2B_4O_7 \cdot 10H_2O$ (0.4 mg l^{-1}), $CaCl_2$ (84 mg l^{-1}), biotin (2 mg l^{-1}), thiamine (20 mg l^{-1}), deferoxamine (3 mg l^{-1}), glycine betaine (2 g l^{-1}).

Polypropylene glycol (1.3 g l^{-1}) was used as antifoaming agent. The additional amounts of glucose were added, as required, during the production phase. Because *C. glutamicum* 2262 pks13::km failed to grow in MCGC without reversion, *C. glutamicum* 2262 and the derivative mutant strain *C. glutamicum* 2262 pks13::km were cultivated in brain heart infusion (BHI) medium (Biokar Diagnostics, France) and in BHI containing kanamycin at 50 μg ml^{-1}, respectively, for anisotropy experiments without temperature shift.

Fermentation conditions

The *C. glutamicum* inoculum was prepared in shake flasks at 33°C containing the modified MCGC medium, supplemented with Na_2HPO_4, 3.8 g l^{-1} and 1.9 g l^{-1} urea. The glucose concentration was diminished to 34 g l^{-1} and pH was initially set at 7.6 with NaOH. For fed-batch cultures, 40 ml of this overnight culture were used to inoculate a 2-l bioreactor (Biolafitte, France) containing 0.75 l of modified MCGC. The culture was grown at 33°C with an air rate of 60 l h^{-1}. The pH set point was 7.6, regulated with NH_3 12 N. When the biomass concentration reached 5.6 g l^{-1}, corresponding to the exponential growth phase, the culture temperature was increased to 39°C to induce glutamate production (it took approximately 15 min to increase the temperature of the fermenter from 33 to 39°C). During the production phase, the culture was fed with a concentrated glucose solution (500 g l^{-1}) to avoid periods of glucose limitation. To prevent oxygen limitation, the dissolved oxygen was kept at least 20% of saturated level by increasing air flow rate and agitation all over the culture.

To study the effect of temperature on fluidity of cell envelope and specific glutamate production rates, batch instead of fed-batch processes were performed with similar medium and inoculum conditions. Nevertheless, to avoid the effect of product (i.e. glutamate) inhibition, a smaller amount of inoculum was used to inoculate the bioreactor (diluted culture), resulting in an initial OD_{570} of 0.4 (Lapujade 2000). The culture temperature was set at 33°C for 4 h to reach the exponential growth phase and was then changed to 33, 36, 38, or 39°C to induce the glutamate excretion.

To verify the binding site of the fluorescent probe, fluorescence anisotropy of a mutant devoid of corynomycolic acids (*C. glutamicum* 2262 pks13::km) and the wild type strain (*C. glutamicum* 2262) were compared. Those bacteria were cultivated in bioreactor (Applikon, NL) batch fermentations (0.3-l working volume) at a constant temperature of 30°C, in BHI medium either without antibiotic or with kanamycin (50 μg ml^{-1}) for the wild-type and the mutant strain, respectively. The pH was set at 7.6 with NH_3 (9N) and the dissolved oxygen was maintained over 20% of air saturation. The bioreactor medium was inoculated with colonies from BHI agar plates to reach an initial OD_{570} of 0.2.

Construction of a pks13::km mutant of *Corynebacterium glutamicum* 2262

The mutant of *C. glutamicum* 2262 defective in mycolic acids synthesis was constructed by inserting a kanamycin resistance cassette into the *pks13* gene encoding for a polyketide synthase responsible for the final condensation step of two fatty acids (Portevin et al. 2004). Design of the forward primer pks1 (ATG GAA CAG AGC CAA TCG TCG GAT C) and reverse pks2 primer (GGC TTG CCC ACG GTG AAC GCG GTG T) was based on the genome sequence of *C. glutamicum* ATCC13032 (GenBank Accession Number BX927147). They were used to amplify a 2.8-kb fragment of the *pks13* gene from the DNA of the unsequenced *C. glutamicum* 2262 genome using a *Pfx* DNA polymerase (Invitrogen). The expected polymerase chain reaction (PCR) product was then inserted into the Zero-Blunt pCR cloning vector (Invitrogen) containing a kanamycin resistance cassette. The resulting plasmid was then transferred into *C. glutamicum* 2262 by electroporation and transformants, in which a site specific crossover event has occurred, were selected on BHI agar plates containing kanamycin at 50 μg ml^{-1}, and incubated at room temperature. The partial nucleotide sequence of the gene encoding the polyketide synthase in *C. glutamicum* 2262 was submitted to GenBank under the accession number DQ813498.

Analytical methods

During the fermentations, samples were collected to determine biomass, glucose, and glutamate concentrations. The cell concentration was determined by absorbance at 570 nm (Spectrophotometer DU7500, Beckman) and by a direct gravimetric method. After centrifugation of the

sample (13,000 rpm, 5 min at room temperature, Microfuge Lite, Beckman), the titers of glutamate and glucose were determined enzymatically on the culture supernatant (r-Biopharm, Germany).

Fluorescence measurements of bacterial cell envelope

Fluorescence intensity (arbitrary units) and anisotropy measurements of cells labeled with a cationic derivative of 1,6-diphenyl-1,3,5-hexatriene (DPH), TMA-DPH were carried out with a T-format continuous excitation device (Fluofluidimeter, Regulest, Florange, France). As recommended by Kuhry et al. (1983 and 1985), the intensities were also measured on blank samples (unlabeled cells) for correction (see below). Samples collected from the culture were diluted in a buffer (pH 7.6) containing Na_2HPO_4 (3 g l^{-1}), KH_2PO_4 (6 g l^{-1}), NaCl (2 g l^{-1}), and $(NH_4)_2SO_4$ (8 g l^{-1}), to obtain a turbidity corresponding to an optical density of 1 at 570 nm. For cultures in BHI, samples were centrifuged at 5,000×*g*, 10 min, 4°C, and the pellets were resuspended in buffer to reach an initial OD_{570} of 1. Diluted cell suspension (3 ml) was labeled with 3 µl of TMA-DPH solution (prepared in *N*,*N* dimethylformamide, 2 mM) for 5 min.

Excitation and emission wavelengths were 365 and 425 nm, respectively. The temperature of labeled and blank samples was rigorously controlled during the measurement and the anisotropy was measured at the culture temperature. Five measurements were performed for each sample. The fluorescence anisotropy $\langle r \rangle$ was calculated from the definition Eq. 1:

$$\langle r \rangle = \frac{\left[\left(\mathrm{If}_{//} - \mathrm{Ib}_{//}\right) - g\left(\mathrm{If}_{\perp} - \mathrm{Ib}_{\perp}\right)\right]}{\left[\left(\mathrm{If}_{//} - \mathrm{Ib}_{//}\right) + 2g\left(\mathrm{If}_{\perp} - \mathrm{Ib}_{\perp}\right)\right]}, \quad (1)$$

where g is an instrumental correction factor accounting for the unequal transmission in the optical system, $If_{//}$ and $If_{\perp}$ are the emission intensities of the labeled sample, $Ib_{//}$ and $If_{\perp}$ the signals from unlabeled samples (blanks) with polarizers parallel and perpendicular, respectively, to the direction of the vertically polarized excitation light. The reproducibility of the anisotropy values within a given fivefold determination of five independent samples was ±0.002.

The steady state fluorescence anisotropy $\langle r \rangle$, which can be considered as a reciprocal measure of fluorophore rotational mobility in the cell membrane, is linked both to microviscosity (η) of the medium (defined as the reciprocal of fluidity, a higher anisotropy reflecting a lower fluidity of the labeled environment) and to fluorescence lifetime τ_f through Perrin's Eq. 2:

$$\left(\frac{r_0}{\langle r \rangle}\right) - 1 = \frac{RT\tau_f}{V\eta}, \quad (2)$$

where R is the gas constant, T the temperature in Kelvin, V the volume of the rotating probe, and r_0 the fundamental anisotropy measured in a rigid medium. Thus, the fluidity can be approached from fluorescence anisotropy only by taking into account fluorescence lifetime, which needs to also measure this parameter. TMA-DPH fluorescence lifetimes (τ_f) were determined on similarly prepared samples by using a SLM-48000 multi-frequency phase and modulation spectrofluorometer (SLM-Aminco, Urbana, Champaign, IL, USA) equipped with a 450-W xenon lamp as a light source. A solution of 2,2′-*p*-phenylene-bis-(5-phenyl) oxazole (POPOP) in ethanol, with a lifetime of 1.35 ns, was used as reference (Ho et al. 1992). The fluorescence was observed through 435 nm interference filters (Schott, Mainz, Germany) with a 10-nm band path. The phase and modulation values were determined at 12 different modulation frequencies between 5 and 90 MHz. The data were accumulated until standard deviations of phase and modulation were below 0.3° and 0.006, respectively. Average fluorescence lifetimes were directly calculated from the SLM software.

Results

TMA-DPH staining and fluorescence anisotropy measurement

TMA-DPH is an amphipathic probe that was used to show rates of flip-flop of lipids from inner to outer layer (Cranney et al. 1983). TMA-DPH at a final concentration of 2 µM was used to assess the fluidity of the envelope of *C. glutamicum* 2262 samples with an optical density (turbidity) of 1 at 570 nm. The measured fluorescence intensities of the samples during incorporation of TMA-DPH are reported in Fig. 1. Whatever the temperature tested, for staining durations below 5 min, the fluorescence varied with time, whereas at higher incubation time it

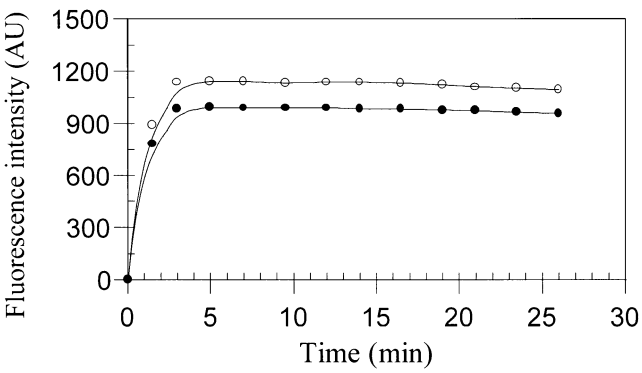

Fig. 1 Incorporation kinetics of TMA-DPH into the cell envelope of *C. glutamicum* 2262 at 33 (*open circles*) and 39°C (*filled circles*) followed by fluorescence intensity

remained constant for more than 25 min. These results are in agreement with data presented by Kuhry et al. (1983). Thus, staining of the cells was thereafter performed during 5 min in presence of TMA-DPH. Fluorescence intensity of TMA-DPH in stained Corynebacteria was shown to vary with the temperature of the assay: 1,150 and 1,000 AU at 33 and 39°C, respectively. The lower fluorescence intensity at 39°C, compared to 33°C, should be due to the known increase in the constant rate of non-radiative decay with temperature.

To test the effect of the incubation temperature, anisotropy of stained cells was determined at 33 and 39°C. It appeared that a 6°C increase in incubation temperature led to a 0.01 decrease in anisotropy value. Besides, we observed that an increase in culture temperature significantly decreased the fluorescence anisotropy, although the measurements were carried out at 33 or 39°C (Table 1). Therefore, staining of the cells and fluorescence anisotropy measurements were performed at a temperature identical to the culture temperature.

Corynebacterium glutamicum 2262 kinetics and envelope anisotropy in glutamate-producing fed-batch process

When *C. glutamicum* was cultivated at 33°C, the specific growth rate increased rapidly and reached a maximum value of 0.58 h^{-1} after 4 h of culture (Fig. 2). During this period, glutamate was not produced and fluidity remained at a low level, corresponding to the highest anisotropy measured value (r # 0.21). The specific rate of glucose consumption increased in a first step with the specific growth rate, and remained constant at its maximal value (i.e., 1.35 g g^{-1} h^{-1}) until 5 h after temperature shift as a consequence of the increase in glutamate specific production rate. When the temperature of the fed-batch was kept at 33°C all over the process, the biomass increased rapidly till the oxygen was limiting in the culture vessel. For these culture conditions, glutamate production was not observed and the fluorescence anisotropy remained constant at about 0.21 during the whole culture duration (data not shown).

On the other hand, after a 4-h period, the temperature was increased from 33 to 39°C to induce the glutamate production.

Table 1 Effect of temperature on fluorescence anisotropy of TMA-DPH-stained *Corynebacterium glutamicum* 2262 cultured at 33 and 39°C

	Anisotropy determined at	
	33°C	39°C
Cells cultured at 33°C (growth exponential phase)	0.213±0.002	0.200±0.002
Cells cultured at 39°C for 4 h	0.195±0.002	0.187±0.002

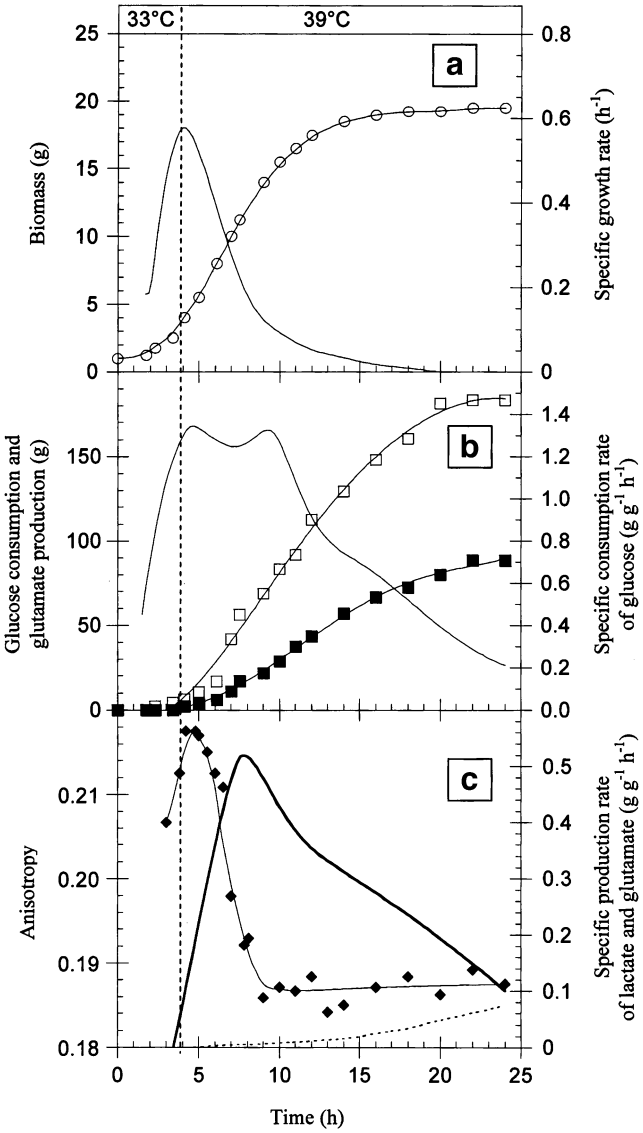

Fig. 2 Effect of a temperature shift on the growth, glucose consumption, glutamate and lactate productions, and cell envelope anisotropy of *C. glutamicum* 2262 in fed-batch culture: biomass (**a**, *open circles*), glucose (**b**, *open squares*) and glutamate (**b**, *filled squares*) productions, anisotropy (**c**, *filled diamonds*), specific growth rate (**a**, *solid line*), specific consumption rate of glucose (**b**, *solid line*), specific production rate of glutamate (**c**, *solid line*) and lactate (**c**, *dashed line*). Biomass, glutamate, and sugar are given as mass values to compensate for the expansion of reactor volume due to feed additions. The *vertical dashed line* indicates the time when the temperature was increased from 33 to 39°C

As a consequence, growth was rapidly reduced and practically abolished between 15 and 24 h of culture and glutamate was excreted to obtain a final amount of 90 g, corresponding to 180 g of glucose consumed after 24 h of culture. At the same time, the specific production rate of lactate slightly increased until the end of culture. The specific rate of glutamate production that increased sharply to its maximum level (0.55 g g^{-1} h^{-1}) after 4 h of culture at 39°C afterward decreased gradually to reach 0.1 g g^{-1} h^{-1} at the end of the

culture. It is important to note that the decrease in the specific rate of glutamate production for the period between 8 and 24 h of culture, during which fluorescence anisotropy remained constant, was mainly due to an inhibition by extracellular glutamate (Lapujade 2000; Nunheimer et al. 1970) as cells collected from the fed-batch culture after 6, 9, and 18 h exhibited the same rate of glutamate excretion when incubated for an additional 4-h period in fresh and diluted culture (i.e., about 0.8 g g^{-1} h^{-1}).

Although the culture temperature was increased from 33 to 39°C, no decrease in fluorescence anisotropy was measured during the first hour after the temperature change. Then, after 5 h of culture, the fluorescence anisotropy decreased from the maximum value of 0.217 to 0.186, and then remained unchanged at this low level until the end of the culture. To check that the fluorescence anisotropy measurement was not affected by the probe lifetime (according to Perrin's equation described in the "Materials and methods" section), the average fluorescence lifetime of TMA-DPH was measured all over the fermentation process. In fact, the fluorescence lifetime remained practically constant (2.95 ns) all over the process, indicating that the fluorescence anisotropy can be directly used as an index of cell envelope fluidity.

Anisotropy of the *Corynebacterium glutamicum* 2262 envelope during glutamate-producing batch cultures performed at different temperatures

According to Fig. 2, the specific glutamate production rate is the only parameter that varies concomitantly to the cell envelope fluidity. Indeed, the specific rate of lactate production remained negligible, and the specific rate of glucose consumption remained constant at its maximal value, while anisotropy strongly decreased to its minimal level. To assess the relationship existing between fluorescence anisotropy, the maximal specific rate of glutamate production and the temperature of the process, *C. glutamicum* 2262 was cultivated in batch process at 33, 36, 38, or 39°C. The cultures were initiated at 33°C by inoculating small amounts of cells to avoid the different effects due to glutamate accumulation in the culture medium and especially the inhibitory effect of extracellular glutamate on glutamate excretion (see "Materials and methods" section).

When the maximum specific growth rate was reached, i.e., 4 h after the bacteria inoculation, the temperature was set at 33, 36, 38, or 39°C. Cell growth and glutamate production were monitored during 10 h. For all cultures, the maximum specific glutamate production rate was reached approximately 4 h upon temperature shift and remained stable for several hours (data not shown). In fact, the highest specific rate of glutamate production correlated with the highest temperature tested and the highest cell envelope fluidity (the lowest anisotropy value). When the maximum specific glutamate production rate of these cultures was plotted against the fluorescence anisotropy (determined at the time of the maximum excretion), a linear relationship was observed (Fig. 3).

On the contrary, when the glutamate non-producing *C. glutamicum* 2262 NP was used, fluorescence anisotropy remained constant at 0.2072±0.0035 and did neither vary over a fermentation performed at 33°C, nor when a temperature upshift was applied from 33 to 36, from 33 to 38 and from 33 to 39°C.

Corynebactrium glutamicum 2262 pks13::km anisotropy in batch cultures

Corynebacteria belong to the CMN group, which have mycolic acids on their outer cell layer. Mycolic acids are synthesized from two fatty acid chains using the condensase, a polyketide synthase enzyme (encoded by *pks13*) responsible for the last condensation step in mycolic acid biosynthesis in mycobacteria and related organisms (Portevin et al. 2004). To know whether the measured anisotropy using TMA-DPH (fluidity) is affected or not by the corynomycomembrane, a mutant devoid of corynomycolates was constructed by interruption of the *pks13* gene in *C. glutamicum* 2262 (pks13::km). Absence of mycolic acids in the cell envelope of the mutant strain was confirmed by thin layer chromatography (data not shown). It can be assumed that the TMA-DPH probe would target the plasma membrane in these mutant cells.

The wild type as well as the mutant bacteria were grown at 30°C, in BHI, for the determination of the fluorescence anisotropy (Fig. 4). As previously reported for other strains of Corynebacterianae, the growth of the pks13::km cells became more difficult as both a lag phase and a reduction of the growth rate appeared (Portevin et al. 2004; Liu and Nikaido 1999). Fluorescence anisotropy increased in both cultures during the growth phase. However, it appeared that the anisotropy was strongly affected by the lack of

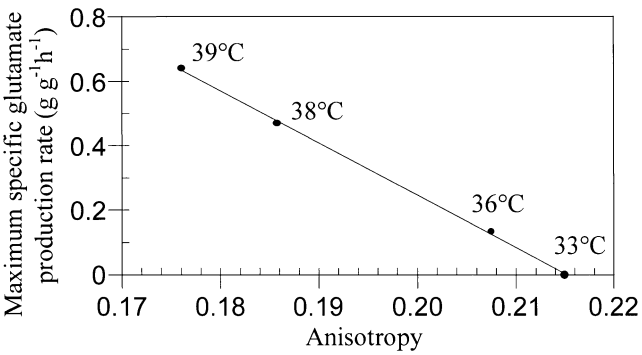

Fig. 3 Relationship between fluorescence anisotropy of the cell envelope and the maximum specific glutamate production rate of *C. glutamicum* 2262 determined at 33, 36, 38, and 39°C

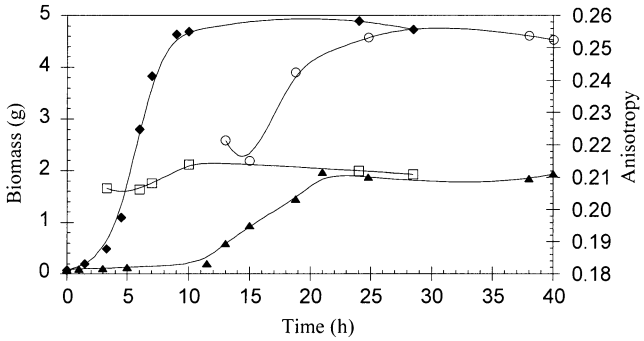

Fig. 4 Kinetics of growth (*filled symbols*) and anisotropy (*open symbols*) of *C. glutamicum* 2262 (*filled diamonds*; *open squares*) and *C. glutamicum* 2262 pks13::km (*filled triangles*; *open circles*) in batch process at 30°C

corynomycolates: whereas the increase in fluorescence anisotropy ranged between 0.208 and 0.220 for the parental strain, a much stronger increase (from 0.215 to 0.254) was measured for the plasma membrane when the strain was devoid of corynomycolates. In addition, the initial anisotropy value was slightly higher when the probe targeted the plasma membrane of the mutant strain.

Discussion

To evaluate the variation in cell envelope fluidity of Corynebacteria cells during the temperature triggered glutamic acid fermentation, TMA-DPH was used as a probe to measure the fluorescence anisotropy of the cell envelope.

A linear relationship appeared between the measured fluidity and the maximum glutamate excretion rate calculated during processes performed at temperatures ranging from 33 to 39°C. In fact, the temperature per se was probably not responsible for the increase in fluidity as no decrease in fluorescence anisotropy occurred during the first 2 h upon temperature upshift from 33 to 39°C. Then, the fluorescence anisotropy of the cell envelope decreased from the maximum value of 0.217 to 0.186; this parameter remained thereafter unchanged at this minimum level until the end of the culture. Similar results were obtained for *Escherichia coli* and *Oenococcus oeni* cells subjected to high temperature shift from 30 to 45°C and 30 to 42°C, respectively; nevertheless, the initial fluidity was restored after a few hours of heat exposure as a consequence of the modification of the membrane composition (Mejía et al. 1999; Liu and Nikaido 1999; Tourdot-Maréchal et al. 2000).

Therefore, during the glutamic acid fermentation, *C. glutamicum* 2262 cell envelope fluidity did not increase as a consequence of the temperature per se, but probably because of a modification of the envelope composition. Interestingly, when experiments were carried out with *C. glutamicum* 2262NP, no difference in fluorescence anisotropy was detected during the culture and the temperature upshift from 33 to 36, 33 to 38 and 33 to 39°C, that is in agreement with a correlation between the envelope fluidity modification and the glutamate excretion rate.

A modification of the composition of the cytoplasmic membrane (Marquet et al. 1986; Clément et al. 1984; Clément and Lanéelle 1986; Huchenq et al. 1984; Hoischen and Krämer 1990) might be involved in the glutamate excretion by *C. glutamicum* 2262. In fact, an increase in the saturated/unsaturated fatty acids ratio was previously observed after the temperature upshock during the glutamic acid fermentation using *C. glutamicum* 2262 (Gourdon 1999). Fatty acids are essentially found as acyl chains of phospholipids in the plasma membrane and such a change in the satured/unsaturated ratio might decrease the fluidity of this membrane. In addition, it was reported for *Pseudomonas putida* and *E. coli* that the saturation degree of fatty acids of growing bacteria increased over the fermentation process (Casadei et al. 2002; Härtig et al. 2005; Denich et al. 2003). We can therefore consider that those biochemical modifications occurred when the Corynebacteria (both wild type and mutant) were grown at a constant temperature of 30°C, as the fluidity decreased over the batch processes (Fig. 4): whereas the increase in anisotropy of the envelope of the parental strain remained quite low over the batch culture (from 0.208 to 0.220), this parameter dramatically increased when TMA-DPH targeted the plasma membrane of the pks13::km Corynebacteria (from 0.210 to 0.250).

Therefore, the fluidity of the envelope of *C. glutamicum* 2262 measured during this work, using TMA-DPH, is definitively not the fluidity of the cytoplasmic membrane. Although corynomycolates do not uniformly cover the cell surface leading to the anchorage of a part of TMA-DPH in the plasma membrane (Kumagai et al. 2005), it can be concluded that TMA-DPH targets mainly the corynomycomembrane of the Corynebacteria.

During the glutamate production phase, anisotropy of the cell envelope decreased as the rate of glutamate production increased. Besides, a strong relationship between the specific glutamate production rate and the envelope fluidity was observed when the temperature of the fermentation was ranging from 36 to 39°C. In fact, Hashimoto et al. (2006) reported an increase in the proportion of short mycolic acids in glutamate-producing conditions. As short mycolic acids possess a lower temperature transition leading to higher fluidity (Liu et al. 1996), our results suggest that a modification of the mycolic acid layer occurred in the temperature-triggered glutamate producing conditions toward a more fluid structure. These observations are consistent with the fact that the corynomycomembrane is the main target of the TMA-DPH molecule.

Therefore, a relationship appears between the fluidity of the corynomycolate membrane, the temperature of the process, and

the specific glutamate production rate by *C. glutamicum* 2262. We can assume that such changes in fluidity of the corynomycomembrane could be required to activate a glutamate carrier; this is consistent with the results of Nampoothiri et al. (2002), which altered the glutamate production of *C. glutamicum*, through modifications of the phospholipids synthesis pathway. Changes in membrane fluidity can be regarded as a mechanism by which bacteria can sense environmental changes, allowing cells to activate different adaptative pathways (Beney and Gervais 2001).

During glutamate production, several authors have also observed a decrease in ODHC activity (Kawahara et al. 1997; Uy et al. 2003 and 2005; Kimura 2005) and deletion for *odh*A encoding the E1o subunit of ODHC led to glutamate overproduction (Kimura 2005; Asakura et al. 2007). Recently, Niebisch et al. (2006) have shown that ODHC activity of *C. glutamicum* is controlled through the phosphorylation status of the protein OdhI. This last protein is phosphorylated by the serine/threonine protein kinase G (pknG) partially located in the cytoplasmic membrane in *Mycobacterium tuberculosis* (Koul et al. 2001; Cowley et al. 2004) and acting as a sensor of environmental signals (Narayan et al. 2007). Therefore, a second hypothesis, that does not exclude the first one, can be: (1) induction of glutamate production (with temperature upshift, biotin limitation, penicillin addition...) leads to changes in cytoplasmic membrane and corynomycomembrane fluidity; (2) these changes could modify activity of some membrane proteins such as DesK, a membrane-associated kinase of *Bacillus subtilis* that senses change in membrane fluidity (Albanesi et al. 2004) leading finally to regulation of enzymatic complexes involved in glutamate synthesis pathway.

A deeper insight into the corynomycomembrane composition of *C. glutamicum* 2262 and its role in signal transduction when glutamate is excreted, as well as the fluidity assessment of the Corynebacteria envelope under different glutamate-triggering stresses (biotin limitation, surfactants addition...), might give clues regarding the relationship between the outer corynomycolate layer and the glutamate excretion mechanism.

Acknowledgments The authors would like to thank Dr. Mohamed Younsi for helpful discussions and providing assistance in performing steady-state anisotropy determinations, and Katharina Groth for her help in generating the *C. glutamicum* 2262 pks13::km mutant. This work was supported in part by the French National Research Agency through the "Corypar" project (ANR-05-JCJC-0069-01).

References

Albanesi D, Mansilla MC, de Mendoza D (2004) The membrane fluidity sensor DesK of *Bacillus* subtilis controls the signal decay of its cognate response regulator. J Bacteriol 186:2655–2663

Asakura Y, Kimura E, Usuda Y, Kawahara Y, Matsui K, Osumi T, Nakamatsu T (2007) Altered metabolic flux due to deletion of *odhA* causes L-glutamate overproduction in *Corynebacterium glutamicum*. Appl Environ Microbiol 73:1308–1319

Beney L, Gervais P (2001) Influence of the fluidity of the membrane on the response of microorganisms to environmental stresses. Appl Microbiol Biotechnol 57:34–42

Börmann-El Kholy ER, Eikmanns BJ, Gutmann M, Sahm H (1993) Glutamate dehydrogenase is not essential for glutamate formation by *Corynebacterium glutamicum*. Appl Environ Microbiol 59:2329–2331

Casadei MA, Mañas P, Niven G, Needs E, Mackey BM (2002) Role of membrane fluidity in pressure resistance of *Escherichia coli* NCTC 8164. Appl Environ Microbiol 68:5965–5972

Clément Y, Lanéelle G (1986) Glutamate excretion mechanism in *Corynebacterium glutamicum*: triggering by biotin starvation or by surfactant addition. J General Microbiol 132:925–929

Clément Y, Escoffier B, Trombe MC, Lanéelle G (1984) Is glutamate excreted by its uptake system in *Corynebacterium glutamicum*? A working hypothesis. J General Microbiol 130:2589–2594

Cowley S, Ko M, Pick N, Chow R, Downing KJ, Gordhan BG, Betts JC, Mizrahi V, Smith DA, Stokes RW, Av-Gay Y (2004) The *Mycobacterium tuberculosis* protein serine/threonine kinase PknG is linked to cellular glutamate/glutamine levels and is important for growth in vivo. Mol Microbiol 52:1691–1702

Cranney M, Cundall RB, Jones GR, Richards JT, Thomas EW (1983) Fluorescence lifetime and quenching studies on some interesting diphenylhexatriene membrane probes. Biochim Biophys Acta 735:418–425

Delaunay S, Gourdon P, Lapujade P, Mailly E, Oriol E, Engasser JM, Lindley ND, Goergen JL (1999) An improved temperature triggered process for glutamate production with *Corynebacterium glutamicum*. Enzyme Microb Tech 25:762–768

Demain AL, Birnbaum J (1968) Alteration of permeability for the release of metabolites from microbial cell. Curr Top Microbiol Immunol 46:1–25

Denich TJ, Beaudette LA, Lee H, Trevors JT (2003) Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. J Microbiol Methods 52:149–182

Eggeling L, Krumbach K, Sahm H (2001) L-glutamate efflux with *Corynebacterium glutamicum*: why is penicillin treatment or tween addition doing the same? J Mol Microbiol Biotechnol 3:67–68

Gourdon P (1999) Etude physiologique d'une souche de *Corynebacterium glutamicum* productrice d'acide glutamique: quantification des flux métaboliques et des contraintes biochimiques en phase de production. Ph.D. Thesis, Institut National des Sciences Appliquées de Toulouse, France

Gutmann M, Hoischen C, Krämer R (1992) Carrier-mediated glutamate secretion by *Corynebacterium glutamicum* under biotin limitation. Biochim Biophys Acta 1112:115–123

Härtig C, Loffhagen N, Harms H (2005) Formation of *trans* fatty acids is not involved in growth-linked membrane adaptation of *Pseudomonas putida*. Appl Environ Microbiol 71:1915–1922

Hashimoto KI, Kawasaki H, Akazawa K, Nakamura J, Asakura Y, Kudo T, Sakuradani E, Shimizu S, Nakamatsu T (2006) Changes in composition and content of mycolic acids in glutamate-overproducing *C. glutamicum*. Biosci Biotechnol Biochem 70:22–30

Hermann T (2003) Industrial production of amino acids by coryneform bacteria. J Biotechnol 104:155–172

Hirasawa T, Wachi M, Nagai K (2000) A mutation in the *Corynebacterium glutamicum ilsA* gene causes susceptibility to lysozyme, temperature-sensitive growth, and L-glutamate production. J Bacteriol 182:2696–2701

Ho C, Williams BW, Stubbs CD (1992) Analysis of cell membrane micro-heterogeneity using the fluorescence lifetime of DPH-type fluorophores. Biochim Biophys Acta 1104:273–282

Hoischen C, Krämer R (1990) Membrane alteration is necessary but not sufficient for effective glutamate secretion in *Corynebacterium glutamicum*. J Bacteriol 172:3409–3416

Huchenq A, Marquet M, Welby M, Montrozier H, Goma G, Lanéelle G (1984) Glutamate excretion triggering mechanism: a reinvestigation of the surfactant-induced modification of cell lipids. Ann Microbiol 135:53–67

Kawahara Y, Takahashi-Fuke K, Shimizu E, Nakamatsu T, Nakamori S (1997) Relationship between the glutamate production and the activity of 2-oxoglutarate dehudrogenase in *Brevibacterium lactofermentum*. Biosci Biotechnol Biochem 61:1109–1112

Kimura E (2002) Triggering mechanism of L-glutamate overproduction by *dtsR1* in coryneform bacteria. J Biosci Bioeng 94:545–551

Kimura E (2005) L-glutamate production. In: Eggeling L, Bott M (eds) Handbook of *Corynebacterium glutamicum*. CRC Taylor and Francis, Boca Raton London New York Singapore, pp 439–463

Kinoshita S, Udaka S, Shimono M (1957) Studies on the amino acid fermentation: Part I. Production of L-glutamic acid by various microorganisms. J Gen Appl Microbiol 3:193–205

Koul A, Choidas A, Tyagi AK, Drlica K, Singh Y, Ullrich A (2001) Serine/threonine kinase PknF and PknG of *Mycobacterium tuberculosis*: characterization and localization. Microbiology 147:2307–2314

Kuhry JG, Fonteneau P, Duportail G, Maechling C, Laustriat G (1983) TMA-DPH: a suitable fluorescence polarization probe for specific plasma membrane fluidity studies in intact living cells. Cell Biophysics 5:129–140

Kuhry JG, Duportail G, Bronner C, Laustriat G (1985) Plasma membrane fluidity measurements on whole living cells by fluorescence anisotropy of trimethylammoniumdiphenylhexatriene. Biochim Biophys Acta 845:60–67

Kumagai Y, Hirasawa T, Hayakawa K, Nagai K, Wachi M (2005) Fluorescent phospholipid analogs as microscopic probes for detection of the mycolic acid-containing layer in *Corynebacterium glutamicum*: detecting alterations in the mycolic acid-containing layer following ethambutol treatment. Biosci Biotechnol Biochem 69:2051–2056

Lambert C, Erdmann A, Eikmanns M, Krämer R (1995) Triggering glutamate excretion in *Corynebacterium glutamicum* by modulating the membrane state with local anesthetics and osmotic gradients. Appl Environ Microbiol 61:4334–4342

Lapujade P (2000) Etude cinétique et physiologique de *Corynebacterium glutamicum* productrice de glutamate: importance du contenu cellulaire et de l'étape d'excrétion de glutamate. Ph.D. thesis, Institut National Polytechnique de Lorraine, France

Lapujade P, Goergen JL, Engasser JM (1999) Glutamate excretion as a major kinetic bottleneck for the thermally triggered production of glutamic acid by *Corynebacterium glutamicum*. Metab Eng 1:255–261

Liu J, Nikaido H (1999) A mutant of *Mycobacterium smegmatis* defective in the biosynthesis of mycolic acids accumulate meromycolates. Proc Natl Acad Sci USA 96:4011–4016

Liu J, Barry CE, Besra GS, Nikaido H (1996) Mycolic acid structure determines the fluidity of the mycobacterial cell wall. J Biol Chem 271:29545–29551

Marquet M, Uribelarrea JL, Huchenq A, Lanéelle G, Goma G (1986) Glutamate excretion by *Corynebacterium glutamicum*: a study of glutamate accumulation during a fermentation course. Appl Microbiol Biotechnol 25:220–223

Mejía R, Gómez-Eichelmann MC, Fernández MS (1999) *Escherichia coli* membrane fluidity as detected by excimerization of dipyrenylpropane: sensitivity to the bacterial fatty acid profile. Arch Biochem Biophys 368:156–160

Miescher GM (1975) Method of producing glutamic acid by fermentation. US Patent 3,929,575

Momose H, Takagi T (1978) Glutamic acid production in biotin-rich media by temperature-sensitive mutants of *Brevibacterium lactofermentum*, a novel fermentation process. Agric Biol Chem 42:1911–1917

Nampoothiri KM, Hoischen C, Bathe B, Möckel B, Pfefferle W, Krumbach K, Sahm H, Eggeling L (2002) Expression of genes of lipid synthesis and altered lipid composition modulates L-glutamate efflux of *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 58:89–96

Narayan A, Sachdeva P, Sharma K, Saini AK, Tyagi AK, Singh Y (2007) Serine threonine protein kinases of mycobacterial genus: phylogeny to function. Physiol Genomics 29:66–75

Neubeck M, Prenner E, Horvat P, Bona R, Hermetter A, Moser A (1993) Membrane fluidity in glutamic acid-producing bacteria Brevibacterium sp. ATCC 13869. Arch Microbiol 160:101–107

Niebisch A, Kabus A, Schultz C, Weil B, Bott M (2006) Corynebacterial protein kinae G controls 2-oxoglutarate dehydrogenase activity via the phosphorylation status of the OdhI protein. J Biol Chem 281:12300–12307

Nunheimer TD, Birnbaum J, Ihnen ED, Demain AL (1970) Product inhibition of the fermentative formation of glutamic acid. Appl Microbiol 20:215–217

Portevin D, de Sousa-D'Auria C, Houssin C, Grimaldi C, Chami M, Daffé M, Guilhot C (2004) A polyketide synthase catalyzes the last condensation step of mycolic acid biosynthesis in mycobacteria and related organisms. Proc Natl Acad Sci USA 101:314–319

Radmacher E, Stansen KC, Besra GS, Alderwick LJ, Maughan WN, Hollweg G, Sahm H, Wendisch VF, Eggeling L (2005) Ethambutol, a cell wall inhibitor of *Mycobacterium tuberculosis*, elicits L-glutamate efflux of *Corynebacterium glutamicum*. Microbiology 151:1359–1368

Sekine H, Shimada T, Hayashi C, Ishiguro A, Tomita F, Yokota A (2001) H^+-ATPase defect in *Corynebacterium glutamicum* abolished glutamic acid production with enhancement of glucose production rate. Appl Microbiol Biotechnol 57:534–540

Shibukawa M, Ohsawa T (1966) L-glutamic acid fermentation with molasses. Part VI. Effect of the saturated-unsaturated fatty acid ratio in the cell membrane fraction on the extracellular accumulation of L-glutamate. Agric Biol Chem 30:750–758

Shibukawa M, Kurima M, Okabe S, Ohsawa T (1968) L-glutamic acid fermentation with molasses. Part X. On the difference in mechanisms for the bacterial extracellular accumulation of L-glutamate between fatty acid derivative and penicillin. Agric Biol Chem 32:641–645

Tourdot-Maréchal R, Gaboriau D, Beney L, Diviès C (2000) Membrane fluidity of stressed cells of *Oenococcus Oeni*. Int J Food Microbiol 55:269–273

Uy D, Delaunay S, Germain P, Engasser JM, Goergen JL (2003) Instability of glutamate production by *Corynebacterium glutamicum* 2262 in continuous culture using the temperature-triggered process. J Biotechnol 104:173–184

Uy D, Delaunay S, Goergen JL, Engasser JM (2005) Dynamics of glutamate synthesis and excretion fluxes in batch and continuous cultures of temperature-triggered *Corynebacterium glutamicum*. Bioprocess Biosyst Eng 27:153–162

Von der Osten CH, Gioannetti C, Sinskey AJ (1989) Design of a defined medium for growth of *Corynebacterium glutamicum* in which citrate facilitates iron uptake. Biotechnol Lett 11:11–16

Wijayarathna CD, Wachi M, Nagai K (2001) Isolation of *ftsl* and *murE* genes involved in peptidoglycan synthesis from *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 55:466–470