Exploring the potential of sigma factors for strain development in *Corynebacterium glutamicum*

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Summary

A sigma factor is one of the components of RNA polymerase holoenzyme and responsible for promoter recognition and transcription initiation. Bacteria usually possess multiple genes encoding sigma factors and strictly control their activity. Activation of a specific sigma factor alters the promoter recognition of the RNA polymerase holoenzyme and enhances the transcription of a specific set of genes with similar promoter sequences. Therefore, sigma factors can be used for activating expression of a set of genes at the same time and could be useful for strain development.

In this study, the potential of sigma factors for strain development was examined in *Corynebacterium glutamicum*. This bacterium was first identified as a natural glutamate producer and has been used in industry for production of amino acids such as L-glutamate and L-lysine. Because of its genetic amenability, various strains have been developed to produce industrial relevant compounds using metabolic engineering.

C. glutamicum possesses seven sigma factor genes, *sigA*, *sigB*, *sigC*, *sigD*, *sigE*, *sigH* and *sigM*. In order to test the effect of sigma factors on the cell behavior, all sigma factor genes were overexpressed in an IPTG-inducible system with different IPTG concentrations. Overexpression of each sigma factor gene affected the growth rate differently, and the effect was dependent on the IPTG concentration, thus, on the strength of expression. The strongest effect was observed for overexpression of *sigD* and *sigH*.

Overexpression of *sigH* led to the strong yellow supernatant. HPLC analysis identified that this color was caused by riboflavin. Transcriptome analysis revealed increased expression of genes of riboflavin biosynthesis, the pentose phosphate pathway and of enzymes requiring FMN (flavin mononucleotide), FAD (flavin adenine dinucleotide) or NADPH as cofactor. Since riboflavin is a precursor of FMN, *sigH* overexpression was applied for the production of FMN. Balanced expression o f *sigH* and the bifunctional riboflavin kinase/FMN adenyltransferase gene *ribF* improved accumulation of riboflavin (20 ± 1 μM) and allowed for its conversion to FMN (33 ± 2 μM).

sigD overexpression in *C. glutamicum* WT reduced foaming possibly due to

secretion of anti-foaming compounds. Transcriptome analysis revealed that expression of genes encoding for proteins which are involved in the cell wall integrity such as mycomembrane synthesis increased as a consequence of *sigD* overexpression. TLC analysis revealed that *sigD* overexpression increased the content of trehalose dicorynomycolate, which is one of the components of the mycomembrane.

In order to explore the potential of sigma factor for strain development, overexpression of sigma factor genes was performed in the recombinant lycopene producing strain LYC5, aiming at an increase of lycopene production. Among seven sigma factor genes, *sigA* was selected as the most effective gene for this purpose. Overexpression of *sigA* in the WT strain also increased production of decaprenoxanthin, which is naturally produced by *C. glutamicum*. Accumulation of lycopene and decaprenoxanthin in the *sigA* overexpressing strain was observed especially in the stationary phase. Transcriptome analysis identified many genes which expression was upregulated under *sigA* overexpression. Based on this transcriptome information, the supplement of thiamine and protocatechuic acid was found to further increase decaprenoxanthin production under *sigA* overexpression. In addition, the effect of *sigA* overexpression was successfully transferred to other carotenoid producing strains.

In this study, it was proven that overexpression of sigma factor genes can perturb the transcriptome profile of *C. glutamicum* by artificially activating the native metabolic pathway and change cell physiology under normal conditions. Artificial activation of sigma factors was helpful to understand regulatory networks in bacteria by connecting genes at different loci with similar promoter sequences. In addition, overexpression of sigma factor and the following screening of the potent sigma factor gene for a specific purpose was shown to be an effective approach for strain development. Therefore, overexpression of genes encoding sigma factors is not only relevant to study the gene regulatory networks, but also effective in strain development of *C. glutamicum* with respect to production of value-added chemicals.

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Abbrebiation

3HB	3-hydroxybutyrate
A-AMP	acyl-AMP
A-CoA	acyl-CoA
AG	arabinogalactan
AMP	adenosine monophosphate
ATCC	American Type Culture Collection
BHI	brain-heart infusion
BLAST	Basic Local Alignment Search Tool
CA-CoA	carboxylated acyl-CoA
cAMP	cyclic adenosine monophosphate
CDW	cell dry weight
ChIP	chromatin immunoprecipitation
CMN group	Corynebacterium, Mycobacterium, Nocardia
CP	cytoplasmic membrane
CRP	cyclic AMP receptor protein
DAD	diode-array detector
DMAPP	dimethylallyl pyrophosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DXP	1-deoxy-D-xylulose-5-phosphate
ECF	extracytoplasmic function
EDTA	ethylenediaminetetraacetic acid
FA	fatty acid
FAD	flavin adenine dinucleotide
FDR	false discovery rate
FLD	fluorescence detectors
FMN	flavin mononucleotide
G6P	glucose 6-phosphate
gTME	global transcription machinery engineering
GTP	guanosine triphosphate
HPLC	high performance liquid chromatography
HTH	helix-turn-helix
IMP	inosine monophosphate
IPP	isopentenyl pyrophosphate

IPTG	isopropyl β-d-1-thiogalactopyranoside
LA	lactate
LC-MS/MS	liquid chromatography–mass spectrometry mass spectrometry
MALDI-TOF/TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometer time-of-flight mass spectrometer mass spectrometry
MEP	methylerythritol 4-phosphate
MM	mycomembrane
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCA	protocatechuic acid
PCC	Pasteur Culture collection
PCR	polymerase chain reaction
PG	peptidoglycan
PHB	Polyhydroxybutyrate
PM	periplasmic membrane
PPP	pentose phosphate pathway
PRPP	phosphoribosyl pyrophosphate
R5P	ribose 5 phosphate
RAISE	random mutation, insertion, deletion
RF	riboflavin
RL5P	ribulose 5 phosphate
RNA	ribonucleic acid
RNAP	RNA polymerase core enzyme
SDS	sodium dodecyl sulfate
TDCM	trehalose dicorynomycolate
TDM	trehalose dimycolate
TLC	thin-layer chromatography
TMCM	trehalose monocorynomycolate
Tre	trehalose
TS	transcription start site
UV	ultraviolet
WT	wild type

1 Introduction

1.1 Strain development

Various chemical compounds are produced through biological processes in nature, and a large number of compounds are useful for human beings as nutritions, drugs and materials (Clardy and Walsh, 2004). For several thousands of years, human beings have used the abilities of microorganisms for production of several compounds such as ethanol through fermentation, however, the practice has been based on unexpected discoveries in nature and long term experiences (Jeffries, 2005).

The emergence of DNA manipulation techniques expanded the potential use of microorganisms for chemicals production and caused a paradigm shift in the usage of microorganisms (Cohen *et al.,* 1973). These techniques made it possible to engineer microorganisms rationally in order to produce a desired compound efficiently. With an increased understanding of biology, researchers started to explore the potential of bioprocess for various applications, and the concept of metabolic engineering arose (Stephanopoulos, *et al.,* 1998).

Microorganisms have evolved by optimizing their cellular mechanisms for survival in nature, therefore, the metabolism of native microorganisms is seldom optimized for the purpose of production. In such cases, strain development is often required to obtain a favorable strain. Strain development has successfully been employed to construct microorganisms suitable for production of desired compounds (Stephanopoulos, 2012). The approaches for strain development can roughly be divided into two categories (Bailey, 1991). One is the random approach, and the other is the rational approach. Both approaches have advantages and disadvantages.

The random approach consists of two parts, the mutant library construction and the following screening/selection. The mutant library construction can be performed with different methods (Packer and Liu, 2015). For example, the random mutant libraries are traditionally constructed by UV irradiation or chemical mutagenesis. The transposon mutant library can be used to evaluate the effect caused by loss of genes (van Opijnen and Camilli, 2013), on the other hand, the shotgun expression library can be used to evaluate the effect of additional copies of genes (Kang *et al.,* 2005). These random mutation, deletion or overexpression are expected to cause perturbation in cellular mechanism and create a mutant library with diverse phenotypes. From

those libraries, microorganisms with a desired phenotype are chosen by screening/selection. Several efficient methods have been invented such as selection based on growth as well as screening based on color phenotype, direct detection of chemical production or indirect detection of chemical production using reporters such as biosensors (Liu and Jiang, 2015). One of the advantages of this approach is that the target is not needed to be specified in advance and unexpected effects can be found. However, a large size of mutant libraries and high throughput screening methods is required for successful screening/selection, and the physical limitation is one of the disadvantages for this approach.

In the rational approach, target genes are engineered using genetic manipulation. A great number of successes have proved that the rational approach is a powerful tool in strain development (Woolston *et al.,* 2013). First step of this approach is to specify a target for genetic engineering and a target is determined base on the biological knowledge. The flux balance analysis using genome-scale metabolic models can be also helpful to identify a new target gene out of several thousand genes (Furusawa *et al.,* 2013). In the rational approach, a bottleneck in a pathway can be overcome by overexpressing genes encoding enzymes relevant for the limiting step (Bailey, 1991). Deletion of genes or downregulation of gene expression can reduce the flux into an undesired pathway and result in the decrease of byproduct formation (Mora-Pale *et al.,* 2014). Furthermore, protein engineering can increase the enzyme activity or eliminate feedback inhibition of enzymes in a pathway (Pleiss, 2011). One of the biggest advantages of this approach is that heterologous pathways can be introduced in addition to the endogenous pathways, which enables a new reaction to take place in cells (Lee *et al.,* 2012b). For example, E. coli was successfully engineered to convert intermediates in amino acid biosynthesis pathways to longer chain alcohols by indroducing non-native steps (Atsumi *et al.,* 2008). The rational approach has been successful, however, the complexity of cellular regulatory networks still hinders precise prediction of a new target for metabolic engineering in some cases.

Both the random and rational approaches have been used successfully for strain development for several decades, however, both of these approaches have disadvantages. In the random approach, insufficient mutant library size and limited number of selection/screening procedures can lead to fail in obtaining a desired strain. In addition, accumulation of unfavorable

mutations changes microorganisms to auxotroph for a certain nutrients, which is not favorable in strain development (Ikeda *et al.,* 2006). In the rational approach, the complex regulatory networks makes it difficult to design a desired pathway with balanced flux, and targeting a wrong gene leads to no or worse effects on strain development. A combination of the random and rational approaches can compensate for those disadvantage by targeting a small number of elements rationally and perturbing their activities or gene expressions randomly (Stephanopoulos *et al.,* 2004). For this purpose, several methods were invented to introduce random mutations in a targeted DNA sequence, aiming to change enzyme activity (Dietrich *et al.,* 2010). Furthermore, randomizing regulatory elements in an operon such as ribosome binding sites was performed to optimize the protein ratio of multiple genes in the heterologous mevalonate pathway (Pfleger *et al.,* 2006). A combination of the random and rational approaches is an efficient strategy for fine tuning metabolic fluxes. However, it is still challenging to optimize the overall performance of cells in a global manner due to the complex interactions among multiple components in the endogenous regulatory networks.

1.2 Strain development using global regulators

As omics analysis reveals the complexity of regulatory networks, the strain development using a global regulators such as transcription factors is drawing attention (Vemuri and Aristidou, 2005). The importance of the proper regulatory control in metabolism is widely acknowledged in terms of toxicity or flux (Farmer and Liao, 2000; Becker *et al.,* 2011). However, the optimization of the cell performance is sometimes difficult because of the complex regulatory networks (Kwok, 2010). In order to optimize the metabolic flux of cells in a global manner and convert microorganisms into microbial cell factories, the balanced control of the endogenous pathways as well as the heterologous pathways are important (Alper and Stephanopoulos, 2009). For those purposes, global regulators can be suitable target. Since global regulators induce or repress a set of genes which works cooperatively in the endogenous pathways, overexpression or deletion of global regulators will perturb the regulatory networks effectively in a global manner (Courchesne *et al.,* 2009). In addition, the strain development by those regulators may shed light on the synergistic effects in the endogenous pathways unexpectedly. In fact, the strain development using transcriptional regulatory proteins have been successfully performed especially in increasing stress

tolerance of microorganisms (Lin *et al.,* 2013b). For example, the knock out mutant of the global regulator cyclic AMP receptor protein (CRP) and the adenyl cyclase (CyaA) successfully increased the tolerance of *E. coli* to several organic solvents (Okochi *et al.,* 2008). Since the effect of global regulators is also complex, it is difficult to predict the effective global regulators for a specific purpose rationally. However, the existence of multiple types of global regulators in cells enables to perturb regulatory networks in different manners and at different levels for a desired purpose. Among those global regulators, sigma factors can be a good candidate for the strain development because of it biological function and the appropriate number of genes.

1.3 Sigma factors

Genes are transcribed by RNA polymerase and the bacterial RNA polymerase is well conserved in terms of its subunit composition and structure over species (Decker and Hinton, 2013). In bacteria, the core of RNA polymerase is composed of two large subunits, β and β′; two α subunits; and one ω subunit. Upon transcription initiation, another factor called sigma factor is required in addition to the RNA polymerase core enzyme (Gruber and Gross, 2003). The sigma factor binds to RNA polymerase core enzyme to form a complex called RNA polymerase holoenzyme. This holoenzyme can recognize the promoter sequences in genome and initiate transcription by melting double helix structure of DNA. Therefore, Sigma factors play an important role in promoter recognition and transcription initiation as one of the components of RNA polymerase holoenzyme.

Most bacteria possess multiple sigma factor genes which products are responsible for the transcription of a set of genes under specific conditions (Sachdeva *et al.,* 2010). RNA polymerase holoenzyme containing a specific sigma factor enhances transcription of genes with the cognate consensus promoter sequence (Staroń *et al.,* 2009). The consensus promoter sequences vary for each sigma factor, and different sigma factors induce different sets of genes, resulting in the different response mechanisms. Therefore, sigma factors have the important role of changing the properties of cells by regulating transcriptional profile (Kazmierczak *et al.,* 2005). For example, the sigma factor RpoF in *E. coli* is responsible for transcription of flagellar and chemotaxis genes and therefore essential for flagella formation (Kundu *et al.,* 1997; Makinoshima *et al.,* 2003). As omics analysis becomes easily accessible, regulation by sigma factors has been evaluated in a global manner by transcriptome analysis and chromatin immuno. For example, sigma factors were shown

to be important hubs of the regulatory architecture in *E. coli* and *B. subtilis* and various cross talks occur between sigma factors as well as sigma factor regulons (Cho *et al.,* 2014; Nicolas *et al.,* 2012).

Sigma factors are divided into two main families, the sigma 70 (σ^{70}) and the sigma 54 (σ^{54}), and these two families are quite distinct both structurally and functionally (Merrick, 1993). Some bacteria do not have a sigma factor of the σ^{54} family, and most sigma factors are classified into the σ^{70} family (Studholme and Buck, 2000). The σ^{70} family sigma factors are further divided into four groups, Group1 to Group4, based on their phylogenetic relatedness, conserved amino acid sequences and protein structures (Gruber and Gross, 2003). From the physiological perspective, Group 1 sigma factors are essential and responsible for the transcription of housekeeping genes. Because of their importance, Group1 sigma factors are also called primary sigma factors. Group 2 sigma factors are closely related to Group 1 sigma factors and responsible for general stress response, however, those sigma factors are often not essential for cell survival (Battesti *et al.,* 2011). Group 3 sigma factors are evolutionarily related to each other and further cluster into groups with similar functions such as heat shock, flagellar biosynthesis and sporulation (Österberg *et al.,* 2011). Group 4 sigma factors are diverse in functions. Group 4 sigma factors are further classified into more than 40 minor groups based on their amino acid sequence similarity and genomic context (Staroń *et al.,* 2009). These sigma factors are also named ECF (extracytoplasmic function) type sigma factors since some of them were initially found to regulate the cell surface integrity. The activity of those sigma factors is sometimes regulated by proteins called anti-sigma factors (Paget, 2015).

From the structural perspective, Group1 sigma factors consist of four regions and those regions are further divided into their subregions (**Figure 1. A**). On the other hand, the crystal structure revealed the existence of three domains in the Group1 sigma factor, σ2 to σ4. The functions of those subregions are well defined. For example, region 2.4 and 4.2 are essential for the recognition of the -10 box and -35 box promoter sequences, respectively. Region 3.0 is necessary for the recognition of the extended -10 box. Group 2 and 3 sigma factors lack the region 1.1, which is present in Group1 sigma factors. In addition to this region, Group 4 sigma factors lack the domain 3 (**Figure 1. B**). Those differences in their structure influences the recognition of

promoter sequences as well as the affinity to RNA polymerase core enzyme (Campagne *et al.*, 2014).

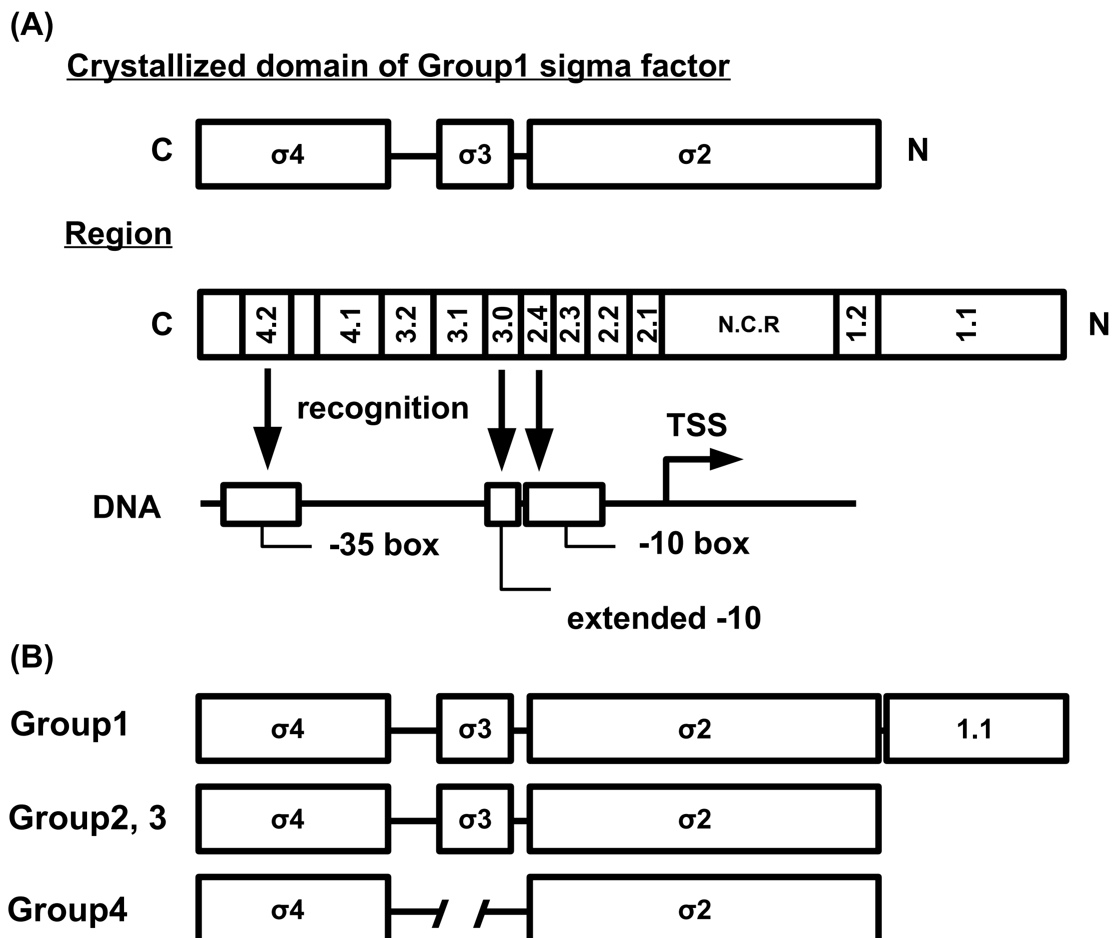

Figure 1: Sigma factor structure. (A) Domains identified by crystal structure and regions conserved in amino acid sequences. Crystallized domains are shown as σ2, σ3, σ4. Regions and subregions are shown by numbers. Functions of subregions important for the promoter recognition are indicated by arrow. C and N represent C-terminus and N-terminus of amino acid sequence. N. C. R represents "Non-conserved region", which is seen in some sigma factors. TSS indicates transcription start site. **(B) Conserved domains and regions in different groups of sigma factors.** The reegion which was not visualized by crystal structure is assigned as subregion 1.1. Figure 1 of Gruber and Gross, 2003 is used as a reference.

1.4 Transcription initiation mechanism by RNA polymerase holoenzyme

The mechanism of transcription initiation is intensively studied for the RNA polymerase holoenzyme containing the σ^{70} type sigma factor (Ruff *et al.,* 2015). First, the holoenzyme binds to promoter sequence recognized by the sigma factor. After initial binding, a conformational change from the closed complex to the open complex occurs through interaction with DNA (Saecker *et al.,* 2011). During the conformational change, the holoenzyme synthesizes several short RNA fragments, pausing at the promoter region. The holoenzyme at the promoter region escapes from the tight binding stochastically, and the stabilized open complex starts transcription elongation, releasing the sigma factor from the core of RNA polymerase (Raffaelle *et al.,* 2005). This promoter escape is often the rate limiting step in transcription initiation rather than recruitment of RNA polymerase to promoter sequence (Wade and Struhl, 2008) (**Figure 2)**.

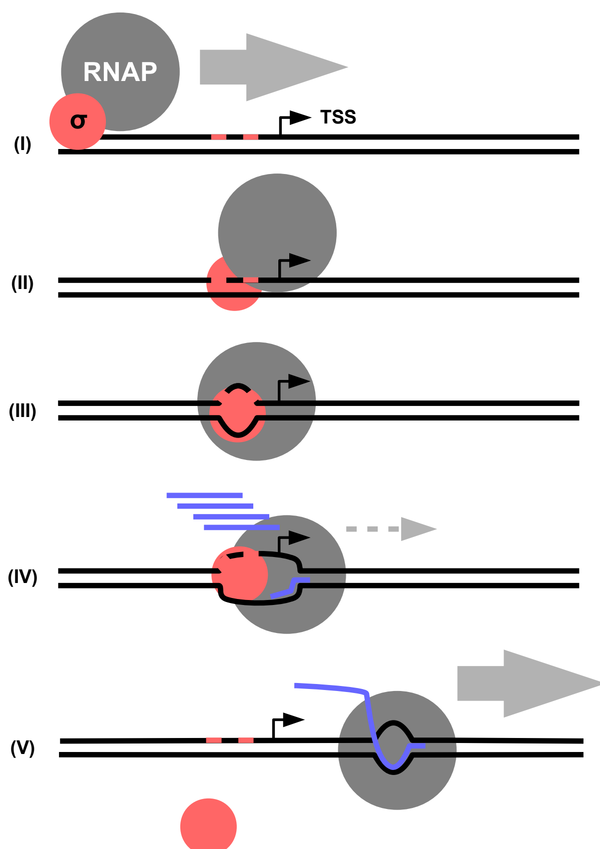

Figure 2: Scheme of transcription initiation by RNA polymerase holoenzyme. (I) RNA polymerase holoenzyme searches for promoter sequence on DNA. (II) The promoter sequence is recognized by the sigma factor (III) The sigma factor (red) unwinds DNA double helix and RNA polymerase (gray) experiences the conformational change. (IV) The open complex transcribes several short RNAs (blue). (V) The open complex is stabilized stochastically and starts transcription elongation by escaping from the tight binding to the promoter region. RNAP represents RNA polymerase core enzyme and TSS is transcription stat site on DNA. Red line on DNA is promoter sequence. Figure 1 of Wade and Struhl, 2008 is used as a reference.

1.5 Sigma factor competition as a regulatory mechanism

In general, bacteria have more than two types of sigma factors that have their specific promoter selectivity. Therefore, RNA polymerase holoenzymes containing different sigma factors recognize different promoter sequences and initiate the transcription of a different set of genes. The number of sigma factor molecules is higher than that of RNA polymerase core enzymes in a cell (Grigorova *et al.,* 2006). Therefore, different sigma factors compete for binding to the limited pool of core enzymes (Mauri and Klumpp, 2014) (**Figure 3**). This competition is one of the strategies of bacteria to respond to stimuli or stress and to cope with the environmental change.

The competition is regulated through multiple mechanisms (**Figure 3**). One of the mechanisms is regulation by the anti-sigma factor. The anti-sigma factor binds to the cognate sigma factor and inhibits the interaction between the sigma factor and the RNA polymerase core enzyme (Paget, 2015). Upon a certain stimulus, the anti-sigma factor releases the cognate sigma factor and allows the sigma factor to cope with the core enzyme. Anti-sigma factors are especially found for ECF type sigma factors. The competition is also influenced by small molecules. For example, guanosine tetraphosphate (ppGpp), which is synthesized under stringent response, enhances the transcription from the promoters dependent on alternative sigma factors (Österberg *et al.,* 2011). ppGpp decreases the binding affinity between the primary sigma factors and RNA polymerase core enzymes, therefore promotes the transcription that is dependent on alternative sigma factors. In addition, the transcriptional levels of the sigma factor genes changes in different growth phases and different environmental conditions (Manganelli *et al.,* 1999). Therefore, several regulations at multiple layers exist for sigma factor competition.

In general, a sigma factor serves as an activator for transcription. However, RNA polymerase holoenzymes can also work as a repressor at overlapping promoters that can be recognized by several sigma factors (Shearwin *et al.,* 2005). Since the pause at promoter region is the rate limiting step in transcription initiation, holoenzymes binding strongly to overlapping promoter sequences inhibit transcription of other holoenzymes with other sigma factors (Bendtsen *et al.,* 2011). In this way, sigma factors can inhibit the transcription from a certain promoter region (**Figure 3**).

It is widely recognized that transcription is regulated by transcriptional repressor and

activator proteins (Lee *et al.,* 2012a). In addition, the regulatory mechanism by sigma factors represent another layer of transcriptional regulation in bacteria.

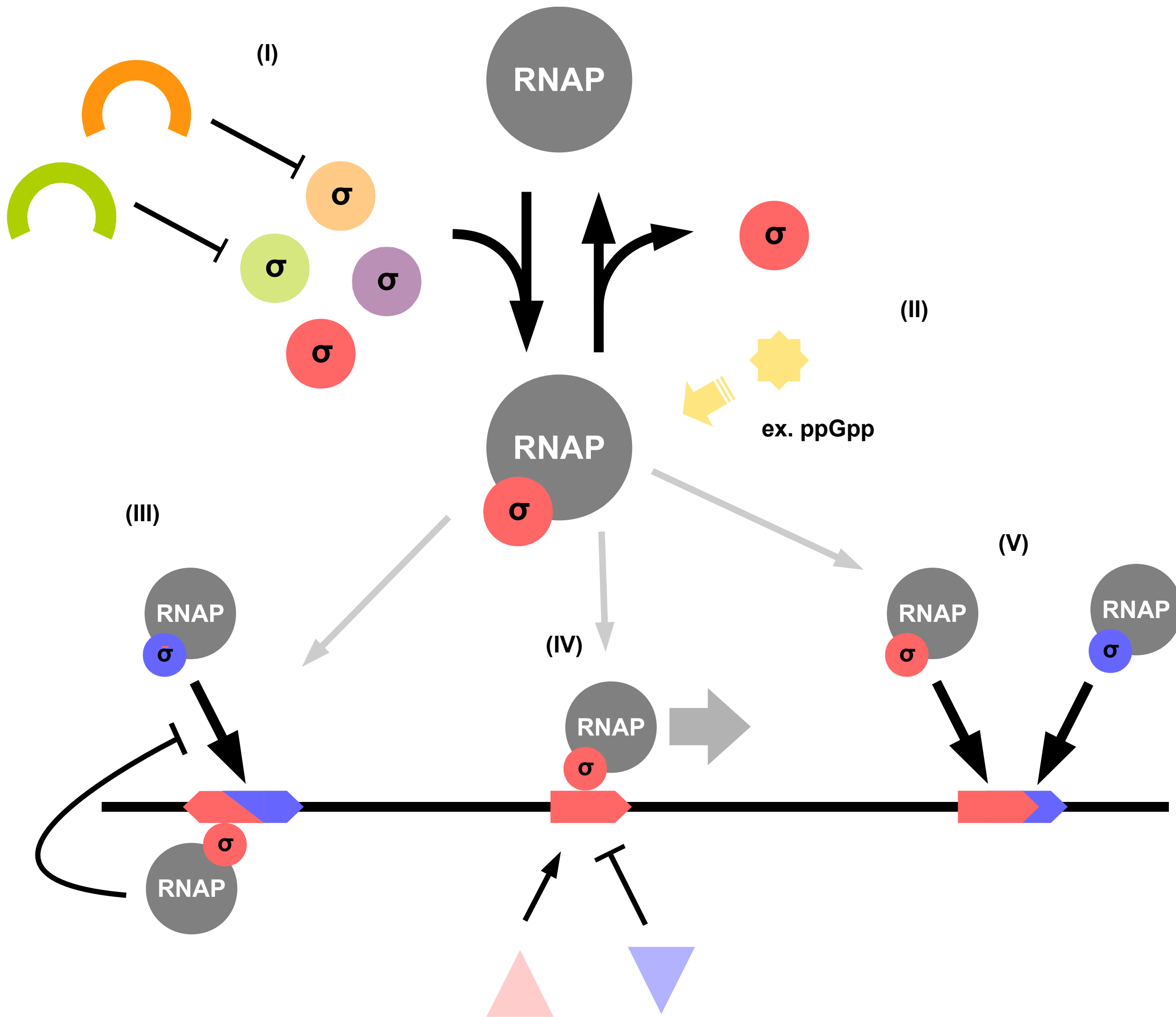

Figure 3: Scheme of sigma factor competition and its regulation. RNAP and σ represent RNA polymerase core enzyme and sigma factor, respectively. **(I)** The fuction of the sigma factor is sometimes repressed by the cognate anti-sigma factor. **(II)** Small molecules such as ppGpp can change the affinity of RNA polymerase core enzyme to sigma factor, and change the equilibrium of sigma factor competition. **(III)** Overlapping promoters especially in the reverse direction can cause transcription inhibition by RNA polymerase containing other sigma factor. **(IV)** Transcription by RNA holoenzyme can also be activated/repressed by other transcription factors. **(V)** Transcription of a single gene is initiated not only by one sigma factor but sometimes by multiple sigma factors.

1.6 Strain development using sigma factors

Regulation mechanism by sigma factors have been optimized during evolution (Cho *et al.,* 2014) Thus, genes with similar promoter sequences are under the control of the same sigma factor and their products are expected to have cooperative functions in an endogenous pathway. The Sigma factor can enhance the transcription of those endogenous genes simultaneously. Therefore, sigma factors are good targets to harness the potential of the endogenous pathways for strain development, aiming at perturbing regulatory networks and boosting some of the pathways. In fact, metabolic engineering using sigma factors induced the transcription of specific sets of genes and successfully influenced cell behavior from various perspectives (Tripathi *et al.,* 2014) (**Table 1**). For example, the potential of the alternative sigma factor for strain development was explored in cyanobacterium, *Synechocystis* sp. PCC 6803. Overexpression of the sigma factor SigE enhanced the expression of multiple sugar catabolic genes simultaneously, and its effect was successfully applied for production of biodegradable polyester, polyhydroxylbutyrate (Osanai *et al.,* 2011, 2013). On the other hand, the potential of the random mutated sigma factors was explored in the global transcription machinery engineering (gTME) approach (Alper and Stephanopoulos, 2007). In this study, the primary sigma factor in *E. coli* RpoD was randomly mutated in order to alter the promoter selectivities. The randomly mutated sigma factors were overexpressed in the wild type stain and the recombinant strain engineered for the lycopene production, and strains with higher ethanol tolerance or higher capability of lycopene production was successfully isolated after multiple selection/screening procedures.

The strain development using sigma factor is a relatively new approach, however, several studies have proven that sigma factors are good target for strain development, aiming at the global changes.

Table 1. Strain development by sigma factor

Target	Method	Purpose	Organism	Reference
RpoN	Gene overexpression	Oxytetracycline production	*E. coli*	(Stevens *et al.,* 2013)
RpoH	Gene overexpression	Protein quality improvement	*E. coli*	(Hsu *et al.,* 2014)
SigE	Gene overexpression	Polyhydroxybutyrate (PHB) production	*Synechocystis sp.* PCC 6803	(Osanai *et al.,* 2013)
SigE	Gene overexpression	Succinate production	*Synechocystis sp.* PCC 6803	(Osanai *et al.,* 2015)
SigB	Gene overexpression	Temperature and butanol tolerance	*Synechocystis sp.* PCC 6803	(Kaczmarzyk *et al.,* 2014)
Orf21	Gene overexpression	Clavulanic acid production	*Streptomyces clavuligerus*	(Jnawali *et al.,* 2011)
σ24	Adaptive evolution, gene overexpression	Ethanol tolerance and production	*Thermoanaerobacter sp.* X514	(Lin *et al.,* 2013a)
AlgU	Activation of sigma factor by mucA deletion	Alginate production	*Pseudomonas aeruginosa*	(Martin *et al.,* 1993)
AlgU	Activation of sigma factor by mucA mutation	Alginate production	*Pseudomonas fluorescens*	(Borgos *et al.,* 2013)
RpoS	Overexpression of 5′ untranslated region of *rpoS* mRNA	Polyhydroxybutyrate (PHB) production	*E. coli*	(Kang *et al.,* 2008)
RpoS	sRNAs overexpression	Acid tolerance	*E. coli*	(Jin *et al.,* 2009)
RpoS	sRNAs overexpression	Carboxylic acid and oxidative stress tolerance	*E. coli*	(Gaida *et al.,* 2013)
RpoS	sRNAs overexpression	Acid tolerance	*E. coli*	(Bak *et al.,* 2014)
RpoD	gTME	Ethanol tolerance, lycopene production	*E. coli*	(Alper and Stephanopoulos, 2007)
RpoD	gTME	Lactic acid tolerance, lactate production	*Lactobacillus plantarum*	(Klein-Marcuschamer and Stephanopoulos, 2008)
RpoD	gTME	Hyaluronic acid production	*E. coli*	(Yu and Stephanopoulos, 2008)
RpoD	gTME	Acrylamide tolerance	*Rhodococcus ruber*	(Ma and Yu, 2012)
RpoD	gTME	Ethanol tolerance	*Zymomonas mobilis*	(Tan *et al.,* 2016)
RpoD	gTME	Furfural tolerance	*Z. mobilis*	(Tan *et al.,* 2015)
RpoD	RAISE (random mutation, insertion, deletion)	Low pH tolerance	*E. coli*	(Gao *et al.,* 2016)
RpoS	Random mutagenesis	Isobutanol production	*E. coli*	(Smith and Liao, 2011)
σHrdB	Random mutation, genome shuffling, point mutation	Teicoplanin production	*Actinoplanes teichomyceticus*	(Wang *et al.,* 2014)
RpoS	Deletion	Putrescine production	*E. coli*	(Qian *et al.,* 2009)
RpoS	Deletion	1-propanol production	*E. coli*	(Choi *et al.,* 2012)
RpoN	Deletion	copolymer of lactate (LA) and 3-hydroxybutyrate (3HB) production	*E. coli*	(Kadoya *et al.,* 2015)
Sig6	Deletion	Avermectin production	*Streptomyces avermitilis*	(Jiang *et al.,* 2011)
ECF σ factors	Chimeric σ factors	Synthetic system construction	*E. coli*	(Rhodius *et al.,* 2013)
Orthogonal σ factors	Bisected T7 polymerase	Synthetic system construction	T7 phage	(Segall-Shapiro *et al.,* 2014)

This table 1 in (Tripathi *et al.,* 2014) is used as reference. Some new studies were added based on the search in Pubmed (http://www.ncbi.nlm.nih.gov/pubmed).

1.7 Corynebacterium glutamicum

C. glutamicum was first isolated as an organism secreting high amounts of L-glutamate (Kinoshita *et al.,* 1957), and has been used for industrial production of L-glutamate and L-lysine in the million tons scale (Eggeling and Bott, 2005). Since its importance in industry, substantial knowledge has been accumulated about its genetic information, metabolic pathways and regulatory networks as well as fermentation conditions (Wendisch *et al.,* 2006). Biologically, *C. glutamicum* is classified as Gram-positive bacteria, and belongs to the CMN (*Corynebacterium*, Mycobacterium, Nocardia) group, which is characterized by the existence of an unique cell envelope structure, mycomembrane (Burkovski, 2013). The CMN group includes pathogenic bacteria such as *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis.* The mycomembrane is suggested to be important for the antibiotics resistance of those organisms (Marrakchi *et al.,* 2014). *C. glutamicum* is easy to handle due to its non pathogenicity, and its metabolic pathway for the mycomembrane synthesis is simple compared to *M. tuberculosis* (Puech *et al.,* 2000). Therefore, *C. glutamicum* is also considered as a model organism for understanding mycomembrane synthesis (Marchand *et al.,* 2012). Furthermore, *C. glutamicum* is known to naturally produces C50 carotenoid, decaprenoxanthin (Krubasik *et al.,* 2001).

For industrial purpose, L-glutamate or L-lysine producing strains of *C. glutamicum* have been intensively developed based on random mutagenesis and/or rational engineering (Becker and Wittmann, 2012). In addition, *C. glutamicum* has been engineered to produce various chemicals such as other α-amino acids, α,ω-diacids, keto acids, α,ω-diamines, ω-amino acids, carotenoids and terpenes, some of which are not produced naturally by this bacterium (Becker and Wittmann, 2012; Zahoor *et al.,* 2012; Wendisch, 2014; Heider and Wendisch, 2015). Metabolic engineering also allows this bacterium to utilize alternative carbon sources such as glycerol, xylose and starch (Zahoor *et al.,* 2012). Therefore, various platform strains are available which can produce or utilize a wide spectrum of chemical compounds.

1.8 Global regulators in *C. glutamicum*

The complete genome sequence of *C. glutamicum* and omics analysis makes it possible to understand the regulatory architecture of *C. glutamicum* from the global point of view (Ikeda and Nakagawa, 2003; Kalinowski *et al.,* 2003). The transcriptional regulatory repertoire of *C. glutamicum* is composed of 129 DNA-binding transcriptional regulators with helix-turn-helix (HTH) motifs, 13 response regulators of two-component systems, 10 not well characterized regulators and seven sigma factors (Brinkrolf *et al.,* 2006, 2010). Several studies suggested that the regulatory networks of *C. glutamicum* is a multi-layered hierarchy with global regulatory factors, local regulatory factors, and sigma factors (Schröder and Tauch, 2010). For example, GlxR is known as a global transcriptional regulator and senses the change of intracellular cAMP concentration (Kim *et al.,* 2004). In addition to GlxR, RamA and RamB serve as transcriptional regulators of acetate metabolism (Auchter *et al.,* 2011). On the other hand, SugR is known as a transcriptional regulator of glycolysis (Engels *et al.,* 2008). Other transcriptional regulators are known such as AmtR for nitrogen metabolism (Jakoby *et al.,* 2000), PhoR for phosphate metabolism (Kocan *et al.,* 2006) and DtxR and RipA for iron metabolism (Wennerhold and Bott, 2006).

Strain development using regulatory proteins has been applied in *C. glutamicum* by deleting pathway-specific regulators such as LbtR (Bückle-Vallant *et al.,* 2014) or ArgR (Hwang *et al.,* 2008) or global regulators such as SugR (Blombach *et al.,* 2009b). Furthermore, random mutagenesis and following screening revealed that the mutation of the global transcription regulator GlxR decreased byproduct formation of L-alanine in the L-valine producing strain (Mahr *et al.,* 2015). However, the strain development using sigma factors has not been reported yet in *C. glutamicum*.

1.9 Sigma factors of *C. glutamicum*

C. glutamicum possesses seven sigma factor genes, *sigA*, *sigB*, *sigC*, *sigD*, *sigE*, *sigH* and *sigM* (Kalinowski *et al.,* 2003) (**Table 2**). Based on their amino acid sequence and conserved domain, SigA and SigB are classified as Group 1 and Group 2 sigma factors, respectively, and the other 5 sigma factors are classified as Group 4 sigma factors. *C. glutamicum* does not have any Group 3 sigma factors (**Figure 4**). The function of each sigma factor is elucidated by several studies (Pátek and Nešvera, 2011).

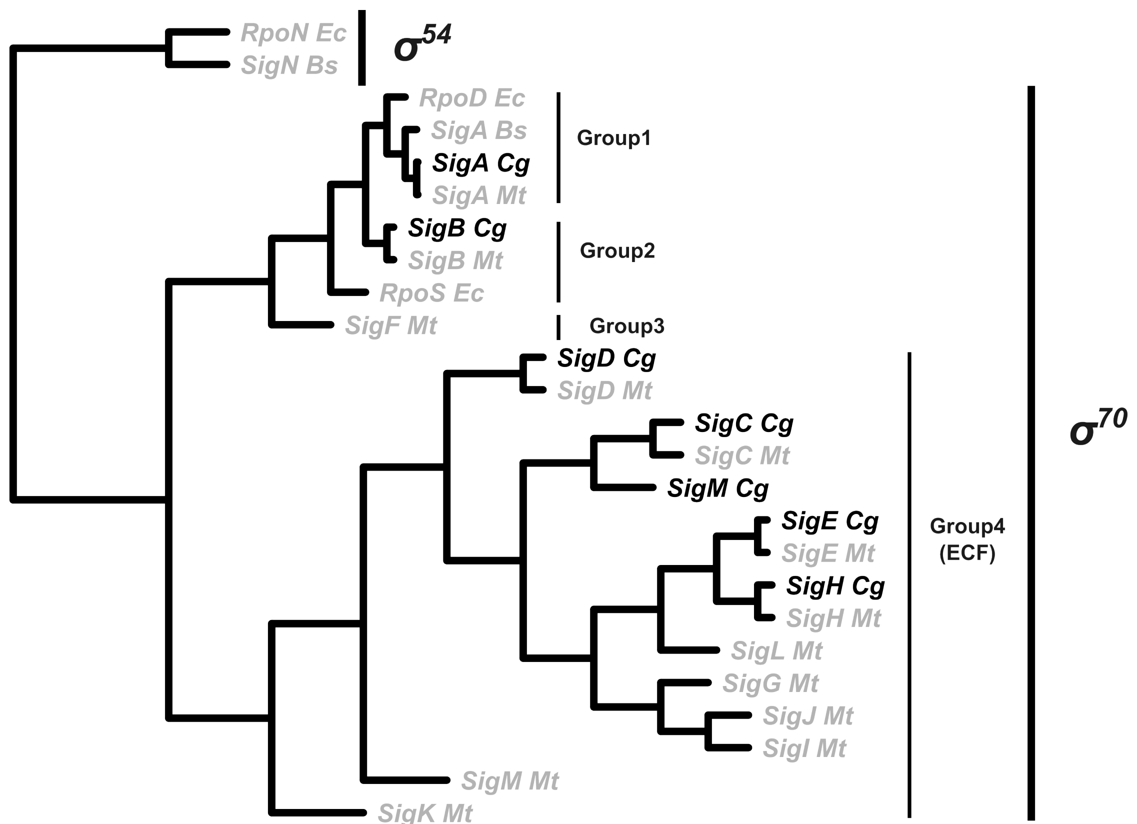

Figure 4: Phylogenetic tree of the sigma factors of *C. glutamicum*. The phylogenetic tree was constructed based on amino acid sequences of all seven sigma factors of *C. glutamicum*, all thirteen sigma factors of *M. tuberculosis*, three sigma factors of *E. coli*, and two sigma factors of *B. subtilis*. Calculation was performed with Clustal Omega and ClustalW2_ Phylogeny (Larkin *et al.,* 2007; Sievers *et al.,* 2011) (http://www.ebi.ac.uk/), and visualization of phylogenetic tree was performed with iTOL (http://itol.embl.de/). The following settings were used. Distance correction: on, Excludes gaps: on, Clustering method: UPGMA. **Ec**: *E. coli*, **Bs**: *B. subtilis*, **Mt**: *M. tuberculosis*, **Cg:** *C. glutamicum*.

Table 2. Sigma factors in *C. glutamicum*

Gene	*sigA*	*sigB*	*sigC*	*sigD*	*sigE*	*sigH*	*sigM*
Gene id	cg2092	cg2102	cg0309	cg0696	cg1271	cg0876	cg3420
ORF size	1485	996	582	567	651	621	606
Initiation	1981923	1990902	274120	614617	1167205	805881	3272969
Termination	1983407	1991897	273539	615183	1167855	806501	3273574
Direction	+	+	-	+	+	+	+
Upstream gene	***ppgK***	**cg2101**	**cg0308**	***whiB3***	**cg1270**	**cg0875**	**cg3419**
Function	glucokinases	putative D-Tyr-tRNA tyr deacylase	putative membrane protein	transcription factor	putative o-methyl transferase	hypothetical protein	virulence factor homolog
Initiation	1980681	1990341	271980	613876	1167080	805713	3269608
Termination	1981433	1990775	273197	614175	1166439	804601	3272715
Direction	+	+	+	+	-	-	+
Space	489	126	341	441	124	167	253
Downstream gene	**cg2094**	***dtxR***	***katA***	**cg0697**	**cg1272**	**cg0877**	***trxB***
Function	hypothetical protein	regulator of iron metabolism	catalase	putative anti-sigma factor	anti-sigma factor	anti-sigma factor	thioredoxin reductase
Initiation	1984044	1992314	274324	615183	1168044	806504	3273679
Termination	1984148	1993000	275874	616211	1168538	806733	3274632
Direction	+	+	+	+	+	+	+
Space	2725	1555	2139	1306	774	787	3965

Genes upstream or downstream of each sigma factor are shown. "ORF size" represents the base number of open reading frame of gene. Initiation and termination base position is shown based on the genomic sequence (Kalinowski *et al.,* 2003). "Direction" shows the gene direction in the genome. For upstream and downstream of each sigma factor gene and their fuctions are shown based on CoryneRegNet (http://coryneregnet.compbio.sdu.dk/v6/index.html). Space represents the nucleotide between sigma factor gene and the gene mentioned.

SigA belongs to Group 1 sigma factors which serves as the primary sigma factor (Pátek *et al.,* 2003). The consensus promoter sequence of SigA was determined from 159 promoters which are assumed to be SigA-dependent (TTGNCA as -35 box sequence and gnTANANTng as -10 box sequence with extended -10 box sequence in lower case, N represents the conservation less than 35%) (Pátek and Nešvera, 2011). SigA is essential for cell survival, and it is responsible for transcription of many housekeeping genes especially in the exponential growth phase. From RNA-seq analysis, more than 2000 transcriptional start sites under unstressed condition were identified, and most of them are expected to be SigA-dependent (Pfeifer-Sancar *et al.,* 2013). Transcriptional regulation of *sigA* is not elucidated very well, however, there are several promoter sequences upstream of *sigA*, and one of them is known to be weakly regulated by SigH (Toyoda *et al.,* 2015). The number of *sigA* transcripts decreases at the transition from the exponential growth phase to the stationary phase (Larisch *et al.,* 2007).

SigB belongs to Group 2 sigma factors, and it recognizes the similar promoter sequence as SigA (Pátek and Nešvera, 2011). SigB is not essential for cell growth, and its transcription level increases during the transition from the exponential growth phase to the stationary phase (Larisch *et al.,* 2007). Therefore, SigB is expected to play an important role especially in the transition and the stationary phase (Cho *et al.,* 2012; Kim *et al.,* 2016). Disruption of *sigB* suggested that SigB is related to the positive regulation of glucose consumption especially under oxygen deprivation (Ehira *et al.,* 2008).

SigC is classified as ECF36 type of Group 4 sigma factors (Staroń *et al.,* 2009). No anti-sigma factor is found for this type of sigma factor. Recently, transcriptome analysis revealed that SigC regulates the aerobic respiratory system in *C. glutamicum* (Toyoda and Inui, 2016). More precisely, SigC activates the compromised aerobic respiration that suppresses the production of reactive oxygen species under low oxygen conditions. Furthermore, SigC is directly or indirectly activated by impaired electron transfer via cytochrome aa3 (Toyoda and Inui, 2016).

SigD belongs to ECF40 type sigma factors (Staroń *et al.,* 2009). This type of sigma factor often has an anti-sigma factor gene at the neighbor locus of a sigma factor gene. SigD in *M. tuberculosis* is also classified as this type, and its relation to virulence is suggested from transcriptome analysis (Raman *et al.,* 2004; Calamita *et al.,* 2005). In *C. glutamicum*, selection of

high oxygen requiring strains from transposon library suggested that SigD is responsible for oxygen limitation response (Ikeda *et al.,* 2009). However, its impact on transcription has not been elucidated yet.

SigE is classified as ECF14 type sigma factors (Staroń *et al.,* 2009). This type of sigma factor is only found in Actinobacteria, and its activity is usually regulated by the cognate anti-sigma factor. Furthermore, O-methyltransferase is located directly upstream of this sigma factor gene, and this genomic context is well conserved (Staroń *et al.,* 2009). Therefore, the involvement of this methyltransferase in regulation of SigE is expected. In *C. glutamicum*, *sigE* is located between genes encoding the putative methyltransferase and the anti-sigma factor for SigE, CseE. The effect of deleting *sigE* or *cseE* on cell behavior was studied in detail (Park *et al.,* 2008). Deletion of *sigE* led to an increase of sensitivity to magnesium deficiency, SDS, lysozyme, EDTA, heat and several antibiotics. Therefore, its role in response to cell surface stresses is implied. Furthermore, deletion of the anti-sigma factor gene, *cseE*, retarded the growth in MCGC minimal medium, so the appropriate regulation of SigE activity seems to be important for growth under the tested condition. The global effect on transcription by SigE has not been elucidated yet .

SigH is one of the most studied sigma factors in *C. glutamicum*, and it belongs to ECF12 type sigma factors (Staroń *et al.,* 2009). Most sigma factors in this group are known to be associated with the cognate anti-sigma factor containing a ZAS domain, which changes the conformation in response to the oxidative stress by reversible disulfide bond formation (Hillion and Antelmann, 2015). In *C. glutamicum*, several studies elucidated the regulation by SigH from different perspectives. Deletion of *sigH* led to the increase of sensitivity to thiol specific oxidation induced by diamide (Kim *et al.,* 2005a). Transcriptome analysis revealed a SigH regulon, which is related to protein quality control, oxidative/disulfide stress response, redox homeostasis, pentose phosphate pathway, riboflavin biosynthesis and zinc uptake (Ehira *et al.,* 2009; Busche *et al.,* 2012; Toyoda *et al.,* 2015; Taniguchi and Wendisch, 2015). Activity of SigH is inhibited by the anti-sigma factor, RshA, containing ZAS domain and transcription of *rshA* is SigH-dependent (Busche *et al.,* 2012).

SigM belongs to ECF27 type sigma factors, and this type of sigma factor is expected to be regulated by an anti-sigma factor (Staroń *et al.,* 2009). However, the anti-sigma factor for SigM in *C. glutamicum* is not yet known. Deletion of *sigM* and following transcriptome analysis revealed

that SigM is related to oxidative/disulfide stress response along with SigH (Nakunst *et al.,* 2007).

1.10 Objectives

The aim of this study is to explore the potsential of sigma factors as targets for strain development in *C. glutamicum*. Sigma factors play important roles in the transcriptional regulation through the sigma factor competition. Therefore, it is hypothesized that the artificial expression of sigma factor genes will change the ratio of sigma factors in cells and perturb the sigma factor competition. Such perturbation of the sigma factor competition will induce the changes of the transcriptional profile by enhancing the expression of different sets of genes. Since the expression of those set of genes is under the control of the endogenous regulations, proteins encoded by those genes are expected to have related functions and work cooperatively as one of the parts of endogenous cellular mechanisms. Thus, overexpression of sigma factor genes has potential to upregulate the expression of a set of genes and harness the endogenous regulations. Since bacteria usually possess multiple genes of sigma factors and each of them recognizes the different promoter sequences, the overexpression of different sigma factor genes can increase the expression of different sets of genes and perturb the regulatory networks differently. Furthermore, the expression levels of sigma factor genes can be tuned by the inducible promoter. Therefore, the perturbation of regulatory networks can be performed effectively using two different means (i) sigma factor genes for overexpression and (ii) the expression level of the sigma factor. In this approach, the number of strains and conditions is capable to be handled, on the other hand, the big effect on cell behavior is expected. In addition, unexpected effects observed by this approach will be helpful to understand the complex regulatory networks and mechanisms to cope with the environmental changes.

2 Result

The result parts are divided into three parts. The first part focuses on the effect of *sigH* overexpression on riboflavin accumulation and its potential use for FMN production. This manuscript is formatted based on “Taniguchi H and Wendisch VF (2015) Exploring the role of sigma factor gene expression on production by *Corynebacterium glutamicum*: sigma factor H and FMN as example. *Front. Microbiol*. 6:740. doi: 10.3389/fmicb.2015.00740”. The second part focuses on the role of SigD for transcriptional regulation and its relation to cell envelope integrity. The third part focuses on the effect of sigma factor overexpression on lycopene production as an example for its potential application in the strain development.

2.1 Exploring the role of sigma factor gene expression on production by *Corynebacterium glutamicum*: sigma factor H and FMN as example

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2.1.1 Abstract

Bacteria are known to cope with environmental changes by using alternative sigma factors binding to RNA polymerase core enzyme. Sigma factor is one of the targets to modify transcription regulation in bacteria and to influence production capacities. In this study, the effect of overexpressing each annotated sigma factor gene in *Corynebacterium glutamicum* WT was assayed using an IPTG inducible plasmid system and different IPTG concentrations. It was revealed that growth was severely decreased when *sigD* or *sigH* were overexpressed with IPTG concentrations higher than 50 µM. Overexpression of *sigH* led to an obvious phenotypic change, a yellow-colored supernatant. High performance liquid chromatography analysis revealed that riboflavin was excreted to the medium when *sigH* was overexpressed and DNA microarray analysis confirmed increased expression of riboflavin biosynthesis genes. In addition, genes for enzymes related to the pentose phosphate pathway and for enzymes dependent on flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), or NADPH as cofactor were upregulated when *sigH* was overexpressed. To test if *sigH* overexpression can be exploited for production of riboflavin-derived FMN or FAD, the endogenous gene for bifunctional riboflavin kinase/FMN adenyltransferase was co-expressed with *sigH* from a plasmid. Balanced expression of *sigH* and *ribF* improved accumulation of riboflavin (19.8 ± 0.3 µM) and allowed for its conversion to FMN (33.1 ± 1.8 µM) in the supernatant. While a proof-of-concept was reached, conversion was not complete and titers were not high. This study revealed that inducible overexpression of sigma factor genes at different level is an interesting approach to switch gene expression profiles and to discover untapped potential of bacteria for chemical production.

2.1.2 Introduction

The sigma factor is a component of RNA polymerase holoenzyme and is important to recognize the promoter sequence in transcription initiation (Vassylyev *et al.,* 2002; Feklístov *et al.,* 2014). In

general, a bacterium possesses two or more sigma factor genes, and RNA polymerase holoenzymes with different sigma factors recognize distinct promoter sequences (Paget and Helmann, 2003; Staroń *et al.,* 2009). Upon environmental stress the vegetative sigma factor may be replaced by an alternative sigma factor, a mechanism wide-spread in bacteria to cope with environmental changes (Kazmierczak *et al.,* 2005; Sharma and Chatterji, 2010). This fundamental mechanism of transcriptional regulation has recently drawn attention as a candidate of metabolic engineering for global transcriptional engineering (Tripathi *et al.,* 2014).

Corynebacterium glutamicum was isolated as a glutamate-producing organism in 1956 and has been used for the large scale production of glutamate and lysine for more than five decades (Burkovski, 2008; Eggeling and Bott, 2005, 2015; Yukawa and Inui, 2013). Amino acid producing strains have been developed based on random mutagenesis and/or rational engineering. For instance, this bacterium has been engineered to produce amino acids such as L-serine (Peters-Wendisch *et al.,* 2005), L-isoleucine (Morbach *et al.,* 1996), L-valine (Radmacher *et al.,* 2002; Blombach *et al.,* 2007), L-proline (Jensen and Wendisch, 2013), L-tryptophan (Ikeda and Katsumata, 1999), L-citrulline (Eberhardt *et al.,* 2014), or L-arginine (Park *et al.,* 2014). It has been also engineered to produce precursors of amino acids such as 2-ketoisovalerate (Krause *et al.,* 2010) and 2-ketoisocaproate (Bückle-Vallant *et al.,* 2014; Vogt *et al.,* 2015) or amino acid-derived compounds such 1,4-diaminobutane (Schneider *et al.,* 2012; Schneider and Wendisch, 2010) or 1,5-diaminopentane (Mimitsuka *et al.,* 2007). Metabolic engineering focused mainly on amino acid biosynthesis, precursor supply, cofactor regeneration and amino acid transport. Concerning metabolic engineering targeting at regulatory network, mainly feedback-resistant versions of key enzymes are in use, however, also transcriptional regulatory engineering has been applied, e.g., by deletion of the genes encoding pathway-specific regulators such as LbtR (Bückle-Vallant *et al.,* 2014) or ArgR (Hwang *et al.,* 2008) or higher order regulators such as SugR (Blombach *et al.,* 2009a). However, global regulatory engineering using sigma factor genes has not yet been explored.

C. glutamicum WT possesses seven sigma factor genes encoded on its chromosome (Kalinowski *et al.,* 2003). These sigma factors are classified into group 1 (SigA), group 2 (SigB) and group 4 (SigC, SigD, SigE, SigH, SigM) according to their conserved structures. *C. glutamicum* lacks group 3 type sigma factors (Pátek and Nešvera, 2011). The regulons of some of

these sigma factors have been studied, e.g., for SigA, SigB, SigE, SigH, and SigM. SigA is the principle sigma factor and related to the transcription initiation of housekeeping genes (Pfeifer-Sancar *et al.,* 2013). The gene *sigA* is essential in *C. glutamicum* as well as in other bacteria (Pátek and Nešvera, 2011). SigB is related to the general stress response and assumed to play an important role at the transition from the exponential to the stationary growth phase (Larisch *et al.,* 2007). Analysis of the *sigB* deletion mutant revealed that SigB is involved in glucose metabolism under oxygen deprivation conditions, thymidylate synthesis and protein secretion (Cho *et al.,* 2012; Ehira *et al.,* 2008; Watanabe *et al.,* 2013). The functions of SigC and SigD have not yet been elucidated. SigE is related to surface stress and its activity is repressed by anti-sigma factor CseE (Park *et al.,* 2008). SigH is involved in the response to heat shock, pH stress and disulfide/oxidative stress (Kim *et al.,* 2005a; Ehira *et al.,* 2008; Barriuso-Iglesias *et al.,* 2013), and its activity is repressed by anti-sigma factor RshA (Busche *et al.,* 2012). Recently, SigH-dependent promoters were studied by ChIP-chip analysis (Toyoda *et al.,* 2015). SigM is involved in transcription of disulfide stress related genes (Nakunst *et al.,* 2007).

In this study, the effects of graded sigma factor gene overexpression on *C. glutamicum* have been characterized. Based on the finding that *sigH* overexpression resulted in riboflavin production, flavin mononucleotide (FMN) producing *C. glutamicum* strains have been constructed.

2.1.3 <u>Material and methods</u>

2.1.3.1 Bacterial strains, plasmid and primer

The strains, plasmids and oligonucleotides used in this work are listed in **Table 3**. Plasmids were constructed based on pEKEx3 and pVWEx1, IPTG inducible *Escherichia coli* – *C. glutamicum* shuttle vectors (Peters-Wendisch *et al.,* 2001; Stansen *et al.,* 2005). The DNA sequence of sigma factor gene was amplified from genomic DNA of *C. glutamicum* WT by polymerase chain reaction (KOD, Novagen, Darmstadt, Germany) with respective primer pairs in **Table 3**. The PCR product was inserted into BamHI-digested pEKEx3 or pVWEx1 plasmid by Gibson assembly (Gibson *et al.,* 2009). *E. coli* DH5α was used for cloning. *E. coli* competent cells were transformed by heat shock method (Sambrook, 2001) or by electroporation method (Nováková *et al.,* 2014). All cloned DNA fragments were confirmed to be correct by sequencing. *C. glutamicum* competent cells were transformed by electroporation at 2.5 kV, 200 Ω, and 25 μF (van der Rest *et al.,* 1999; Eggeling and Bott, 2005)

Table 3 . Bacterial strains, plasmids and oligonucleotides used in this study

Bacterial strain	Relevant characteristic	Reference or source
E. coli		
DH5α	F−thi-1 *endA*1 *hsdR*17(r−, m−) *supE*44 Δ*lacU*169 (Φ80*lacZ*ΔM15) *recA*1 *gyrA*96 *relA1*	Bethesda Research Laboratories
C. glutamicum		
WT	Wild type, ATCC 13032	ATCC
Plasmid	**Relevant characteristic**	**References**
pEKEx3	SpecR; *E. coli*-*C. glutamicum* shuttle vector for regulated gene expression (Ptac, *lacIq*, pBL1 oriVCg)	(Stansen *et al.,* 2005)
pVWEx1	KanR; *E. coli*-*C. glutamicum* shuttle vector for regulated gene expression (Ptac, *lacIq*, pCG1 oriVCg)	(Peters-Wendisch *et al.,* 2001)
pEKEx3-*sigA*	SpecR, pEKEx3 with *sigA* from *C. glutamicum* WT	this study
pEKEx3-*sigB*	SpecR, pEKEx3 with *sigB* from *C. glutamicum* WT	this study
pEKEx3-*sigC*	SpecR, pEKEx3 with *sigC* from *C. glutamicum* WT	this study
pEKEx3-*sigD*	SpecR, pEKEx3 with *sigD* from *C. glutamicum* WT	this study
pEKEx3-*sigE*	SpecR, pEKEx3 with *sigE* from *C. glutamicum* WT	this study
pEKEx3-*sigH*	SpecR, pEKEx3 with *sigH* from *C. glutamicum* WT	this study
pEKEx3-*sigM*	SpecR, pEKEx3 with *sigM* from *C. glutamicum* WT	this study
pVWEx1-*sigH*	KanR, pVWEx1 with *sigH* from *C. glutamicum* WT	this study
pEKEx3-*ribF*	SpecR, pEKEx3 with *ribF* from *C. glutamicum* WT	this study
pVWEx1-*ribF*	KanR, pVWEx1 with *ribF* from *C. glutamicum* WT	this study
Oligonucleotide	Sequence (5'-3')	References
sigA-fwd	GCCTGCAGGTCGACTCTAGAGGAAAGGAGGCCCTTCAGATGGTAGAAAAC AACGTAGCAAAAAAGACGGTCG	this study
sigA-rev	CGGTACCCGGGGATCTTAGTCCAGGTAGTCGCGAAGGACCTG	this study
sigB-fwd	GCCTGCAGGTCGACTCTAGAGGAAAGGAGGCCCTTCAGATGACAGCACCG TCCACGCAG	this study
sigB-rev	CGGTACCCGGGGATCTTACTGGGCGTACTCACGAAGACGTG	this study
sigC-fwd	GCCTGCAGGTCGACTCTAGAGGAAAGGAGGCCCTTCAGGTGAAGTCAAAA GAGCGTAACGACGC	this study
sigC-rev	CGGTACCCGGGGATCCTAACCTTGGGCGGATTTGCCATCTTCG	this study
sigD-fwd	GCCTGCAGGTCGACTCTAGAGGAAAGGAGGCCCTTCAGTTGGCTGATACTG AGCGCGAGCTC	this study
sigD-rev	CGGTACCCGGGGATCTTACTTGTTCTCCTGCTGCTCAAGTGTGCTTC	this study
sigE-fwd	GCCTGCAGGTCGACTCTAGAGGAAAGGAGGCCCTTCAGATGACTTATATGA AAAAGAAGTCCCGAGATGACGCAC	this study
sigE-rev	CGGTACCCGGGGATCTTAGTGGGTTGGAACCAACAAAGAAACTTCCTCG	this study
sigH-fwd	GCCTGCAGGTCGACTCTAGAGGAAAGGAGGCCCTTCAGATGGCTGAAAAC CGAACCGGCAC	this study
sigH-rev	CGGTACCCGGGGATCTTATGCCTCCGAATTTTTCTTCATGTCGGGATG	this study
sigM-fwd	GCCTGCAGGTCGACTCTAGAGGAAAGGAGGCCCTTCAGATGACAGTACTGC CTAAAAACCATGACCTAAGC	this study
sigM-rev	CGGTACCCGGGGATCTCAGTTGCTTTCGCACTGTATGGAGCC	this study
ribF-fwd	GCCTGCAGGTCGACTCTAGAGGAAAGGAGGCCCTTCAGGTGGATATTTGGA GTGGACT	this study
ribF-rev	CGGTACCCGGGGATCTTAAGCGCTGGGCTGGGTGT	this study

Underlined sequences represent the overlap region with vector plasmid; sequences in bold italic represent ribosome binding sites; sequences in bold represents the translational start codons.

2.1.3.2 Medium and growth condition

C. glutamicum was precultured in BHI or LB medium overnight, washed once with CGXII medium (Eggeling and Bott, 2005) without carbon source and inoculated in CGXII with 222 mM of glucose at initial OD at 600 nm of 1. The OD was measured with UV-1202 spectrophotometer (Shimadzu, Duisburg, Germany) with suitable dilutions. When appropriate, 100 µg/mL of spectinomycin, 25 µg/mL of kanamycin and IPTG were added. Growth experiment with BioLector® cultivation system (m2pLabs, Baesweiler, Germany) was performed in 1 mL of CGXII using FlowerPlate® (m2pLabs, Baesweiler, Germany) at 30°C, 1,100 rpm. Cell growth was monitored online every 10 min for 48 h. Maximum growth rate µ (h-1) was calculated from 20 measuring points of arbitrary unit of backscattering light (620 nm). Plate image was scanned with Perfection V750-M Pro scanner (Epson, Ludwigshafen am Rhein, Germany). Color balance of blue against yellow was set to +70.

2.1.3.3 Riboflavin production experiments

Riboflavin production experiments were performed at 30°C, 120 rpm in 50 mL of GCXII with 222 mM of glucose and 15 µM of IPTG using 500 mL baffled flasks. Supernatant was separated by centrifugation after 48 h of cultivation. Riboflavin concentration of cell-free supernatant was analyzed using high performance liquid chromatography (HPLC; Agilent Technologies Sales & Services GmbH & Co. KG, Waldbronn, Germany). The confirmation and quantification of riboflavin was performed using diode array detector (DAD). Samples were separated with a column system consisting of a precolumn (LiChrospher 100 RP18 EC-5µ (40 mm × 4 mm), CS-Chromatographie Service GmbH, Langerwehe, Germany) and a main column (LiChrospher 100 RP18 EC-5µ (125 mm × 4 mm), CS Chromatographie Service GmbH, Langerwehe, Germany) with 0.1 M sodium acetate, pH 7.2 supplemented with 0.03% sodium azide (A) and methanol (B) as the mobile phase. The following gradient was used at a flow rate of 1.2 mL/min; 0 min B: 20%, 0.5 min B: 38%, 2.5 min B: 46%, 3.7 min B: 65%, 5.5 min B: 70%, 6 min B: 75%, 6.2 min B: 85%, 6.7 min B: 20%, 8.9 min B: 20%.

2.1.3.4 Transcriptome analysis of *sigH* overexpressing strain using DNA microarray

C. glutamicum strains WT(pEKEx3) and WT(pEKEx3-*sigH*) were cultured in BHI medium and inoculated into CGXII medium with 222 mM of glucose for adaptation. Cells were cultured overnight and inoculated into 50 mL of CGXII medium with 222 mM of glucose and 10 or 15 µM of IPTG at the initial OD of 1. Cells were harvested in the early exponential growth phase (OD between 6 and 8) and RNA isolation was performed as described previously (Wendisch, 2003).

The purified RNA was analyzed by spectrophotometer (NanoDrop) for quantity and gel electrophoresis for quality. The RNA sample was stored at -80°C until further use. cDNA synthesis from total RNA as well as DNA microarray hybridization were performed as described previously (Netzer *et al.,* 2004; Polen *et al.,* 2007). Normalization and evaluation of the microarray data was done with the software package EMMA 2 (Dondrup *et al.,* 2009). Genes which were expressed more in WT(pEKEx3-*sigH*) under both 10 and 15 µM of IPTG concentration were taken into account for further analysis (p-value < 0.05, M-value > 1).

2.1.3.5 Measurement of glucose-6-phosphate 1-dehydrogenase enzyme activities

Enzyme activities of glucose-6-phosphate 1-dehydrogenase in *C. glutamicum* WT (pEKEx3) and *C. glutamicum* WT(pEKEx3-*sigH*) were measured in cell free crude extracts, which were prepared as described previously (Stansen *et al.,* 2005) with some modification. Shortly, cells grown in CGXII medium with 222 mM of glucose and 15 µM of IPTG were harvested in the exponential growth phase (OD around 6), washed once with disruption buffer (50 mM Tris-HCl pH 8.5, 10 mM MgCl2, and 1 mM DTT) and stored at -20°C until use. Protein concentrations were determined with the Bradford reagent using bovine serum albumin as a standard. Enzyme activities were measured spectrophotometrically following NADPH formation at 30°C in final volume of 1 mL. The concomitant formation of NADPH was measured at 340 nm and absorption coefficient of 6.3 mM-1 cm-1 at 340 nm was used for calculating enzyme activities. The assay contained 50 mM Tris-HCl pH 8.5, 10 mM MgCl2, 100 mM NADP+ and 100 mM glucose-6-phosphate.

2.1.3.6 FMN and FAD production experiments

FMN and FAD production experiment was performed at 30°C with 120 rpm in 50 mL of GCXII with 222 mM of glucose using 500 mL baffled flasks. 100 µM of IPTG was added after OD reached around 10. Supernatant was separated by centrifugation after 48 h of cultivation. FMN and FAD concentration of cell-free supernatant was analyzed as described previously with some modifications (Barile *et al.,* 1997). Shortly, signal was detected with fluorescent detector (FLD; excitation and emission wavelengths of 450 and 520 nm, respectively) and samples were separated with the same column systems used in riboflavin production experiments with 20 mM potassium phosphate, pH 6.0 (A) and methanol (B) as the mobile phase. The following ratio was used at a flow rate of 1.0 mL/min; 0–5 min B: 25%, 5–10 min B: 50%.

2.1.4 Results

2.1.4.1 Effect of overexpressing sigma factor genes in *C. glutamicum*

To investigate the influence of overexpressing sigma factor genes in *C. glutamicum*, each sigma factor gene (*sigA*, *sigB*, *sigC*, *sigD*, *sigE*, *sigH*, and *sigM*) was cloned into IPTG-inducible expression vector pEKEx3 and transformed into *C. glutamicum* WT. Growth of these strains and of a control strain containing the empty vector pEKEx3 was monitored in the presence of different IPTG concentrations (0, 5, 15, 50, or 250 µM) in CGXII medium containing 222 mM of glucose. Growth of the control strains was not affected by IPTG, while sigma factor gene transformants grew with lower growth rates at higher IPTG concentrations. In particular, *sigD* and *sigH* transformants exhibited strongly reduced growth rates with 50 and 250 µM IPTG and did not reach the stationary phase during 48 h of cultivation (**Figure 5.** A). Interestingly, the cultures of the *sigH* transformant with up to 15 µM IPTG were colored yellow (**Figure 5.** B). Therefore, the supernatants of all cultures were analyzed by recording absorbance spectra from 350 to 600 nm (**Figure 6**). While absorbance of the different supernatants varied to some degree when comparing the different transformants, the supernatant of *sigH* transformant induced with 10 µM IPTG showed a strong absorbance centered at about 450 nm. Since the *sigH* transformant did not grow when induced with higher IPTG concentrations, this absorbance peak was not observed under these conditions.

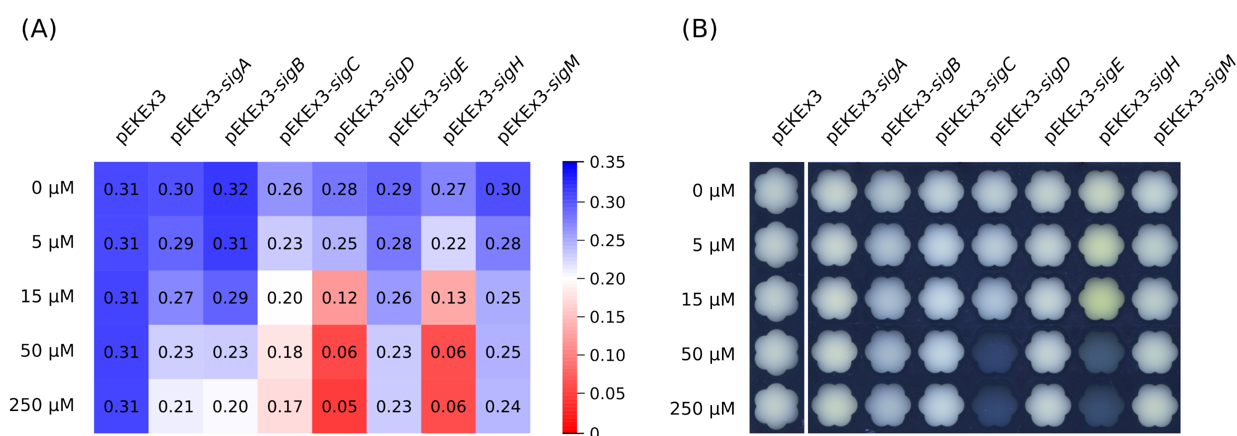

Figure 5. Maximum growth rates of cultivations of *C. glutamicum* wild type (WT) transformed with plasmids for overexpression of various sigma factor genes (A) and images of these cultures taken after 48 h of cultivation (B). CGXII with 222 mM of glucose was used as growth medium. The different IPTG concentrations used to induce sigma factor gene overexpression are indicated. The growth rates (h^{-1}) depicted in (A) are color-coded from red to blue according to the given scale.

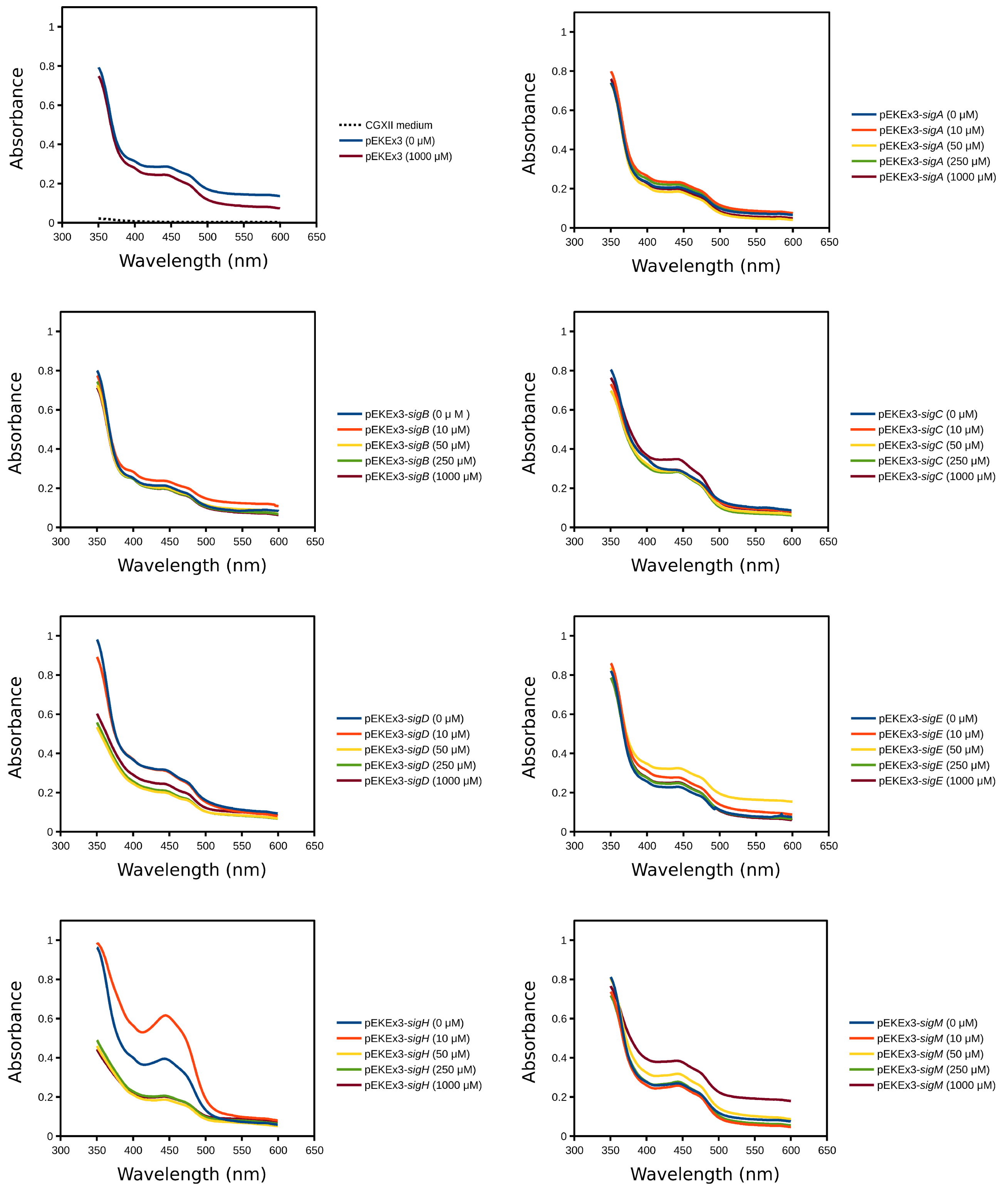

Figure 6. Absorption spectra of supernatants of cultures of *C. glutamicum* WT transformed with plasmids for overexpression of various sigma factor genes. CGXII with 222 mM of glucose was used as growth medium. The IPTG concentrations and the plasmids used for overexpression of sigma factor genes are indicated. Supernatants were analyzed after 48 h of cultivation.

2.1.4.2 Overexpression of *sigH* resulted in riboflavin secretion

To verify the yellow color phenotype of WT(pEKEx3-*sigH*) in a different cultivation setting, this strain was grown in shake flasks and induced with 15 µM IPTG immediately after inoculation. The cultures in shake flasks and the supernatants showed yellow color. Spectrophotometric analysis of the supernatant from the culture of WT(pEKEx3-*sigH*) revealed maximal absorption at 450 nm as well as yellow fluorescence under UV irradiation (data not shown). Since the spectral properties of riboflavin fit well to those observed here, the supernatant and riboflavin as standard were analyzed by HPLC. Co-elution at around 3.2 min of riboflavin with the compound in the supernatant of WT(pEKEx3-*sigH*; **Figure 7.** A) and comparable absorption spectra (300–550 nm; **Figure 7.** B) revealed that riboflavin was produced by *C. glutamicum* WT(pEKEx3-*sigH*). No other significant peak was detected. Quantification based on a series of suitable riboflavin concentrations indicated that the accumulation of riboflavin in the supernatant of WT(pEKEx3-*sigH*) was about seven times as high as that of control strain WT(pEKEx3), (68.0 ± 1.3 µM and 10.4 ± 1.5 µM, respectively, biological triplicates). When expression of *sigH* was induced by addition of 100 µM of IPTG in the middle of the exponential growth phase (OD ~10) about 35 µM riboflavin accumulated (**Figure 8**).

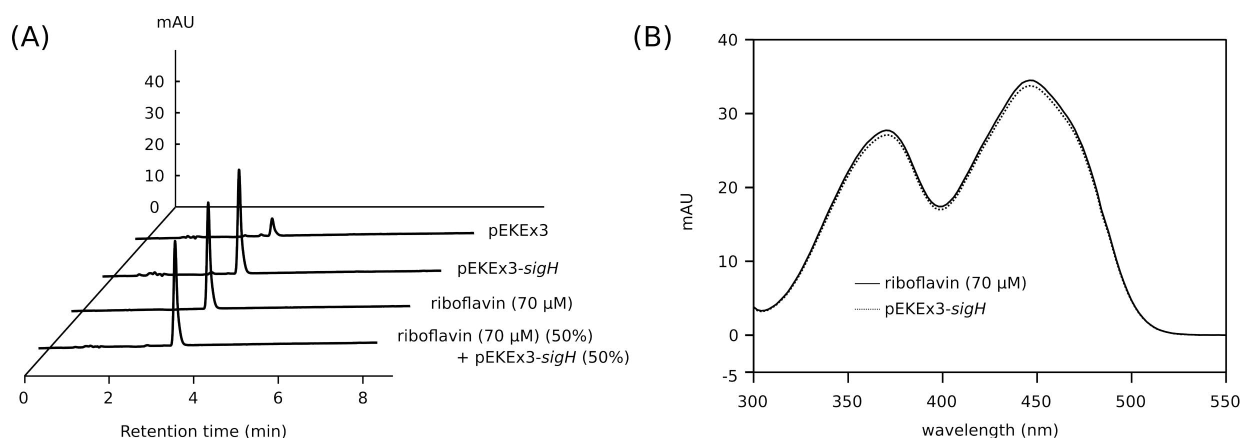

Figure 7. Analysis of supernatants of *C. glutamicum* WT(pEKEx3-*sigH*) cultures by (A) high performance liquid chromatography (HPLC) and (B) spectrophotometry. (A) HPLC chromatograms of supernatants of *C. glutamicum* WT(pEKEx3) and WT(pEKEx3-*sigH*) after 48 h in CGXII with 222 mM of glucose. Expression of *sigH* was induced by addition of 15 µM of IPTG at the start of the cultivation. A standard of pure riboflavin (70 µM) and a 50%/50% mixture of this standard and the supernatant of the culture of WT(pEKEx3-*sigH*) are given for comparison. Absorbance at 450 nm is shown. **(B)** Spectra recorded at the retention time of 3.2 min of the HPLC samples of *C. glutamicum* WT(pEKEx3-*sigH*) and the riboflavin standard from **(A)**.

2.1.4.3 Global gene expression changes due to *sigH* overexpression

To determine if *sigH* overexpression affects riboflavin biosynthesis genes, DNA microarray experiments with *C. glutamicum* WT(pEKEx3-*sigH*) were performed and global gene expression at two different IPTG concentrations (10 and 15 μM) was compared to the control strain WT(pEKEx3). Statistically significant gene expression increases of at least two fold were observed for 193 and 142 genes, respectively, upon induction with 10 and 15 μM of IPTG (M-value > 1, p-value < 0.05; **Table 4**). Fifty genes were considered further as they were upregulated in both IPTG concentrations. Among these, genes related to riboflavin synthesis [*ribH* (cg1797), *ribA* (cg1798), *ribC* (cg1799)] and the pentose phosphate pathway [*zwf* (cg1778), *opcA* (cg1779)] were found. In addition, many genes encoding NADPH-dependent or FAD/FMN-dependent oxidoreductases were upregulated upon *sigH* overexpression (**Table 4**).

To confirm the observed gene expression changes of *zwf* and *opcA*, the specific enzyme activity of glucose-6-phosphate dehydrogenase encoded by *zwf* and *opcA* was measured. The specific activity of glucose-6-phosphate dehydrogenase in the crude extracts of *C. glutamicum* WT(pEKEx3-*sigH*) was three times as high as in those of *C. glutamicum* WT(pEKEx3) (117 ± 7 and 35 ± 4 mU/mg, respectively, biological triplicates). Thus, *sigH* overexpression led to increased *zwf* and *opcA* mRNA level and increased specific activity of the encoded glucose-6-phosphate dehydrogenase.

Table 4. Genes differentially expressed upon *sigH* overexpression

50 genes		M valuer for pEKEx3-*sigH*/pEKEx3 >1	M-value[b]		p-value[c]	
gene ID[a]	gene name[a]	Function of protein[a]	10 µM	15 µM	10 µM	15 µM
cg0184		Conserved hypothetical protein	1.9	1.2	1.6E-2	2.6E-2
cg0186		Putative methylated-DNA-protein-cysteine methyltransferase	1.3	1.1	4.3E-4	3.8E-3
cg0614		Hypothetical protein	2.9	2.2	2.1E-3	5.6E-3
cg0616	***fdhD***	Putative formate dehydrogenase, FdhD-family	2.7	3.4	4.8E-4	1.1E-2
cg0617		Hypothetical protein	2.1	2.6	3.2E-4	8.8E-4
cg0876	***sigH***	RNA polymerase sigma factor, ECF-family	4.1	4.4	1.4E-5	6.0E-4
cg1081		ABC-type putative daunorubicin transporter, ATPase subunit	1.2	1.4	1.6E-2	2.9E-2
cg1127		Putative mycothiol S-conjugate amidase	1.3	2.6	1.3E-3	2.7E-3
cg1386	***fixA***	Putative electron transfer flavoprotein, beta subunit	1.1	2.0	1.7E-3	1.4E-2
cg1397	***trmU***	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase	1.5	1.7	6.6E-4	4.8E-3
cg1398		Conserved hypothetical protein	1.7	2.4	1.9E-2	2.3E-2
cg1432	***ilvD***	Dihydroxy-acid dehydratase	1.9	1.9	2.0E-4	6.7E-4
cg1628		Putative hydrolase, alpha/beta superfamily	2.5	1.9	4.8E-2	9.1E-3
cg1671		Putative membrane-associated GTPase	1.7	1.3	3.9E-2	1.4E-2
cg1687		Putative transcriptional regulatory protein	1.4	1.3	1.9E-2	9.7E-3
cg1688		Putative proteasome component	2.2	2.2	2.0E-4	7.9E-3
cg1689		Conserved hypothetical protein	2.3	3.0	9.5E-4	1.4E-2
cg1709	***mshC***	Putative 1-D-myo-inositycl-2-amino-2-deoxy-alpha-D-glucopyranoside-L-cysteine ligase	2.9	1.9	1.2E-4	1.5E-3
cg1764	***sufB***	FeS assembly membrane protein, SufB-family	1.2	1.0	1.8E-3	9.1E-3
cg1776	***tal***	Transaldolase	1.0	1.7	1.6E-2	1.2E-3
cg1778	***zwf***	Glucose-6-phosphate 1-dehydrogenase	1.2	2.0	4.1E-3	4.6E-3
cg1779	***opcA***	Glucose-6-phosphate 1-dehydrogenase subunit	1.6	1.2	1.5E-3	5.0E-2
cg1796	***ribX***	Conserved putative membrane protein, RibX-like	1.2	1.7	6.7E-3	3.5E-3
cg1797	***ribH***	Riboflavin synthase, beta chain	1.7	1.3	6.4E-5	1.7E-3
cg1798	***ribA***	Putative GTP cyclohydrolase II/3,4-dihydroxy-2-butanone-4-phosphatesynthase	2.0	1.0	4.6E-5	8.0E-3
cg1799	***ribC***	Riboflavin synthase, alpha chain	1.9	3.0	8.8E-4	2.7E-2
cg2078		Peptide methionine sulfoxide reductase	3.3	2.8	4.4E-5	4.2E-5
cg2079		Conserved hypothetical protein	1.4	1.1	2.2E-3	7.7E-3
cg2106		Conserved hypothetical protein	2.7	5.0	3.1E-3	4.5E-3
cg2127		Hypothetical protein	1.1	1.8	8.7E-3	3.5E-2
cg2194	***mtr***	Putative NADPH-dependent mycothiol reductase	3.1	2.9	2.9E-8	2.2E-3

Table 4. continues from the previous page

cg2206	***ispG***	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	1.3	1.2	7.5E-3	2.7E-2
cg2247		Hypothetical protein	1.8	2.0	2.4E-5	3.1E-4
cg2296	***hisI***	Phosphoribosyl-AMP cyclohydrolase	1.2	1.1	3.1E-3	8.4E-3
cg2297	***hisF***	Imidazole glycerol phosphate synthase subunit HisF	1.4	1.4	1.6E-2	9.7E-4
cg2411		Conserved hypothetical protein, HesB/YadR/YfhF family	2.1	2.8	4.0E-4	4.2E-4
cg2423	***lipA***	Lipoyl synthetase	1.6	1.7	1.9E-4	8.5E-3
cg2538		Alkanal monooxygenase (FMN-linked)	3.3	4.2	7.9E-4	1.6E-5
cg2644	***clpP2***	Endopeptidase Clp, proteolytic subunit	1.1	1.1	7.6E-4	9.4E-5
cg2661		Putative dithiol-disulfide isomerase	1.5	1.9	1.1E-4	4.7E-3
cg2665		Hypothetical protein	1.4	1.4	4.6E-3	7.8E-3
cg2762	***murI***	Glutamate racemase	2.0	2.4	5.0E-2	2.4E-2
cg2835		Putative acetyltransferase	1.0	3.3	4.7E-2	2.6E-2
cg2838		Putative dithiol-disulfide isomerase	3.6	3.2	3.9E-6	2.9E-3
cg3236	***msrA***	Protein-methionine-S-oxide reductase	1.4	3.4	3.4E-3	1.1E-2
cg3372		Conserved hypothetical protein	1.1	1.3	2.5E-5	4.2E-2
cg3405		NADPH:quinone reductase Zn-dependent oxidoreductase	2.6	2.8	7.8E-4	1.7E-2
cg3422	***trxB***	Thioredoxin reductase	1.8	2.2	3.1E-4	1.8E-4
cg3423	***trxC***	Thioredoxin	1.5	2.1	4.1E-4	6.8E-5
cg3424	***cwlM***	N-Acetylmuramoyl-L-alanine amidase	1.3	1.7	8.2E-3	1.4E-2

a Gene ID, gene name and function of proteins are given according to CoryneRegNet (http://coryneregnet.de/). b Relative RNA levels in a strain overexpressing *sigH* as compared to the empty vector control are shown as log 2 values (M-values). To induce *sigH* overexpression either 10 µM or 15 µM IPTG were added. c P-values were determined by Student's t test.

2.1.4.4 FMN production by *C. glutamicum* established as proof-of-concept based on overexpression of endogenous genes *sigH* and *ribF*

Riboflavin is the precursor of FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide), which are biologically important as redox cofactor for many flavoenzymes and have an advantage as food additives over riboflavin due to much higher solubility in water (Kirk-Othmer, 1984). *C. glutamicum* possesses one gene, *ribF* (cg2169), encoding putative bifunctional riboflavin kinase / FMN adenylyltransferase, which converts riboflavin to FMN and FAD. In *C. glutamicum*, *ribF* is located about 350 kb downstream of the def2-fmt-fmu-rpe-ribGACH operon that contains the riboflavin biosynthesis genes ribG, *ribA*, *ribC*, and *ribH*. Since the *ribF* mRNA level was not affected notably by *sigH* overexpression (**Table 4**), simultaneous overexpression of *ribF* and *sigH* was tested. However, severely retarded growth was observed already with only 15 µM IPTG (data not shown). Therefore, expression of *ribF* or/and *sigH* was induced in the middle of the exponential growth phase (OD ~10) using two compatible IPTG inducible plasmids, pEKEx3 and pVWEx1, with 100 µM of IPTG. After 48 h, neither riboflavin, FMN nor FAD were detected (<5 µM) in the supernatants of the control strain carrying the empty vectors. Expression of only *ribF* from pVWEx1 did not result in accumulation of riboflavin, FMN nor FAD. When only *sigH* was overexpressed from plasmid pEKEx3 riboflavin was secreted to the medium (32.4 ± 1.8 µM), but neither FMN nor FAD accumulated (**Figure 8**). However, when both genes were overexpressed in *C. glutamicum* WT(pEKEx3-*sigH*, pVWEx1-*ribF*) secretion of FMN (17.0 ± 0.6 µM) in addition to riboflavin (11.8 ± 0.4 µM) was detected, while FAD was not detected (<5 µM). To test if a different gene dosage affects FMN production, *sigH* was expressed from low copy number plasmid pVWEx1 and *ribF* from medium copy number plasmid pEKEx3. *C. glutamicum* WT(pEKEx3-*ribF*, pVWEx1-*sigH*) accumulated about two times higher concentrations of riboflavin (19.8 ± 0.3 µM) and FMN (33.1 ± 1.8 µM).

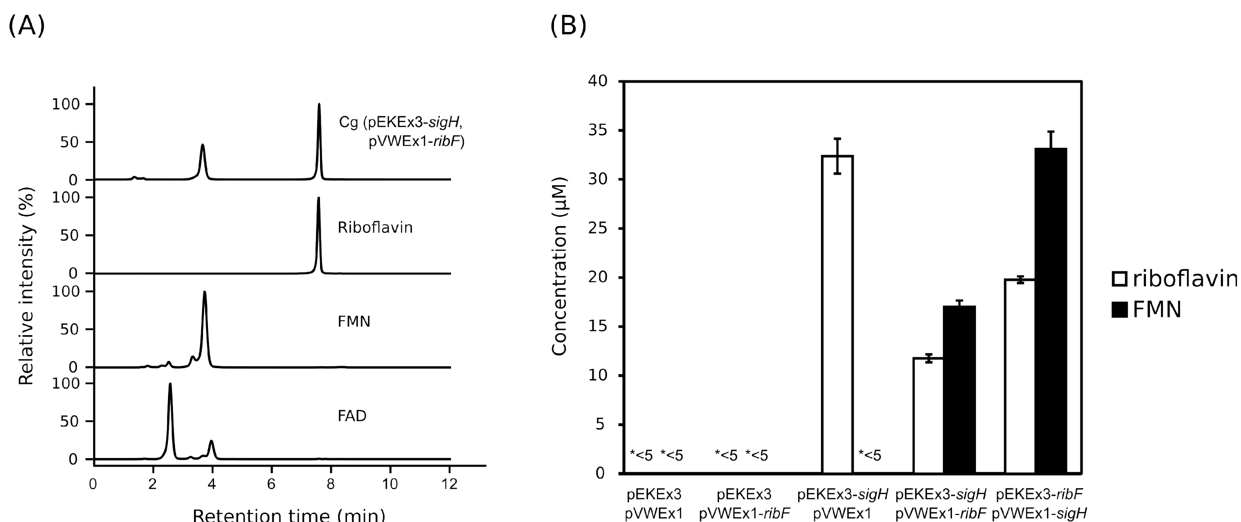

Figure 8. Analysis of supernatants of *C. glutamicum* WT(pEKEx3-*sigH*, pVWEx1-*ribF*) cultures by HPLC (A) and riboflavin and FMN concentrations in supernatants of various strains (B). (A) HPLC chromatograms of supernatants of *C. glutamicum* WT(pEKEx3-*sigH*, pVWEx1-*ribF*) after 48 h in CGXII with 222 mM of glucose. Expression of *sigH* was induced by addition of 100 µM of IPTG in the middle of the exponential growth phase (OD ~10). Standards of commercial preparations of riboflavin, FMN and FAD are given for comparison. Absorbance at 450 nm is shown. **(B)** Concentrations of riboflavin and FMN in supernatants of cultures of *C. glutamicum* WT transformed with the indicated plasmids. FAD was not detectable (<5 µM) in the analyzed supernatants. *<5 indicates that riboflavin or FMN in these supernatants were below 5 µM. Biological triplicates.

2.1.5 Discussion

In this study the potential of overexpressing sigma factor genes for metabolic engineering of *C. glutamicum* was tested. Sigma factors are related to the promoter selectivity during transcription initiation and are expected to affect expression of larger groups of genes, e.g., *rpoS* of *E. coli* regulates 481 genes under different growth and stress conditions (Weber *et al.,* 2005). However, there are examples of sigma factors relevant for expression of only few genes, e.g., FecI of *E. coli* that is involved in expression of only seven genes (Cho *et al.,* 2014). The functions of the seven sigma factors of *C. glutamicum*, which for comparison possesses 127 DNA-binding transcriptional regulators (Brune *et al.,* 2005), have not been studied in detail although SigB, SigE, SigH, and SigM have been studied by several groups (Kim *et al.,* 2005a; Larisch *et al.,* 2007; Nakunst *et al.,* 2007; Ehira *et al.,* 2008, 2009; Park *et al.,* 2008; Pátek and Nešvera, 2011; Busche *et al.,* 2012; Holátko *et al.,* 2012; Toyoda *et al.,* 2015). Here, we have determined the growth response of *C. glutamicum* to sigma factor gene overexpression. Overexpression of every sigma factor gene slowed growth in glucose minimal medium, however, the effects varied. The smallest effects were found when the general sigma factor genes *sigA* and *sigB* or the genes for SigE and SigM were overexpressed (**Figure 5**). Overexpression of *sigC* in glucose minimal medium with 250 µM IPTG reduced the growth rate by about one third (**Figure 5**). The growth was severely inhibited (about half-maximal inhibition with IPTG concentrations as low as 15 µM) as consequence of overexpressing *sigD* or *sigH*. The functions and promoter selectivities of SigC and SigD remain to be studied, however, it is known that deletion of *sigD* retarded growth under microaerobic conditions (Ikeda *et al.,* 2009). The observed growth inhibitory effects of overexpressing *sigD* or *sigC* described in this study suggested that these sigma factors are not negligible and proper expression levels of those sigma factors are important for expression of genes required for fast growth in glucose minimal medium.

Analysis of the supernatants of *C. glutamicum* overexpressing sigma factor genes (**Figure 6**) revealed that only *sigH* overexpression led to the production of a colored compound, which was identified to be riboflavin (**Figure 7**). Moreover, *sigH* overexpression slowed growth (**Figure 5**). Regulation by SigH in *C. glutamicum* is known to some detail. The alternative sigma factor SigH is controlled by anti-sigma factor RshA, which possibly shuts down the SigH-dependent stress

response after the cells have overcome the stress condition (Busche *et al.,* 2012). SigH has been shown to be involved in expression of *trxB* encoding thioredoxin reductase (Kim *et al.,* 2005a), *whcE* encoding transcriptional regulator WhiB (Kim *et al.,* 2005b), *sigM* (Nakunst *et al.,* 2007), small antisense RNA gene arnA (Zemanová *et al.,* 2008), the F0F1-ATP synthase operon atpBEFHAGDC (Barriuso-Iglesias *et al.,* 2013), mycothiol peroxidase gene mpx (Si *et al.,* 2015b), mycothiol S-conjugate amidase gene mca (Si *et al.,* 2014), and methionine sulfoxide reductase A gene msrA (Si *et al.,* 2015a). In addition, promoter selectivity of SigH has been studied using an in vitro transcription system (Holátko *et al.,* 2012). Moreover, the SigH regulon has been studied by DNA microarray and ChIP-chip analyses involving deletion and overexpression of *sigH* as well as deletion of the anti-sigma factor gene *rshA* (Busche *et al.,* 2012; Ehira *et al.,* 2009; Toyoda *et al.,* 2015). The strong growth inhibition as a result of overexpression of *sigH* shown here is commensurate with the described functions of SigH. In our DNA microarray analysis, 50 genes were upregulated when *sigH* overexpression was induced with 10 and 15 µM IPTG (**Table 4**). These data generally agree with previous data on control by SigH (Busche *et al.,* 2012; Ehira *et al.,* 2009; Toyoda *et al.,* 2015). Notably, overexpression of *sigH* in the wild type, i.e., in the presence of its anti-sigma factor RshA, elicited similar expression changes as deletion of *rshA*, i.e., 43 out of 50 genes upregulated as consequence of *sigH* overexpression were also upregulated in the absence of anti-sigma factor RshA (Busche *et al.,* 2012). A motif search with the 50 upregulated genes (**Table 4**) using UniProt database (http://www.uniprot.org/) identified putative iron sulfur cluster-containing proteins encoded by cg0616 (*fdhD*), cg1432 (*ilvD*), cg2206 (*ispG*) and cg2423 (*lipA*), proteins predicted to contain NAD(P)H binding sites encoded by cg0184, cg0616 (*fdhD*), cg1778 (zwf), cg2194 (*mtr*), and cg3405, and proteins with predicted FMN/FAD binding sites encoded by cg0616 (*fdhD*), cg1386 (*fixA*), cg2194 (*mtr*), cg2538 and cg3422 (*trxB*). Iron sulfur clusters are sensitive to oxidative stress and NAD(P)H, FMN, FAD are important electron donor/acceptors. Upregulation of genes related to riboflavin synthesis under *sigH* overexpression observed here (**Table 4**) was consistent with a very recent ChIP-chip data on SigH-dependent promoters in *C. glutamicum* R (Toyoda *et al.,* 2015).

In *C. glutamicum*, riboflavin biosynthesis was shown to be dependent on *ribA*-encoded bifunctional GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase, since in its

absence efficient growth required supplemental riboflavin (Takemoto *et al.,* 2014). Uptake of supplemental riboflavin occurs via the transporter RibM (Vogl *et al.,* 2007) and both RibM protein levels and *ribM* mRNA were reduced in FMN rich cells due to the FMN riboswitch (Takemoto *et al.,* 2014). The FMN riboswitch has been observed in an RNA- seq based analysis of the transcriptional landscape of *C. glutamicum* (Pfeifer-Sancar *et al.,* 2013) and control by the FMN riboswitch was shown to involve Rho and RNase E/G (Takemoto *et al.,* 2015). However, riboflavin biosynthesis appears not to be controlled by the FMN riboswitch. Instead, transcription of the riboflavin biosynthesis operon depends on SigH and deletion of *rshA* and overexpression of *sigH* resulted in riboflavin secretion as recently reported in the *rshA* deletion mutant (**Figure 7**; Toyoda *et al.,* 2015). Neither FMN nor FAD accumulated under these conditions, which may be explained by the fact that *ribF* expression has not been found to be influenced by deletion of *rshA* and overexpression of *sigH* (Busche *et al.,* 2012; Toyoda *et al.,* 2015) (**Table 4**).

Riboflavin concentrations in supernatants of wild-type *C. glutamicum* cultures were low, but traces may be present (**Figure 7**). *Eremothecium ashbyii* and *Ashbya gossypii* are known as natural producers of riboflavin (Osman and Soliman, 1963; Kato and Park, 2011) and *Bacillus subtilis*, *E. coli*, and *Corynebacterium ammoniagenes* were selected and/or metabolically engineered to overproduce riboflavin (Koizumi *et al.,* 2000; Stahmann *et al.,* 2000; Lin *et al.,* 2014). The role of extracellular riboflavin is still unclear. However, iron limitation resulted in riboflavin secretion by *Candida guilliermondii* and other organisms (Enari and Kauppinen, 1961; Neilands, 2014) and it has been suggested that excreted riboflavin may play an important role for ferric iron reduction and iron acquisition (Worst *et al.,* 1998; Crossley *et al.,* 2007). A riboflavin export system is currently unknown.

This study showed that FMN overproduction by *C. glutamicum* is possible. Simultaneous overexpression of *sigH* and *ribF* resulted in the secretion of riboflavin and FMN into the medium, while FAD was not detected (**Figure 8**). Currently, FMN is synthesized chemically involving phosphorylation of riboflavin. However, FMN preparations typically contain ~25% impurities such as isomeric riboflavin phosphates, riboflavin cyclophosphates, and riboflavin bisphosphates, which can act as antimetabolites and thus be toxic (Abbas and Sibirny, 2011). Enzyme-catalyzed biotransformation of riboflavin and metaphosphate using a crude enzyme preparation from

genetically engineered C. ammoniagenes yielded 40 µM of FMN without concomitant FAD formation (Nakagawa *et al.,* 1995). Fermentative production of 0.5 mM of FMN using genetically engineered *Candida famata* has also been reported (Yatsyshyn *et al.,* 2010). Although conversion from riboflavin to FMN in the present study was not complete and titers were not high, a proof-of-principle demonstration of fermentative FMN production by *C. glutamicum* could be shown. Future work will address conversion of FMN to FAD and strain development to improve riboflavin, FMN and FAD yields and productivities.

This and work by others (Toyoda *et al.,* 2015) showed that analysis of sigma factor gene overexpression in *C. glutamicum* wild type helped discover the potential of this bacterium for riboflavin production. In *Synechocystis* sp. PCC 6803, overexpression of *sigE* activated expression of sugar catabolic genes and increased polyhydroxybutyrate (PHB) during nitrogen starvation (Osanai *et al.,* 2011, 2013). SigE from *Synechocystis* sp. PCC 6803 and SigB from *C. glutamicum* belong to group 2 sigma factors and SigB from *C. glutamicum* positively regulates glucose catabolism genes (Ehira *et al.,* 2008). Overexpression of SigF in *Mycobacterium smegmatis* enhanced carotenoid biosynthesis by upregulating the carotenoid biosynthesis operon (Kumar *et al.,* 2015), however, *C. glutamicum* does not possess the same type of sigma factor. Future studies will have to establish if and to what extent the approach of sigma factor gene overexpression is transferable to classically obtained or metabolically engineered *C. glutamicum* strains and/or to other bacteria. This may also pertain to “awakening” silent or orphan gene clusters relevant for secondary metabolite production, e.g., silent antibiotic biosynthesis gene clusters.

2.2 Characterization of the physiological role and the regulatory mechanism of sigma factor D in *Corynebacterium glutamicum*

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2.2.1 Abstract

Sigma factor is one of the components of RNA polymerase holoenzyme and important for transcriptional regulation in bacteria. *Corynebacterium glutamicum* posseses 7 types of sigma factors. The regulation by most of the sigma factors except SigD has been studied, and therefore, the knowledge on transcriptional regulation by SigD is still limited until now.

In this study, disruption and overexpression of *sigD* were performed to understand the SigD regulon. Overexpression of *sigD* decreased the growth rate of the transformant and led to unusual phenotypes such as less foaming of the cell culture, more turbid supernatant and cell aggregation. From RNA-seq and real-time PCR analysis, we elucidated that *sigD* overexpression activats the expression of multiple genes related to the cell envelope integrity. Among those, four genes for corynomycolyl transferase (*cop1*, *cmt1*, *cmt2*, *cmt3*), two genes related to corynomycolic acid synthesis (*fadD2*, *pks*), genes for L, D,-transpeptidase (*lppS*), the subunit of the major cell wall channel (*porH*) and envelope lipid regulation factor (*elrF*) were found. Overexpression of *sigD* also changed the pattern of secreted proteins in the supernatant of the culture. Furthermore, the *sigD* overexpressing strain excreted carbohydrate containing compounds into the supernatant and accumulated more trehalose dicorynomycolate in the cell envelope.

This study expands the knowledge about transcriptional regulation by sigma factors in *C. glutamicum*. In addition, this study elucidated that SigD regulates the synthesis of corynomycolate and corynomycolate-related compounds.

2.2.2 Introduction

A sigma factor is an essential component of bacterial RNA polymerase for promoter recognition and transcription initiation (Feklístov *et al.,* 2014). Most bacteria encode multiple sigma factors, and each sigma factor initiates transcription from the cognate promoter sequences in conjunction with RNA polymerase (Rodrigue *et al.,* 2006; Staroń *et al.,* 2009; Nicolas *et al.,* 2012; Cho *et al.,* 2014). By replacing a sigma factor in RNA polymerase, bacteria can activate transcription of different gene sets under different conditions (Österberg *et al.,* 2011). Therefore, sigma factors play an important role in transcriptional regulation in a global manner, and the knowledge on regulations by sigma factors is helpful to elucidate regulatory architecture and its control of the organism.

C. glutamicum was first isolated as an organism secreting high amounts of glutamate (Kinoshita *et al.,* 1957). Nowadays, this bacterium has been used for production of L-glutamate and L-lysine in million tons per year (Eggeling and Bott, 2005). Because of its genetic amenability and knowledge about its metabolic pathways, *C. glutamicum* is a promising organism for production of chemicals especially for amino acid as well as their derivatives (Wendisch, 2014). *C. glutamicum* is a Gram-positive bacterium and belongs to the CMN (*Corynebacterium*, *Mycobacterium*, *Nocardia*) group, which is characterized by the unique molecular constituents of the cell envelope (Barksdale, 1970). Because of its similar structure of cell envelope and non-pathogenicity, *C. glutamicum* serves as a good model organism for understanding the cell wall biosynthesis and resistance to antibiotics of *Corynebacterineae*, which includes human pathogenic bacteria such as *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae (Portevin et al., 2004)*. The cell envelope of *C. glutamicum* consists of plasma membrane, peptidoglycan, arabinogalactan, and mycomembrane (Burkovski, 2013). Its mycomembrane is composed of a monolayer of corynomycolates (α-alkyl, β-hydroxy fatty acid) which are covalently linked to arabinogalactan or form other lipids such as trehalose monocorynomycolate (TMCM) and trehalose dicorynomycolate (TDCM) (Lanéelle *et al.,* 2013). The mycomembrane is suggested to work as a permeability barrier for hydrophilic molecules such as antibiotics, sugars or amino acids (Eggeling and Bott, 2005; Marrakchi *et al.,* 2014). Therefore, understanding the regulation of mycomembrane synthesis in *C. glutamicum* can be helpful to improve production of chemical compounds as well as to give some clues for antibiotics resistance acquisition of pathogenic

bacteria.

C. glutamicum has one circular chromosome and no plasmid by nature. Genomic DNA of *C. glutamicum* ATCC13032 was sequenced individually from two groups and is available to public (Ikeda and Nakagawa, 2003; Kalinowski *et al.,* 2003). *C. glutamicum* has seven types of sigma factor genes in its chromosome, *sigA*, *sigB*, *sigC*, *sigD*, *sigE*, *sigH*, and *sigM*. SigA belongs to Group 1 sigma factors and SigB belongs to Gorup 2 sigma factors. The other five sigma factors belong to Group 4 sigma factors, also called ECF (extracytoplasmic function) sigma factors. *C. glutamicum* possesses no Group 3 sigma factors. For SigA, SigB, SigE, SigH and SigM, its physiological function and consensus promoter sequence has been studied in detail (Pátek and Nešvera, 2011). Recently, the transcriptional regulation by SigC and its consensus promoter sequence was elucidated (Toyoda and Inui, 2016). For SigD, the selection of high oxygen requiring mutants from transposon library accidentally revealed that *sigD* is somehow important in oxygen limiting static culture (Ikeda *et al.,* 2009). Furthermore, the overexpresison of *sigD* decreased the growth rate (Taniguchi and Wendisch, 2015). However, the regulation by SigD is still not fully understood in *C. glutamicum*

SigD of *C. glutamicum* is classified as ECF40 type sigma factor by ECFfinder (Staroń *et al.,* 2009). This group contains SigD of *M. tuberculosis*, which is linked to virulence (Calamita *et al.,* 2005; Raman *et al.,* 2004). SigD is well conserved among corynebacteria, and 17 out of 19 examined corynebacteria species possess *sigD* gene (Pátek and Nešvera, 2011). Therefore, it is assumed that SigD plays an important role in transcriptional regulation.

In this study, we evaluated the effect of *sigD* overexpression on cellular phenotype and elucidated the regulon of SigD by transcriptome analysis in *C. glutamicum* ATCC13032. Furthermore, the relation between SigD overexpression and regulation of mycomembrane synthesis was investigated.

2.2.3 Material and methods

2.2.3.1 Bacterial strains, plasmids and oligonucleotides

The strains, plasmids and oligonucleotides used in this work are listed in **Table 5**. A plasmid for *sigD* overexpression was constructed based on pVWEx1, an IPTG inducible *Escherichia coli* - *C. glutamicum* shuttle vector (Peters-Wendisch *et al.,* 2001). Plasmids for gene disruption were constructed based on pK18mobsacB, a suicide vector for *Escherichia coli* and *C. glutamicum* (Schäfer *et al.,* 1994). DNA fragments were amplified from the genomic DNA of *C. glutamicum* ATCC13032 WT by PCR with respective primer pairs in **Table 5**. The fragments were inserted into digested plasmid by ligation or Gibson assembly (Sambrook, 2001; Gibson *et al.,* 2009). *E. coli* DH5α was used for cloning. *E. coli* competent cells were transformed by heat shock method (Sambrook, 2001) or by electroporation method (Nováková *et al.,* 2014). The DNA sequence of all cloned DNA fragments was confirmed to be correct by sequencing. *C. glutamicum* competent cells were transformed by electroporation at 2.5 kV, 200 Ω, and 25 µF (van der Rest *et al.,* 1999; Eggeling and Bott, 2005). Gene disruption via two-step homologous recombination and the following selections were carried out as described previously (Eggeling and Bott, 2005). Disruption was verified by PCR with respective primer pairs and gel electrophoresis. The genomic DNA sequence of *C. glutamicum* ATCC13032 (NCBI:NC_006958.1) was used as a reference sequence (Kalinowski *et al.,* 2003).

2.2.3.2 Medium and conditions for growth experiments

As far as not specified, *C. glutamicum* was precultured in LB medium (Sambrook, 2001) supplemented with 56 mM of glucose overnight, washed once with CGXII medium (Eggeling and Bott, 2005) without carbon source and inoculated in CGXII with 222 mM of glucose at initial OD of 1 (600 nm). The OD was measured with UV-1202 spectrophotometer (Shimadzu, Duisburg, Germany) with suitable dilutions. When necessary, IPTG and 25 µg/mL of kanamycin were added. For the growth experiment in BioLector® cultivation system (m2pLabs, Baesweiler, Germany), cells were cultivated in 1 mL of CGXII with 222 mM of glucose using FlowerPlate® (m2pLabs, Baesweiler, Germany) at 30°C, 1,100 rpm. Cell growth was monitored online every 10 min, and the maximum growth rate (h^{-1}) was determined from the growth rate µ (h^{-1}) which was calculated by regression analysis from arbitrary unit of backscattering light (wavelength of 620 nm) at 20 consecutive measuring points.

Table 5. Bacterial strains, plasmids and oligonucleotides used in this study

Bacterial strain	**Relevant characteristic**	**Reference or source**
<u>*E. coli*</u>		
DH5α	F−*thi*-1 *endA*1 *hsdR*17(r−, m−) *supE*44 Δ*lacU*169 (Φ80*lacZ*ΔM15) *recA*1 *gyrA*96 *relA*1	Bethesda Research Laboratories
<u>*C. glutamicum*</u>		
WT	Wild type, ATCC 13032	ATCC
Δ*sigD*	Δ*sigD* (Δcg0696)	this study
Δcg0697	Δcg0697 (Δ*rsdA*)	this study
<u>**Plasmid**</u>	Relevant characteristic	References
pVWEx1	KanR; *E. coli*-*C. glutamicum* shuttle vector for regulated gene expression (Ptac, *lacIq*, pCG1 oriVCg)	(Peters-Wendisch *et al.,* 2005)
pVWEx1-*sigD*	KanR, pVWEx1 with *sigD* from *C. glutamicum* WT	this study
pK18mobsacB	KanR, *E. coli* - *C. glutamicum* shuttle vector for construction of insertion mutants in *C. glutamicum* (pK18 oriVec *sacB lacZ*α)	(Schäfer *et al.,* 1994)
pK18mobsacB-Δ*sigD*	KanR, pK18mobsacB with a Δ*sigD* (Δcg0696) disruption construct	this study
pK18mobsacB-Δcg0697	KanR, pK18mobsacB with a Δcg0697 (Δ*rsdA*) disruption construct	this study
<u>**Oligonucleotide**</u>	Sequence (5'-3')	References
sigD-fwd	GCCTGCAGGTCGACTCTAGAG***GAAAGGAGG***CCCTTCAG**TTG**GCTGATACTGAGCGCGAGCTC	(Taniguchi and Wendisch, 2015)
sigD-rev	CGGTACCCGGGGATCTTACTTGTTCTCCTGCTGCTCAAGTGTGCTTC	(Taniguchi and Wendisch, 2015)
Δ*sigD*_1_fwd	GATCTATCTAGAGATAGAACACGTCGGAGGTC	this study
Δ*sigD*_1_rev	CTGTGAGCATCTGCGACCTTCAAGTTCTCGCACCTTCCTG	this study
Δ*sigD*_2_fwd	CAGGAAGGTGCGAGAACTTGAAGGTCGCAGATGCTCACAG3	this study
Δ*sigD*_2_rev	GATCTATCTAGATAACCGTGGTGTCCGAAGTG	this study
Δ*sigD*_genome_fwd	GATGCAGGAAGGTGCGAGAA	this study
Δ*sigD*_genome_rev	CTGCTCACCACCATGTAGAC	this study
*sigD*_qPCR_fwd	GCTGGTCAGCGATGGAAGTA	this study
*sigD*_qPCR_rev	TGCTGCTCAAGTGTGCTTCG	this study
cg0413_qPCR_fwd	CCTCGGTGGCAAGCAAATGT	this study
cg0413_qPCR_rev	TGTTGTGGGAAGAGTAGGGAAGTAG	this study
cg0420_qPCR_fwd	GATCGCTTTAGAGTTCCATCCCTTG	this study
cg0420_qPCR_rev	TCCTCCCTTATCTTTATTTATTCTGCCG	this study

Table 5. continues from the previous page

cg0532_qPCR_fwd	GTGGGTTGGTTATGCGGTTCG	this study
cg0532_qPCR_rev	CTCGATTTCGCGCTGCTAGTG	this study
cg1052_qPCR_fwd	ACCGACCTTGATGAGTTGACCAG	this study
cg1052_qPCR_rev	TGCCCAGTCAGCGTAGAAACTAAA	this study
cg1181_qPCR_fwd	CATCACACCACAAACGACACGG	this study
cg1181_qPCR_rev	TAATGACTGCGAAATAGGCGACCAA	this study
cg2720_qPCR_fwd	AATCCTCGCAGCAAAGTACAGAAG	this study
cg2720_qPCR_rev	CACATCCTCATCACCATCATCAACA	this study
cg3009_qPCR_fwd	TCAAGGAAACCCTCGGCAACTA	this study
cg3009_qPCR_rev	GAAGTTAAGGATGGAATCGAGCAGG	this study
cg3178_qPCR_fwd	AGGTTATGTCTCGGAAGATGGAAGA	this study
cg3178_qPCR_rev	AGACCAAAGAACTCCGCATCAAAG	this study
cg3179_qPCR_fwd	GCTAACAACAGCCCTGAGTACATTT	this study
cg3179_qPCR_rev	TCGTTTGGATCATAAAGCGGCAC	this study
cg3180_qPCR_fwd	ACCGCGAAGATGAATGAGGAACT	this study
cg3180_qPCR_rev	CTGGGAGAAACCAACGATGATGAAC	this study
cg3182_qPCR_fwd	CTGAAATCTGGGCACTTGACGG	this study
cg3182_qPCR_rev	ATGGCGTTCTTATCGGCGTAGTA	this study
cg3186_qPCR_fwd	CTCTACGCACCAAACAACATCACC	this study
cg3186_qPCR_rev	ATGACAGGATCGGAGGAGGAAAGA	this study
Δcg0697_1_fwd	GGTGGTGAATTCTGGCGGTAGCCACCTCCATT	this study
Δcg0697_1_rev	TATTTTTACCTAGTTTCCACGCCGCGGGACAGGTCAGT	this study
Δcg0697_2_fwd	ACTGACCTGTCCCGCGGCGTGGAAACTAGGTAAAAATA	this study
Δcg0697_2_rev	GGTGGTTCTAGACACAACCGGAAATCTGGCAG	this study
Δcg0697_genome_fwd	TTATGCTCGCGCTCGTATTG	this study
Δcg0697_genome_rev	GACAGTTCCTCCTCATAGTG	this study
cg0697_qPCR_fwd	GGCACGCGACATTCTTATCT	this study
cg0697_qPCR_rev	ATCCTGCTCACCACCATGTA	this study

Sequences in bold italic represent ribosome binding sites; sequences in bold represents the translational start codons.

2.2.3.3 Photometric determination of supernatant turbidity

For quantifying turbidity in the supernatant, cell culture was centrifuge for 30 min with 15,000 x g at room temperature. The supernatant was carefully transferred, and the absorption at wavelength of 600 nm was measured using CGXII without carbon source as blank.

2.2.3.4 Confirmation of cell aggregation by microscopic and flow cytometric analysis

For microscopic imaging, each strain in the stationary phase was diluted in CGXII without carbon source to OD around 1, and cell shapes and aggregations were observed under microscopy with 100 x oil immersion objective lens and 10 x ocular lens. For confimation of cell size distribution by flow cytometry, each strain was grown until the stationary phase and resuspended in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7) to OD around 0.3. The forward and side scatter light of resuspension was measured by flow cytometry, Gallios™ (Beckman Coulter, USA) until the number of analyzed particles reached 20,000. The data was analyzed with Kaluza (Beckman Coulter, USA).

2.2.3.5 RNA extraction

Cells were precultured in CGXII with 222 mM of glucose for adaptation, inoculated in CGXII with 222 mM of glucose at OD 1 and harvested at OD around 6. 1 mL of cell culture was centrifuged for 30 sec at 20,000 x g and immediately frozen with liquid nitrogen after removing supernatant. RNA isolation was performed using the RNeasy mini kit along with an RNase-free DNase set (Qiagen, Hilden, Germany) and a DNase I kit (Roche Diagnostics, Mannheim, Germany) as described previously (Hüser *et al.,* 2003). Purity of genomic DNA was confirmed by PCR with multiple primer pairs specific to genomic DNA.

2.2.3.6 RNA-seq analysis and real-time PCR analysis

For RNA-seq analysis, RNA samples isolated individually from biological triplicates were mixed for each stain. Quality check of the isolated RNA, library preparation, RNA sequencing and data analysis was performed by the whole transcriptome protocol described previously with some modification (Pfeifer-Sancar *et al.,* 2013). Briefly, RNA quality was checked by Trinean Xpose (Gentbrugge, Belgium) and Agilent RNA Nano 6000 kit with Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). Ribo-Zero rRNA Removal Kit (Bacteria) from Illumina (San Diego, CA, USA) was used to remove the ribosomal RNA molecules from the isolated total RNA. Removal of rRNA was checked by Agilent RNA Pico 6000 kit on Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). TruSeq Stranded mRNA Library Prep Kit from Illumina (San Diego, CA, USA) was used to prepare cDNA libraries. The cDNA was sequenced paired end on an Illumina MiSeq system (San Diego, CA, USA) using 75 bp read length.

For differential gene expression analysis, ReadXplorer and Bioconductor package DESeq implemented in ReadXplorer were used (Anders and Huber, 2010; Hilker *et al.,* 2014). Genes with the mean of signal intensity value less than 30 were discarded. A-value and M-value were calculated based on the intensity value of the strain of interest and the control strain.

For real-time PCR analysis, relative abundance of mRNA of each gene was quantified using the same amount of total RNA. RNA sample extracted from biological triplicate were quantified individually. The experiment and analysis were performed as described previously with respective primer pairs in **Table 5** (Busche *et al.,* 2012).

2.2.3.7 Protein analysis in supernatant

Supernatant was taken from the culture in the stationary phase. 800 µL of acetone was added to 200 µL of the supernatant and stored at -20 °C overnight. After centrifugation at 4 °C, 20,000 x g for 15 min, supernatant was removed, and precipitates was resuspended in 20 µL of 20 mM Tris-HCl (pH 7). 20 µL of the samples were used for analysis. SDS-PAGE and following visualization was performed as described previously using Tris-glycine discontinuous buffer (Sambrook, 2001).

Protein bands with different intensities between the control strain and the *sigD* overexpressing strain were excited from SDS-PAGE gel and transferred into a tube which was previously washed with TFA:ACN:H_2O (0.1:60:40 v/v). The digestion of protein in the band was performed with trypsin overnight as described previously (Shevchenko *et al.,* 2007). Protein sequence was identified using an ultrafleXtreme MALDI-TOF/TOF mass spectrometry (Bruker, Bremen, Germany) and Mascot search engine (Matrix Science, London, UK) as described previously with some modifications for *C. glutamicum* ATCC13032 WT (Musa *et al.,* 2013).

2.2.3.8 Quantification of carbohydrate in acetone precipitates

Supernatant was precipitated with acetone as mentioned in the section of protein analysis in supernatant. Precipitates was resuspended with 100 µL of 20 mM Tris-HCl (pH 7), placed at room temperature for 30 min and the insoluble fraction was recovered by centrifugation. Insoluble fraction was hydrolyzed as described previously (Dallies *et al.,* 1998). Briefly, the precipitates were resuspended with 75 µL of 72% (w/w) H_2SO_4 and incubated at room temperature for 3 h. The slurry was diluted to 1 mL with H_2O, heated at 100 °C for 4 h and cooled down on ice. Colorimetric quantification for carbohydrates was performed by the phenol sulfuric acid method as described previously with some modification (DuBois *et al.,* 1956; Masuko *et al.,* 2005). Briefly, 200 µL of hydrolysate was mixed with 600 µL of concentrated sulfuric acid rapidly, and 120 µL of 5% phenol (w/v) in water was added immediately. The mixture was incubated for 5 min at 90 °C and cooled to room temperature for 5 min. The absorption at 490 nm was measured by photometer and compared to the absorption of the control samples with different concentrations of arabinose.

2.2.3.9 Detection of TDCM by TLC

Lipid extraction and thin layer chromatography (TLC) was performed based on the method described previously (Brand *et al.,* 2003; Puech *et al.,* 2000). Briefly, the crude lipid was extracted from cell pellet first with $CHCl_3/CH_3OH$ (1:1 v/v) once and with $CHCl_3/CH_3OH$ (2:1 v/v) for three times. All the extracts were pooled for each strain, concentrated by evaporation and mixed with $CHCl_3/CH_3OH/H_2O$ (8:4:2 v/v). The lower organic phase was collected and evaporated to dryness. Dried lipid was weighted and suspended with $CHCl_3/CH_3OH$ (4:1 v/v). TLC was performed with ALUGRAM SIL G/UV254 (Macherey-Nagel, Germany) with $CHCl_3/CH_3OH/H_2O$ (30:8:1 v/v) as development solvent. 600 µg of total lipid was developed for each strain. The mobility of TDCM or TMCM was determined by Rf value from previous study (Brand *et al.,* 2003; Puech *et al.,* 2000)

2.2.4 Results

2.2.4.1 Disruption and overexpression of *sigD* influenced the maximum growth rate

The *sigD* disrupted mutant and the *sigD* overexpressing strain were cultivated in CGXII medium containing 222 mM of glucose. *sigD* was overexpressed from the plasmid, pVWEx1-*sigD*, using an IPTG inducible promoter. Different IPTG concentrations (0, 10, 50, 250 or 1000 μM) were used aiming at the induction of *sigD* at different levels.

The *sigD* disrupted mutant could grow but slightly slower than the wild type strain (**Figure 9. A**). For WT(pVWEx1-*sigD*), the maximum growth rate decreased in an IPTG- dependent manner, which was not observed for WT(pVWEx1) (**Figure 9. B**). Higher concentrations of IPTG (250 and 1000 μM) severely inhibited the growth of WT(pVWEx1-*sigD*). The growth retard by *sigD* overexpression was consistent with the result shown before (Taniguchi and Wendisch, 2015). No difference in final biomass was observed between the wild type strain and the *sigD* disrupted strain or between WT(pVWEx1) and WT(pVWEx1-*sigD*) with 0, 10 or 50 μM of IPTG (data not shown). The slower growth of the *sigD* disrupted mutant indicates that *sigD* is not essential for cell survival but necessary for the optimal growth in CGXII minimal medium. In addition, the excessive *sigD* expression is harmful to cells by disturbing the cellular regulation. Considering the effect of *sigD* overexpression on transcription and the growth rate, 50 μM of IPTG was used for further experiments.

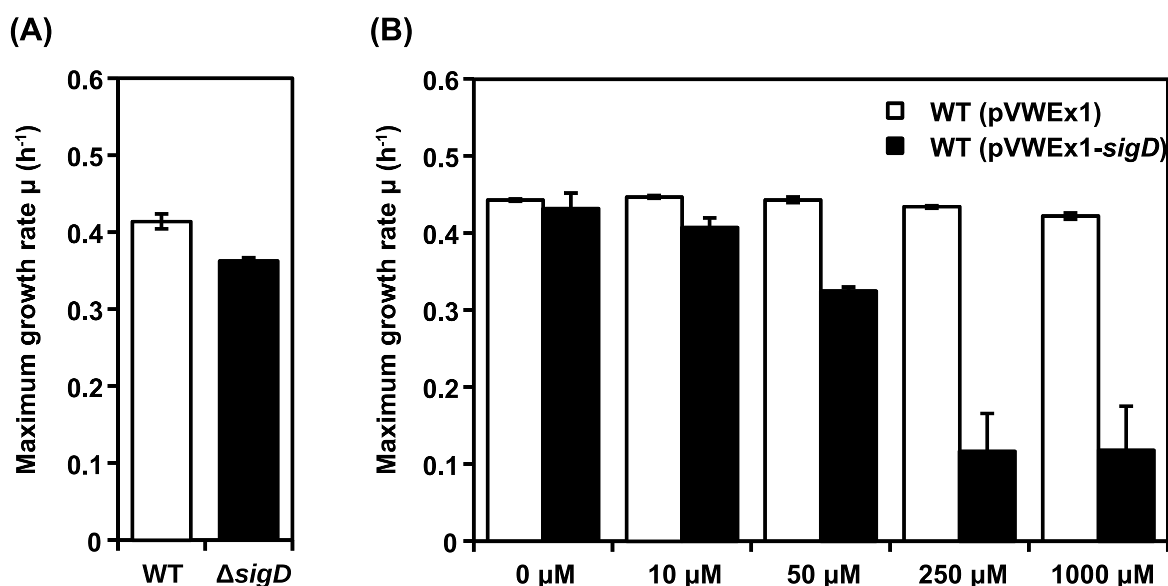


Figure 9. The maximum growth rate of (A) the *sigD* disrupted mutant and (B) *sigD* overexpressing strain with different IPTG concentrations. The maximum specific growth rate (h^{-1}) was shown for (A) WT (the wild type strain) and Δ*sigD* (the *sigD* disrupted mutant) and (B) WT(pVWEx1) and WT(pVWEx1-*sigD*) with different IPTG concentrations (0, 10, 50, 250, 1000 μM). Error bar stands for standard deviation calculated from biological triplicates.

2.2.4.2 *sigD* overexpression influenced the characteristic of cell culture

WT(pVWEx1-*sigD*) with 50 µM of IPTG showed distinct characteristics compared to WT(pVWEx1). Culture of WT(pVWEx1-*sigD*) foamed significantly less than that of WT(pVWEx1) (**Figure 10. A**). Furthermore, the supernatant from cell culture of WT(pVWEx1-*sigD*) showed higher turbidity (**Figure 10. B**). The effect of different IPTG concentrations (0, 10, 50 µM) was tested in 1mL of CGXII medium containing 222 mM using FlowerPlate. As a result, the turbidity in the supernatant of WT(pVWEx1-*sigD*) increased in an IPTG- dependent manner (**Figure 10. C**).

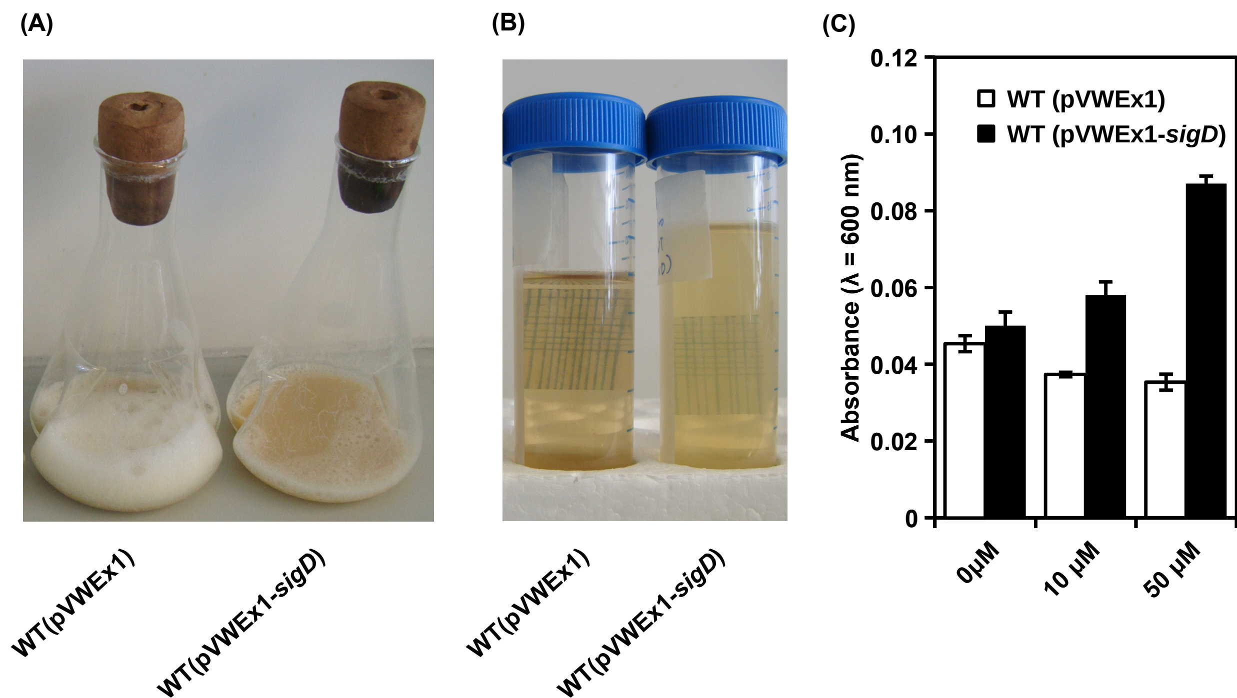


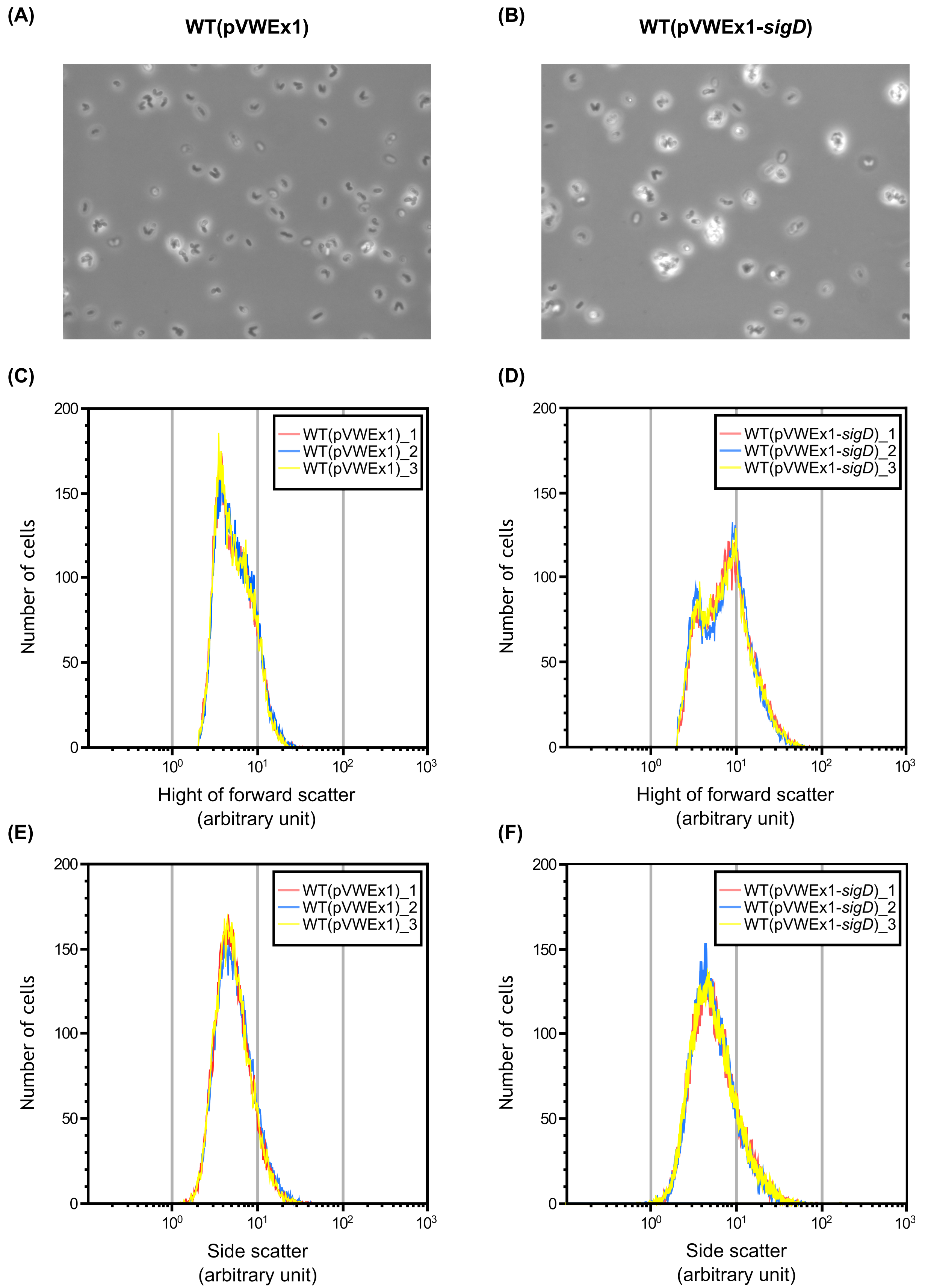
Figure 10. The influence of *sigD* overexpression on cell culture. (A) Cell culture with 50 µM of IPTG after 36 h of cultivation **(B)** Supernatant of cell culture with 50 µM of IPTG after 36 h. **(C)** Supernatant turbidity with different concentrations of IPTG (0 µM, 10 µM, 50 µM) after 36 h. Error bar represents standard deviation calculated from biological triplicates.

2.2.4.3 *sigD* overexpression induced cell aggregation

In addition to the distinct characteristic of cell culture, cell aggregation was observed more often for WT(pVWEx1-*sigD*) with IPTG under the microscope (**Figure 11. A, B**). To measure the aggregation occurrence semiquantitatively, particle size in the culture was compared between WT(pVWEx1) and WT(pVWEx1-*sigD*) by flow cytometry using forward and side scatter. In flow cytometry, it is known that the value of forward scatter is correlated to the particle size and the side scatter value is correlated to the granularity or complexity of particles (Tzur *et al.,* 2011). Therefore, the value of forward scatter increases due to bigger particle size, when cells form aggregation.

A single peak at the arbitrary units of 2 to 3 was detected for the forward scatter signal of the control strain (**Figure 11. C**). For WT(pVWEx1-*sigD*), two peaks were detected, the one at the arbitrary units of 2 to 3 and the othre at the arbitrary units of 10 (**Figure 11. D**). The peak at the arbitrary units of 2 to 3 was observed both for WT(pVWEx1) and WT(pVWEx1-*sigD*), however the peak at the arbitrary units of 10 was observed only for WT(pVWEx1-*sigD*). For the signal of side scatter light, a similar spectrum with the peak at the arbitrary units of 4 to 5 was obtained in both strains (**Figure 11. E, F**). Considering the microscopic images and the result from flow cytometry, overexpression of *sigD* induced cell aggregation.

Figure 11. Morphological change by *sigD* overexpression. Microscopic image of the wild type strain **(A)** and the *sigD* overexpressing strain **(B)**. Cells in the stationary phase were observed under the microscope with magnification of 1000. Distribution of forward scatter signal by flow cytometric analysis for the wild type strain **(C)** and the *sigD* overexpressing strain **(D)**. Distribution of side scatter signal by flow cytometric analysis for the wild type strain **(E)** and the *sigD* overexpressing strain **(F)**. Number of particles was plotted against the value of hight of forward scatter or side scatter. Large value of hight of forward scatter indicates bigger size of particle and large value of side scatter indicates higher granularity or complexity of particles. Red, yellow and blue color indicate the distribution of each biological triplicate.



(A)
WT(pVWEx1)
(B)
WT(pVWEx1-sigD)
(C)
WT(pVWEx1)_1
WT(pVWEx1)_2
WT(pVWEx1)_3
Number of cells
Hight of forward scatter
(arbitrary unit)
(D)
WT(pVWEx1-sigD)_1
WT(pVWEx1-sigD)_2
WT(pVWEx1-sigD)_3
Number of cells
Hight of forward scatter
(arbitrary unit)
(E)
WT(pVWEx1)_1
WT(pVWEx1)_2
WT(pVWEx1)_3
Number of cells
Side scatter
(arbitrary unit)
(F)
WT(pVWEx1-sigD)_1
WT(pVWEx1-sigD)_2
WT(pVWEx1-sigD)_3
Number of cells
Side scatter
(arbitrary unit)

2.2.4.4 SigD regulated transcription of several genes especially related to cell envelope integrity

In order to understand the effect of disruption or overexpression of *sigD* at the transcriptonal level, RNA-seq and real-time PCR analysis were performed. For the effect of *sigD* overexpression, the relative abundance of mRNA was compared in WT(pVWEx1-*sigD*) without IPTG or with 50 μM of IPTG. For the effect of *sigD* disruption, the abundance was compared between the *sigD* disrupted mutant and the wild type strain. RNA-seq analysis showed that expression of 29 genes increased under *sigD* overexpression (M-value > 1). Out of this 29 genes, most genes are annotated as genes related to cell wall integrity, 6 genes (*cop1*, *cmt1*, *cmt2*, *cmt3*, *elrF* and *fadD2*) as corynomycolyl related protein, 6 genes (cg0486, cg0606, g1056, cg2317, cg2318 cg3138) as membrane associated protein, 6 genes (cg0079, cg0607, cg1247, *lppS* and cg3181) as secreted protein and 3 genes (cg0420, cg0532, cg1181) as glycosyltransferase. Expression of one putative transcriptional regulator (cg2320) also increased. Expression of 9 out of 29 genes decreased in the *sigD* disrupted strains (M-value <-1). Furthermore, closer look into the RNA-seq results showed that expression of two genes, *porH* (cg3009) and *pks* (cg3178), increased under *sigD* overexpression (M-value >$\log_2(1.5)$) and at the same time decreased in the *sigD* disrupted mutant (M-value <-$\log_2(1.5)$). Based on the RNA-seq result, expression of genes annotated as mycomembrane related protein (*cop1*, *cmt1*, *cmt2*, *cmt3*, *elrF*, *fadD2*, *porH* and *pks*) and genes annotated as glycosyltransferase (cg0420, cg0532, cg1181) were further confirmed by real-time PCR (**Figure 12**). All these twelve genes were confirmed to be upregulated significantly under *sigD* overexpression. These results suggest that SigD is related to the regulation of cell envelope integrity such as mycomembrane synthesis and cell wall synthesis.

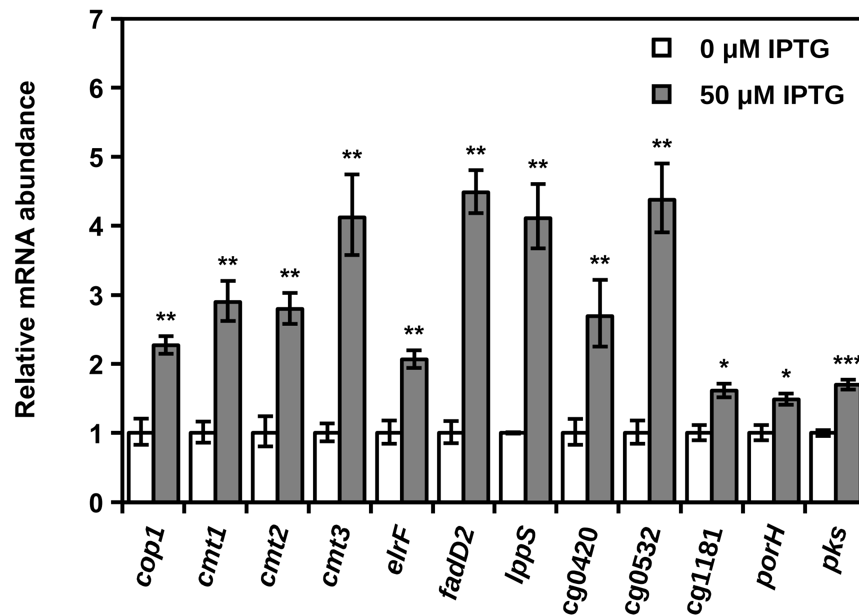


Figure 12. Relative mRNA abundance of genes upregulated under *sigD* overexpression. Relative abundance of mRNA of each gene was quantified by real-time PCR. The white and gray columns show the abundance in WT(pVWEx1-*sigD*) without IPTG (0 µM IPTG) and in WT(pVWEx1-*sigD*) with 50 µM of IPTG (50 µM IPTG), respectively. Error bar represents standard deviation calculated from biological triplicates. p-value was calculated by Student's t-test (two-tail, unpaired) between 0 µM and 50 µM of IPTG, and the value is shown by *, ** and *** for <0.05, <0.01 and <0.001, respectively.

Table 6. Genes differentially expressed upon *sigD* overexpression or *sigD* disruption in RNA-seq analysis

29 genes in total			**M valuer for pVWEx1-*sigD*/pVWEx1 >1**	***sigD* over-expression**	***sigD* disruption**
NCgl	**gene ID[a]**	**gene name[a]**	**Function of protein[a]**	**M-value[b]**	**M-value[b]**
NCgl0059	cg0079	-	Putative secreted protein, CotH homologue	1.2	0.74
NCgl0336	cg0413	***cmt1***	Trehalose corynomycolyl transferase	1.6	-0.41
NCgl0343	cg0420	-	Putative glycosyltransferase	1.0	-0.55
NCgl0394	cg0486	-	ABC-type transporter, ATPase and permease subunit	2.6	-0.53
NCgl0434	cg0532	-	Putative glycosyltransferase	1.9	-0.41
NCgl0497	cg0606	-	Putative membrane protein	2.5	-0.73
NCgl0498	cg0607	-	Putative secreted protein	3.5	-1.8
NCgl0575	cg0696	***sigD***	RNA polymerase sigma factor, ECF-family	3.3	-4.5
NCgl0576	cg0697	-	Conserved hypothetical protein	2.7	-4.7
NCgl0885	cg1052	***cmt3***	Corynomycolyl transferase	2.2	-0.06
NCgl0889	cg1056	-	Putative membrane protein	1.2	-0.34
NCgl0995	cg1181	-	Glycosyltransferase, probably involved in cell wall biogenesis	1.1	-0.21
NCgl1051	cg1246	-	Conserved hypothetical protein	2.2	-1.5
NCgl1052	cg1247	-	Putative secreted protein	2.3	-1.5
NCgl1751	cg2047	-	Putative secreted protein	3.1	-1.3
NCgl2032	cg2317	-	ABC-type putative iron(III) dicitrate transporter, permease subunit	1.6	0.01
NCgl2033	cg2318	-	ABC-type putative iron(III) dicitrate transporter, substrate-binding lipoprotein	2.9	-1.1
NCgl2034	cg2320	-	Putative transcriptional regulator, ArsR-family	3.1	-4.5
NCgl2260	cg2572	-	Conserved hypothetical protein	1.6	-0.92
NCgl2366	cg2693	-	Conserved hypothetical protein	1.1	0.03
NCgl2388	cg2720	***lppS***	Conserved putative secreted lipoprotein, ErfK/YbiS/YcfS/YnhG-family	2.1	-2.0
-	cg2875	-	Hypothetical protein	1.1	-1.1
NCgl2737	cg3138	-	Band 7 domain-containing protein, stomatin/prohibitin homolog	1.4	-0.22
-	cg3139	-	Conserved hypothetical protein	1.2	0.02
NCgl2774	cg3179	***fadD2***	Putative long-chain-fatty-acid--CoA ligase	2.2	-0.41

Table 6. continues from the previous page

NCgl	**gene ID[a]**	**gene name[a]**	**Function of protein[a]**	**M-value[b]**	**M-value[b]**
NCgl2775	cg3180	***elrF***	Envelope lipids regulation factor	1.1	-0.10
NCgl2776	cg3181	**-**	Putative secreted protein	1.2	-0.14
-	cg3182	***cop1***	Trehalose corynomycolyl transferase	1.3	-0.04
NCgl2779	cg3186	***cmt2***	Trehalose corynomycolyl transferase	1.0	0.47

2 genes in total			**M valuer for pVWEx1-*sigD*/pVWEx1 >$\log_2(1.5)$, Δ*sigD*/WT <-$\log_2(1.5)$**	***sigD* overexpres sion**	***sigD* disruption**
NCgl	**gene ID[a]**	**gene name[a]**	**Function of protein[a]**	**M-value[b]**	**M-value[b]**
NCgl2773	cg3178	***pks***	Polyketide synthase, PksM-like	0.98	-0.68
-	cg3009	***porH***	Porin, cation-specific	0.95	-0.57

[a] Gene ID, gene name and function of proteins are given according to CoryneRegNet (http://coryneregnet.de/) or from references. [b]Relative RNA levels in WT(pVWEx1-*sigD*) with 50 μM of IPTG compared to WT(pVWEx1-*sigD*) without IPTG or the *sigD* disrupted mutant compared to the wild type strain are shown as log 2 values (M-values). IPTG was added at the beginning of the cultivation. Number of mapped reads was 7.58 million for the wild type, 7.77 million for the cg0697 disrupted mutant, 7.93 million for WT(pVWEx1-*sigD*) without IPTG, 7.22 million for WT(pVWEx1-*sigD*) with IPTG, respectively.

2.2.4.5 *sigD* overexpression changed the pattern of secreted proteins

The RNA-seq analysis revealed that the expression of many genes encoding secreted proteins and membrane associated proteins increased. Therefore, the proteins in the supernatant of culture were concentrated by acetone and analyzed by 1D SDS-PAGE. The protein sequence of intense bands was further identified by MALDI-TOF/TOF MS with tryptic digestion.

Patterns of secreted proteins in the supernatant were different between WT(pVWEx1) and WT(pVWEx1-*sigD*) (**Figure 13**). Band 3 identified as Cmt1 was more abundant when *sigD* was overexpressed. On the other hand, band 1 identified as Psp3 was less abundant. Band 2 was identified as LppS and band 4 was identified as mixture of two proteins, Cmt2 and Cg2052. Band 5 appeared lower than the dye front of electrophoresis only in the supernatant of the *sigD* overexpressing strain, and its protein sequence could not be identified after several attempts. Overexpression of Cmt1 is consistent with RNA-seq analysis and real-time PCR result. In addition, overexpression of Cmt2 and LppS was also confirmed by RNA-seq and real-time PCR result. On the other hand, *psp3* expression was suggested to be slightly downregulated by the RNA-seq result (M-value, -0.67 under *sigD* overexpression). These result showed that overexpression of *sigD* changed not only the transcriptome but also the secreted protein profile.

The cultivation, the culture of *sigD* overexpressing strain showed specific characteristics such as less foaming and higher turbidity of the supernatant (**Figure 11**). Since Cmt1 was the only protein that was secreted more into the supernatant under *sigD* overexpression, the overexpression of *cmt1* was performed. However, the characteristic of less foaming was not confirmed (data not shown). Therefore, it was concluded that less foaming of the culture occurs not because of protein abundance but from other unknown reasons.

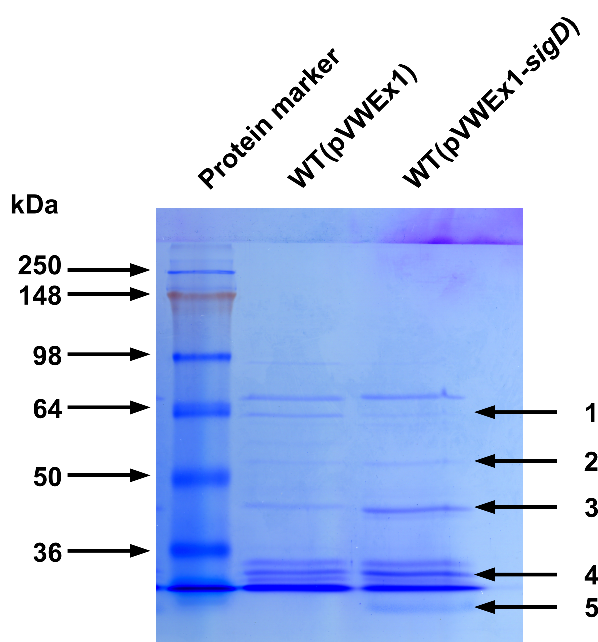


Figure 13. 1D-SDS PAGE of proteins in the supernatant The molecular size of proteins in the marker was shown in kDa. WT(pVWEx1) and WT(pVWEx1-*sigD*) indicates the protein sample which was obtained by acetone of supernatant. Protein from 200 µL of supernatant was loaded on each lane. For the protein band with number, the sequence was identified by MALDI-TOF/TOF MS. 1: Psp3 (Cg2061), 2: LppS (Cg2720), 3: Cmt1 (Cg0413), 4: Cmt2 (Cg3186) and Cg2052, 5: Not identified

2.2.4.6 *sigD* overexpression altered the pattern of secreted metabolites

Acetoen was performed in order to concentrate the proteins in the supernatants. After dissolving the acetone precipitates again, an insoluble fraction was recognized only in the *sigD* overexpressing strain. This fraction is soluble in DMSO/H_2O (9:1 v/v) but not to H_2O, 20 mM of Tris-HCl (pH 7), chloroform, methanol nor chloroform/methanol (2:1 v/v). Furthermore, this insoluble fraction still existed after protease K treatment overnight. For the strain *C. glutamicum* CGL2005, it was reported that the ethanol precipitated fraction of extracellular components consisted primarily of carbohydrates (Puech *et al.,* 2001). These facts suggested that the insoluble fraction can be polysaccharides or carbohydrate containing compounds. Therefore, determination of carbohydrate content in this fraction was performed based on the phenol sulfuric acid method (DuBois *et al.,* 1956), which detects all classes of carbohydrates (Nielsen, 2010). Arabinose, which is one of the components of peptidoglycan in *C. glutamicum,* was used as control. Colorimetric determination after hydrolysis confirmed that the insoluble fraction of WT(pVWEx1-*sigD*) contained carbohydrates (arabinose equivalent of 1.3 mM). On the other hand, the same treatment for supernatants of WT, Δ*sigD* or WT(pVWEx1) resulted in carbohydrate content below the detection limit (less than arabinose equivalent of 0.1 mM) (**Figure 14**). These result suggested that change of transcription by *sigD* overexpression leads to the secretion of polysaccharides or carbohydrate containing compounds into the supernatant.

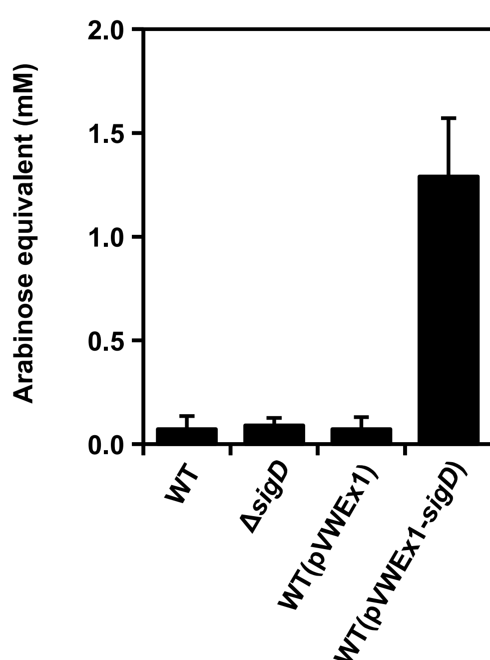


Figure 14. Carbohydrate content in the acetone precipitates of supernatant. Carbohydrate content was measured by the phenol sulfuric acid method. Arabinose samples with known concentrations were used for standard. WT, Δ*sigD*, WT (pVWEx1) and WT(pVWEx1-*sigD*) indicates the sample from the wild type strain, the *sigD* disrupted strain, the wild type strain with the empty vector pVWEx1, and the wild type strain with the plasmid, pVWEx1-*sigD*. Content was calculated to the concentration (mM) of arabinose equivalent carbohydrate. 0.1 mM was the detection limit. Error bar indicates standard deviation calculated from three biological replicates.

2.2.4.7 Overexpression of *sigD* increased the amounts of trehalose dicorynomycolate

The RNA-seq results and the following real-time PCR confirmed that the expression of several genes annotated as corynomycolyl transferase (*cop1*, *cmt1*, *cmt2*, *cmt3*) and genes related to corynomycolic acid production (*pks* and *fadD2*) increased under *sigD* overexpression. Overexpression of *cop1* is know to increase TDCM (trehalose dicorynomycolate) content in the cells (Puech *et al.,* 2000). To confirm the effect of *sigD* overexpression to the TDCM content, crude lipid was extracted from cells, and its composition was analyzed by TLC (thin layer chromatography) with following visualization of carbohydrate containing compounds (**Figure 15**).

After visualization, several bands were detected. The intensity of the band corresponding to TDCM was significantly stronger in WT(pVWEx1-*sigD*) than in WT (pVWEx1). The intensity of the band corresponding to TMCM (trehalose monocorynomycolate) did not show notable difference between the two strains. Interestingly, the faint band appeared between the solvent front and the band corresponding to TDCM only in the *sigD* overexpressing strain. The result indicates that upregulation of multiple genes by *sigD* overexpression increases the flux toward corynomycolic acid synthesis and result in the alteration of TMCM/TDCM ratio.

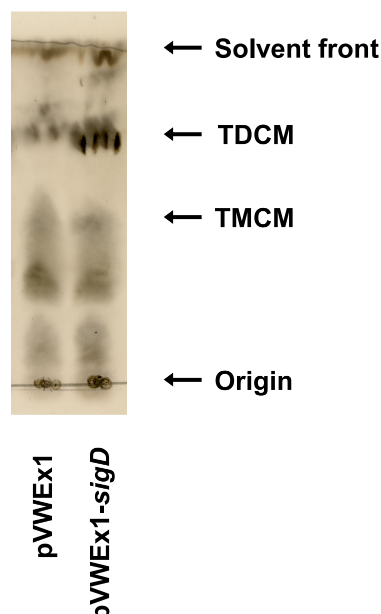


Figure 15. TLC analysis of lipid crude extract. The mobility of TDCM (trehalose dicorynomycolate) and TMCM (trehalose monocorynomycolate) were determined by Rf value from previous study. $CHCl_3/CH_3OH/H_2O$ (30:8:1 v/v) was used as development solvent.

2.2.4.8 cg0697 is a candidate for the anti-sigma factor of SigD

SigD is classified as a ECF40 type sigma factor. Most of ECF sigma factors possesses an anti-sigma factor which controls the activity of the cognate sigma factor. An anti-sigma factor gene is often encoded next to the cognate sigma factor gene. In *C. glutamicum*, a gene encoding hypothetical protein (Cg0697) exists next to *sigD*, and the last nucleotide of *sigD* and the first nucleotide of cg0697 overlap. Therefore, cg0697 is strongly suggested to be the anti-sigma factor of SigD. In order to examine the activity of Cg0697 as the anti-sigma factor of SigD, the disrupted mutant of cg0697 was constructed (**Figure 16**).

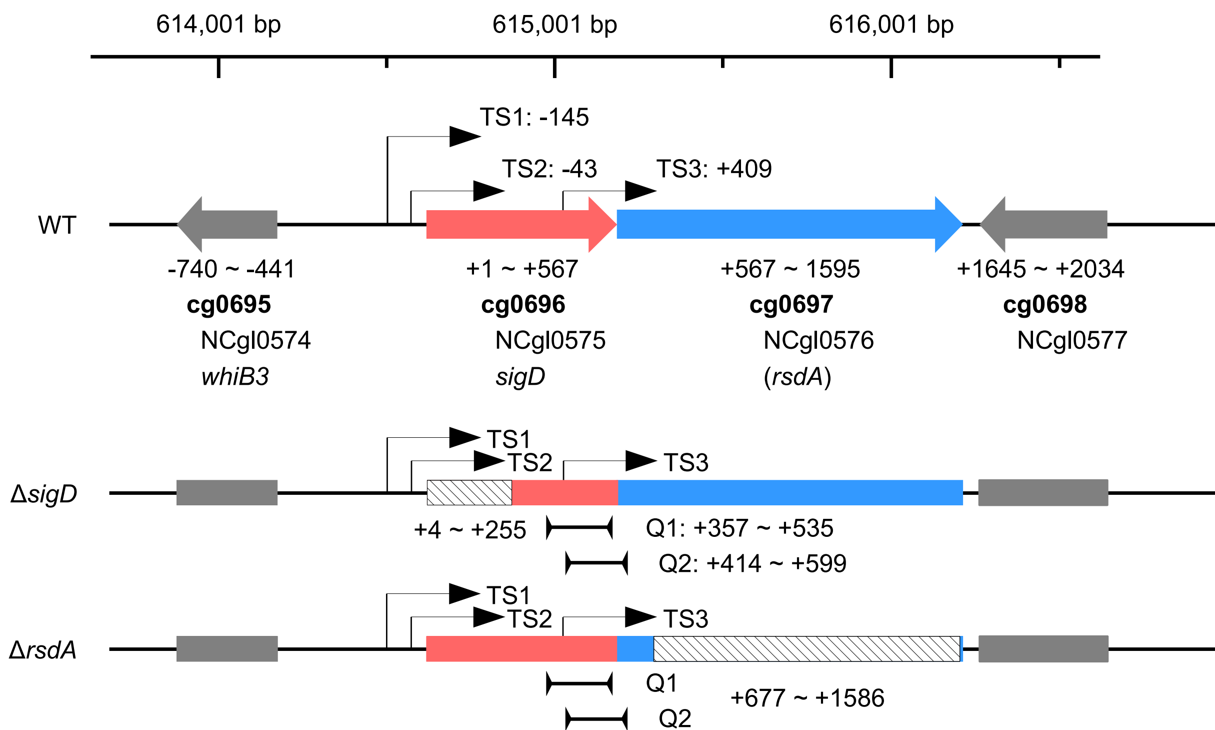
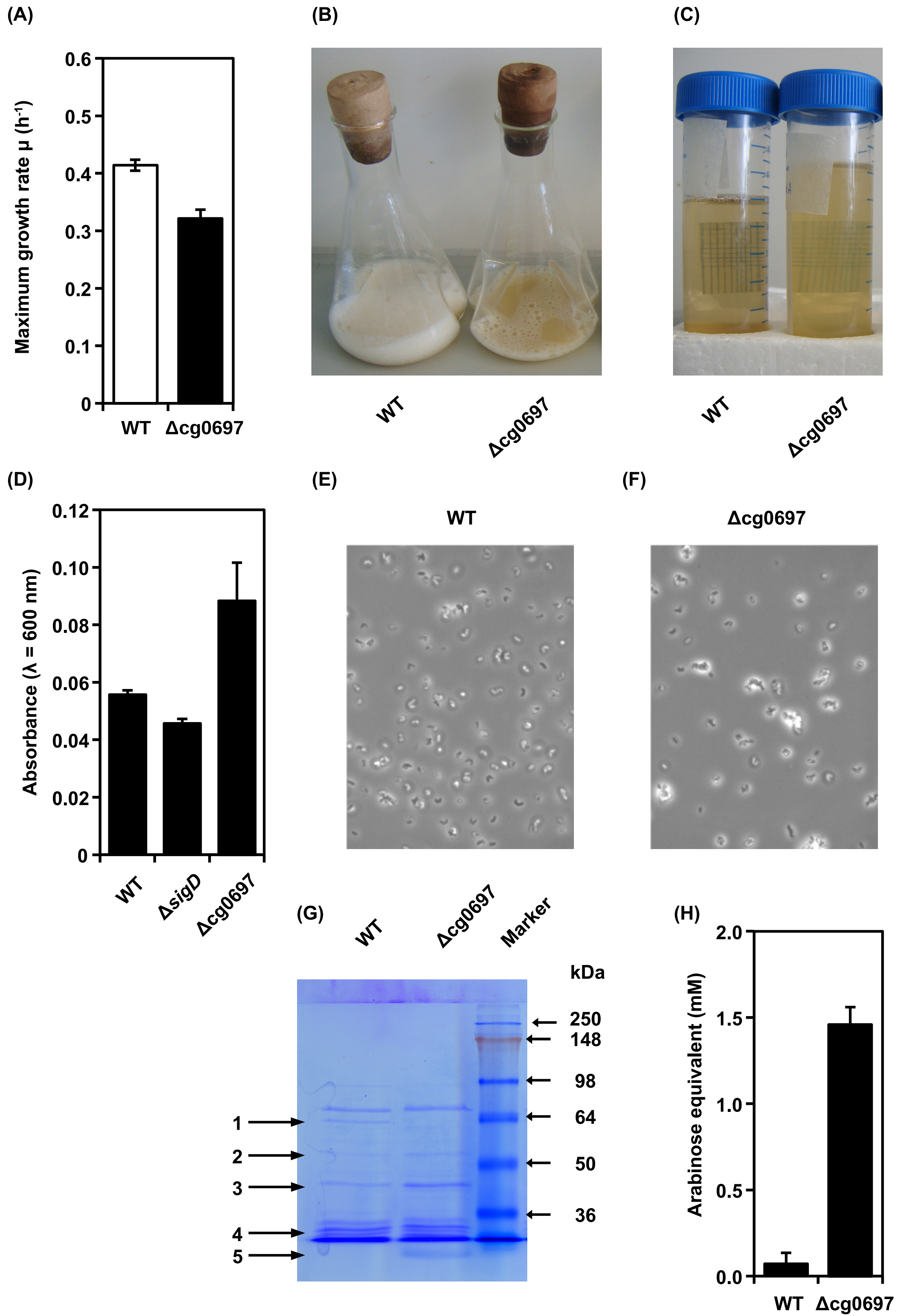


Figure 16. Genomic locus of *sigD* and cg0697 in *C. glutamicum*. Genomic locus of *sigD* (red) and cg0697 (blue) are shown for the each strain, WT (the wild type strain), Δ*sigD* (the *sigD* disrupted mutant) and Δcg0697 (the cg0697 disrupted mutant). Scale bar indicates the nucleotide position in the chromosome (Reference sequence, NCBI:NC_006958.1). Gene loci are shown based on the first nucleotide of *sigD* ORF (+1). TS1 (-145), TS2 (-43) and TS3 (+409) indicate transcription start sites identified in the previous research (Pfeifer-Sancar *et al.,* 2013). Q1 (+357 ~ +535) and Q2 (+414 ~ +599) indicate the targeted region of real-time PCR in this study. Hatched boxes indicate the region disrupted in each strain (Δ*sigD*: +4 ~ +255, Δcg0697: +677 ~ +1586).

2.2.4.9 The cg0697 disrupted mutant showed a similar phenotype as the *sigD* overexpressing strain

The cg0697 disrupted mutant was cultivated in CGXII medium with 222 mM of glucose. The disrupted strain grew slower than WT strain, and the maximum growth rate was comparable to the rate under moderate expression of *sigD* from plasmid (**Figure 17. A**, **Figure 9. B**). In addition, the cg0697 disrupted mutant showed a similar phenotype as the *sigD* overexpressing strain such as less foaming, turbidity in the supernatant and cell aggregation (**Figure 17**, **Figure 10**) The pattern of secreted proteins in supernatant is also similar to that of the *sigD* overexpressing strain (**Figure 17. G**, **Figure 13**). After precipitating the supernatant with acetone and dissolving with 20 mM of Tris-HCl (pH 7), the insoluble fraction was confirmed for the cg0697 disrupted mutant, which was also consistent with the *sigD* overexpressing strain. Carbohydrate content was measured with the phenol sulfuric acid method, and the insoluble fraction of the mutant contained carbohydrates (arabinose equivalent of 1.5 mM), which is comparable to the insoluble fraction of WT(pVWEx1-*sigD*) (**Figure 17. E**, **Figure 14**). These results shows that disruption of cg0697 leads to the same phenotype as overexpression of *sigD*.

Figure 17. (A) Maximum growth rate of WT and Δcg0697. (B) Cell culture after 36 hours of cultivation of WT and Δcg0697. (C) Supernatant of cell culture of WT and Δcg0697 after 36 hours. (D) Supernatant turbidity of cell culture of WT, Δ*sigD* and Δcg0697 after 36 hours. (E) Microscopic image of wild type strain in the stationary phase. (F) Microscopic image of Δcg0697 strain in the stationary phase. (G) 1D-SDS PAGE of proteins in the supernatant. The molecular size of proteins in the marker is shown in kDa. WT and Δcg0697 indicates the protein sample which was obtained by acetone of supernatant. Protein from 200 μL of supernatant was loaded on each lane. The protein band with number is the same as ; 1: Psp3 (Cg2061), 2: LppS (Cg2720), 3: Cmt1 (Cg0413), 4: Cmt2 (Cg3186) and Cg2052, 5: Not identified. **(H) Carbohydrate content in the acetone precipitates of supernatant.** Carbohydrate content was measured by the phenol sulfuric acid method. Arabinose samples with known concentrations were used as standard. Content was calculated to the concentration (mM) of arabinose equivalent carbohydrate. 0.1 mM was detection limit. Error bar indicates standard deviation calculated from three biological replicates.


(A)
Maximum growth rate μ (h⁻¹)
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
Δcg0697
(B)
WT
Δcg0697
(C)
WT
Δcg0697
(D)
Absorbance (λ = 600 nm)
0.12
0.10
0.08
0.06
0.04
0.02
0
WT
ΔsigD
Δcg0697
(E)
WT
(F)
Δcg0697
(G)
WT
Δcg0697
Marker
kDa
250
148
98
64
50
36
1
2
3
4
5
(H)
Arabinose equivalent (mM)
2.0
1.5
1.0
0.5
0.0
WT
Δcg0697

2.2.4.10 Transcription of cg0697 is SigD-dependent and Cg0697 regulates SigD activity

In *C. glutamicum*, it was shown that one of the transcription start sites of an anti-sigma factor gene, *rshA*, is dependent on the cognate sigma factor, SigH (Busche *et al.,* 2012). This regulation works as a negative feedback loop for SigH activity and is reasonable for quick activation and deactivation of SigH-dependent transcription. For transcription of cg0697 and *sigD*, two transcription start sites (TS1, TS2) were identified in front of *sigD* start codon and one transcription start site (TS3) was identified inside of *sigD* and in front of the cg0697 coding sequence (**Figure 16**) (Pfeifer-Sancar *et al.,* 2013). Open reading frames of *sigD* and cg0697 overlap one nucleotide and no transcription termination sequence is found within the two genes. These facts suggest that *sigD* and cg0697 are co-transcribed as one operon from TS1 and TS2 and cg0697 is transcribed individually from TS3 in addition. This arrangement is similar to *sigH* and *rshA* (Busche *et al.,* 2012). Therefore, the involvement of SigD on transcription of *sigD* and cg0697 was examined by real-time PCR, using the *sigD* disrupted strain and the *sigD* overexpression strain as well as the cg0697 disrupted strain (**Figure 18**). In order to distinguish transcripts from different transcription start sites (TS1, TS2 and TS3), two regions (Q1, Q2) were targeted for real-time PCR. The Q1 region is only amplified from transcripts starting from TS1 or TS2, and the Q2 region is amplified from transcripts starting from TS1, TS2 or TS3.

First, comparison between WT and WT(pVWEx1*sigD*) without IPTG confirmed that the existence of plasmid or IPTG did not affect the relative abundance of the Q1 region and the Q2 region. An increase of the Q1 signal in WT(pVWEx1-*sigD*) with IPTG compared to WT(pVWEx1-*sigD*) without IPTG confirmed that *sigD* is overexpressed from plasmid. On the other hand, an increase of the Q2 signal in WT(pVWEx1-*sigD*) with IPTG compared to WT(pVWEx1-*sigD*) without IPTG indicates that overexpression of *sigD* enhances transcription from TS3, since transcript of *sigD* from plasmid does not contain sequence that is necessary for amplification of the Q2 region. A decrease of the Q2 signal in Δ*sigD* compared to WT indicates that transcription from TS3 is SigD-dependent. Therefore, the data from overexpression and disruption of *sigD* strongly support the SigD-dependent transcription from TS3. Furthermore, an increase of the Q2 signal in Δcg0697 compared to WT indicates that absence of Cg0697 influences SigD activity positively. This result strongly supports that Cg0697 is the anti-sigma factor for SigD. A slight decrease of the Q1 signal in Δ*sigD* was seen, and it may be because the disruption of *sigD* sequence in the genome

influences 5' stability of mRNA.

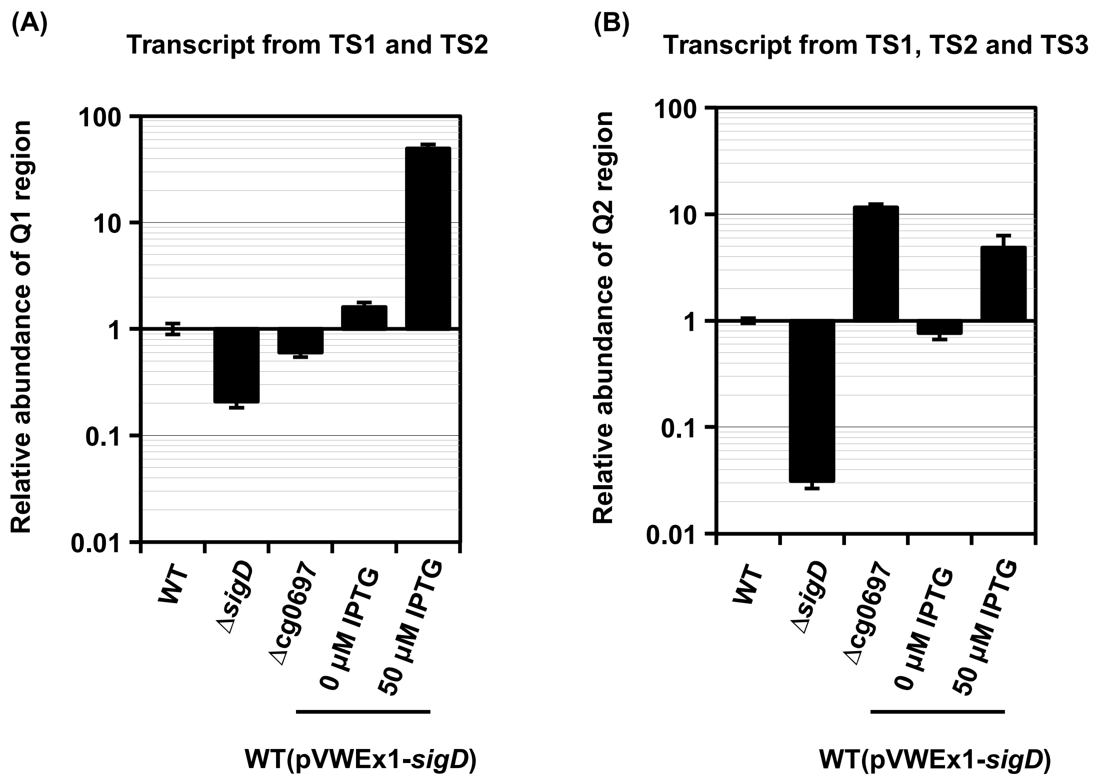


Figure 18: (A) Relative amount of mRNA containing the Q1 region. The Q1 region represents the transcript from the transcription start site TS1 and TS2 (**Figure 16**). **(B) Relative amount of mRNA containing the Q2 region.** The Q2 region represents the transcript from the transcription start site TS1, TS2 and TS3 (**Figure 16**). Error bar represents standard deviation from biological triplicates .

2.2.4.11 Disruption of cg0697 led to similar transcriptome changes as *sigD* overexpression

The RNA-seq result of the cg0697 disrupted mutant revealed that expression of 42 genes increased compared to the wild type strain (M-value > 1) (**Table 7**). Among those, four corynomycolyl transferase genes (*cop1*, *cmt1*, *cmt2*, *cmt3*), 1 corynomycolate synthesis gene (*fadD2*) and one putative L, D-transpeptidase gene (*lppS*) were found. Furthermore, some genes with a known or predicted function increased their expression such as *pknB* (cg0057) encoding serine/threonine protein kinase, *wzx* (cg0421) encoding putative oligosaccharidyl lipid translocase, *rpf2* (cg1037) encoding resuscitation-promoting factor 2, *ctpC* (cg1329) encoding putative Cd^{2+} transporting P-type ATPase, *ugpB* (cg1570) encoding an extracytoplasmic glycerol-3-phosphate-binding protein, *sufR* (cg1765) encoding iron-sulfur cluster biogenesis transcriptional regulator, *idhA3* (cg2313) encoding myo-inositol-2-dehydrogenase and *tagA1* (cg3140) encoding putative DNA-3-methyladenine glycosylase I. Expression of *sigD* and cg0697 was shown to increase, however, this was an artifact occurred by the transcription start site of cg0697 and the disrupted region of cg0697. By disruption of cg0697, the amount of transcript from TS3 increased, and transcript from TS3 overlaps with the open reading frame of *sigD* (+409 to +567) (**Figure 16**). Since this signal is very high, *sigD* was found to be upregulated. No significant increase of *sigD* transcript from TS1 and TS2 was confirmed by visualization of the RNA-seq result by ReadXplorer. Transcript from TS3 also increased the signal from the open reading frame of cg0697 (+567 to +677), even after disruption of this gene. No transcript was detected for the disrupted region of cg0697 from visualization by ReadXplorer. 27 out of these 42 genes are also upregulated in the *sigD* overexpression strain, and the disruption of cg0697 has a similar effect on the transcriptome profile as overexpression of *sigD*. This fact further confirms the inhibition of SigD activity by Cg0697.

Table 7. Genes differentially expressed upon cg0697 disruption in RNA-seq analysis

42 genes in total			M valuer for Δ*cg0697*/WT >1	cg0697 disruption	*sigD* over-expression	*sigD* disruption
NCgl	**gene ID[a]**	**gene name[a]**	**Function of protein[a]**	**M-value[b]**	**M-value[b]**	**M-value[b]**
NCgl0040	cg0057	***pknB***	Serine/threonine protein kinase	1.1	0.88	-0.19
NCgl0059	cg0079	**-**	Putative secreted protein, CotH homolog	2.0	1.2	0.74
NCgl0076	cg0105	**-**	Hypothetical protein	1.1	0.71	1.1
NCgl0077	cg0107	**-**	Putative secreted protein	1.1	0.82	0.93
NCgl0336	cg0413	***cmt1***	Trehalose corynomycolyl transferase	1.7	1.7	-0.41
NCgl0343	cg0420	**-**	Putative glycosyltransferase	1.2	1.0	-0.55
NCgl0344	cg0421	***wzx***	Putative PST O-antigen protein, multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) translocase	1.0	0.93	-0.46
NCgl0394	cg0486	**-**	ABC-type transporter, ATPase and permease subunit	2.9	2.6	-0.53
NCgl0434	cg0532	**-**	Putative glycosyltransferase	1.9	1.9	-0.41
NCgl0497	cg0606	**-**	Putative membrane protein	2.9	2.5	-0.73
NCgl0498	cg0607	**-**	Putative secreted protein	3.6	3.5	-1.8
NCgl0575	cg0696	***sigD***	RNA polymerase sigma factor, ECF-family	3.1*	3.3	-4.5
NCgl0576	cg0697	**-**	Conserved hypothetical protein	1.8*	2.7	-4.7
NCgl0623	cg0753	**-**	Putative secreted protein	1.1	0.87	0.79
NCgl0872	cg1037	***rpf2***	RPF2 precursor, secreted protein	1.2	0.30	1.4
NCgl0885	cg1052	***cmt3***	Corynomycolyl transferase	2.3	2.2	-0.06
NCgl0889	cg1056	**-**	Putative membrane protein	1.5	1.2	-0.34
NCgl0933	cg1109	**-**	Hypothetical protein	1.3	0.81	1.1
NCgl0996	cg1182	**-**	Putative membrane protein	1.1	0.97	0.02
NCgl1051	cg1246	**-**	Conserved hypothetical protein	2.5	2.2	-1.5
NCgl1052	cg1247	**-**	Putative secreted protein	2.4	2.3	-1.5
NCgl1129	cg1329	***ctpC***	Putative Cd^{2+} transporting P-type ATPase	1.2	0.40	0.74
NCgl1331	cg1570	***ugpB***	ABC-type sn-glycerol-3-phosphate transporter, substrate-binding lipoprotein (TC 3.A.1.1.3)	1.4	0.49	1.2
NCgl1504	cg1765	***sufR***	Transcriptional repressor of suf operon	1.2	0.57	1.6
NCgl1751	cg2047	**-**	Putative secreted protein	3.1	3.1	-1.3
NCgl2029	cg2313	***idhA3***	Myo-inositol 2-dehydrogenase	1.2	0.66	0.44

Table 7. continues from the previous page

NCgl2032	cg2317	-	ABC-type putative iron(III) dicitrate transporter, permease subunit	1.8	1.6	0.01
NCgl2033	cg2318	-	ABC-type putative iron(III) dicitrate transporter, substrate-binding lipoprotein	2.8	2.9	-1.1
NCgl2034	cg2320	-	Putative transcriptional regulator, ArsR-family	3.2	3.1	-4.5
NCgl2260	cg2572	-	Conserved hypothetical protein	1.5	1.6	-0.92
NCgl2388	cg2720	***lppS***	Conserved putative secreted lipoprotein, ErfK/YbiS/YcfS/YnhG-family	2.4	2.1	-2.0
NCgl2411	cg2747	-	Putative secreted peptidase, M23/M37-family	1.3	0.98	3.3
-	cg2875	-	Hypothetical protein	1.2	1.1	-1.1
NCgl2737	cg3138	-	Band 7 domain-containing protein, stomatin/prohibitin homolog	1.9	1.4	-0.22
-	cg3139	-	Conserved hypothetical protein	2.0	1.2	0.02
NCgl2739	cg3140	***tagA1***	DNA-3-methyladenine glycosylase I	1.6	0.93	-0.19
NCgl2774	cg3179	***fadD2***	Putative long-chain-fatty-acid--CoA ligase	2.3	2.2	-0.41
NCgl2775	cg3180	***elrF***	Envelope lipids regulation factor	1.1	1.1	-0.10
NCgl2776	cg3181	-	Putative secreted protein	1.2	1.2	-0.14
-	cg3182	***cop1***	Trehalose corynomycolyl transferase	1.3	1.3	-0.04
NCgl2779	cg3186	***cmt2***	Trehalose corynomycolyl transferase	1.0	1.0	0.47
NCgl2907	cg3338	-	Putative membrane protein	1.5	0.87	1.3

[a]Gene ID, gene name and function of proteins are given according to CoryneRegNet (http://coryneregnet.de/) and references. [b]Relative RNA levels in the cg0697 or *sigD* disrupted mutant compared to the wild type strain or WT(pVWEx1-*sigD*) with 50 µM of IPTG compared to WT(pVWEx1-*sigD*) without IPTG are shown as log 2 values (M-values). Number of mapped reads was 7.58 million for the wild type, 7.48 million for the *sigD* disrupted mutant, 7.77 million for the cg0697 disrupted mutant, 7.93 million for WT(pVWEx1-*sigD*) without IPTG, 7.22 million for WT(pVWEx1-*sigD*) with IPTG, respectively.

2.2.4.12 Consensus promoter sequence for SigD

Pátek an coworkers confirmed that transcription of cg0697 is SigD-dependent *in vivo* by a two plasmid system. They also confirmed the SigD-dependent transcription of cg0697 by in vitro transcription experiments and identified the transcription start site of this transcript as well as the putative -35 box and -10 box sequences (GTAAC - 15 nt - CTCGAT). The existence of this putative consensus sequence was analyzed in 29 corynebacteria species which posesses the gene homologous to cg0697. Interestingly, 28 out of 29 strains have an identical consensus sequence for -35 box, spacer and -10 box in front of the gene homologous to cg0697. Only *C. kroppenstedtii* does not have an identical but a similar sequence (GTAAC - 15 nt – CTCGA**<u>C</u>**). From this sequence, we searched for this putative promoter sequence upstream of the upregulated gene in the RNA-seq analysis. Four genes (*cmt1*, *cmt2*, *lppS* and cg1056) have a similar sequence to the consensus sequence upstream of the predicted transcription start site (**Table 8**). Dr. Jiří Holátko from the group of Dr. Miroslav Pátek showed that RNA polymerase containing SigD is able to initiate transcription from these four promoters by in vitro transcription experiment (data not shown).

Table 8. Putative promoter sequence identified from RNA-seq result

Gene name	Gene ID	60 bases upstream of the TSS (-59 to +1) with -10 and -35 regions in upper case letters
-	cg0697	ACTTTGCGCTGGTCAGCGATGGAA**<u>GTAAC</u>**AGAGTTAGGGAACTT**<u>CTCGAT</u>**CTACTGAGTG
cmt1	cg0413	TCGAAAAGGTAAAGCGCCTGT**<u>TAAC</u>**GTAATAGCTTGAAATATA**<u>GAT</u>**GTAAATTAAAGGTG
-	cg1056	CAGTAATTTGTTTTGACGACGCA**<u>GTAAC</u>**GCAATCGGGGATTGTGG**<u>TCGAT</u>**TCTTTAAGCA
lppS	cg2720	AACCATTGAAAAGGCATTCTGGAC**<u>GTAAC</u>**GCTCCGGCATCTACAAGG**<u>GAT</u>**GATCAAAATA
cmt2	cg3186	ACTACCCAAGAGTGTCACAACTTG**<u>GTAAC</u>**GTGTGGGCGGAAAAACAA**<u>GAT</u>**AGGCATCGAG

Sequence which has similarity to the consensus promoter sequence derived from the promoter of cg0697 was shown in bold letter with underline.

2.2.5 Discussion

In this study, the effect of overexpression of *sigD* was elucidated. Overexpression of *sigD* led to the phenotypic change of slower growth, cell aggregation, less foaming of the culture and turbidity of supernatant. The influence of *sigD* overexpression on the transcriptome was analyzed by RNA-seq as well as real-time PCR, and the expression of many genes related to cell envelope integrity increased. Overexpression of *sigD* caused an increase of trehalose dicorynomycolate in lipid extract and secretion of carbohydrate containing compounds in the supernatant.

Mycomembrane synthesis pathway and related enzymes in *C. glutamicum* have been studied in detail (Lanéelle *et al.,* 2013). First, one molecule of fatty acid is carboxylated via carboxylation complex (Gande *et al.,* 2007), and a second fatty acid is activated to a fatty acyl-CoA by FadD2 (Cg3179, NCgl2774) (Portevin *et al.,* 2005). These two molecules are condensed and attached to trehalose by Pks (Cg3178, NCgl2773) (Gavalda *et al.,* 2014; Portevin *et al.,* 2004). The product is further reduced to TMCM (trehalose monocorynomycolate) by CmrA (Lea-Smith *et al.,* 2007), and TMCM is exported from cytoplasm though MmpL1 and MmpL3 with acetylation by TmaT (Varela *et al.,* 2012; Yamaryo-Botte *et al.,* 2015). Corynomycolate is transferred from TMCM onto arabinogalactan, TMCM itself and proteins such as PorH and PorA by several corynomycolyl transferases (Puech *et al.,* 2000; Brand *et al.,* 2003; De Sousa-D'Auria *et al.,* 2003; Huc *et al.,* 2013). *C. glutamicum* ATCC13032 possesses six types of corynomycolyl transferase genes, *cop1*, *cmt1*, *cmt2*, *cmt3*, *cmt4* and *cmt5* (Brand *et al.,* 2003). Cop1, Cmt1 and Cmt2 catalyze TDCM synthesis from TMCM, and their redundant function was suggested (Brand *et al.,* 2003; De Sousa-D'Auria *et al.,* 2003). Cop1 is also reported to transfer corynomycolate from TMCM to arabinogalactan in the cell wall in *C. glutamicum* CGL 2005 (Puech *et al.,* 2000). PorA and PorH form the major cell wall channel penetrating mycomembrane, and this channel works in transport of hydrophobic compounds (Burkovski, 2013). Corynomycolation of PorA and PorH was shown to be necessary for the pore forming activity, and its corynomycolation is catalyzed by Cmt1 (Barth *et al.,* 2010; Huc *et al.,* 2013). ElrF (Cg3180, NCgl2775) was identified as envelope lipids regulation factor and shown to regulate lipid composition of corynomycolic acid and phospholipid in cell envelope under stress condition (Meniche *et al.,* 2009).

Interestingly, the RNA-seq and real-time PCR analysis confirmed that the expression of nine genes related to mycomembrane synthesis (*fadD2*, *pks*, *cop1*, *cmt1*, *cmt2*, *cmt3*, *porH*, *elrF* and *porH*) increased under *sigD* overexpression. TLC analysis showed that overexpression of *sigD*

accumulated more TDCM in the lipid crude extract. These results indicate that SigD controls the integrity of cell envelope, especially for mycomembrane in *C. glutamicum*. Furthermore, upregulation of *porH* and *cmt1* indicates that synthesis of the functional cell wall channel increases under *sigD* overexpression. A low ratio of TDM (trehalose dimycolate), which is the main component in the mycomembrane of *M. tuberculosis*, to other lipids in mycomembrane leads to the increase of sensitivity to antibiotics in *M. tuberculosis* (Nguyen *et al.,* 2005), therefore, a high TDCM ratio is expected to work as a stronger permeability barrier. However, excess permeability barrier also reduces the uptake rate of nutrients. Therefore, it is reasonable for bacteria to increase the expression of the functional cell wall channel in order to cope with the high TDCM ratio and the strong permeability barrier.

The expression level of *lppS* also increased under *sigD* overexpression. The function of LppS in *C. glutamicum* is not determined yet, but its amino acid sequence shows 48% identity and 62% similarity to Ldt_{Mt2} of *M. tuberculosis* by NCBI's BLAST (http://blast.ncbi.nlm.nih.gov/). Ldt_{Mt2} is a transpeptidase that generates non-classical 3 → 3 transpeptide linkages (Gupta *et al.,* 2010). These facts suggest that LppS in *C. glutamicum* may work in peptidoglycan reorganization and cell envelope integrity.

Several glycosyltransferases were upregulated under *sigD* overexpression. The exppression of cg0420 encoding putative glycosyltransferase increased under *sigD* overexpression. Cg0420 is predicted to be co-transcribed with the downstream gene, *wzx* (cg0421) (Pfeifer-Sancar *et al.,* 2013). In the RNA-seq analysis, the expression of *wzx* was slightly upregulated under *sigD* overexpression (M-value, 0.93) and slightly downregulated in the *sigD* disrupted mutant (M-value, -0.46). Although there is no study about function of *wzx* in *C. glutamicum*, protein domain search revealed that Wzx of *C. glutamicum* possesses RfbX domain, which is involved in the export of polysaccharides. Wzx translocates polysaccharides from inner membrane to outer membrane (Islam and Lam, 2013). Therefore, several glycosyltransferases and Wzx may play a role in regulation of cell envelope integrity by increasing polysaccharide synthesis and its export to outer membrane.

C. glutamicum is closely related to *M. tuberculosis*. SigD of *C. glutamicum* and *M. tuberculosis* are in the same group of ECF type sigma factor ECF40 (Staroń *et al.,* 2009). SigD in *M. tuberculosis* was shown to be essential for virulence, and disruption of *sigD* decreased the expression of some mycolyl transferase genes as well as other genes related to lipid metabolism

and cell wall process (Raman *et al.,* 2004; Calamita *et al.,* 2005). However, the involvement of SigD in regulation of cell wall integrity was not clear in *M. tuberculosis*. In this study, disruption of *sigD* in *C. glutamicum* showed no or little influence on expression of the genes related to cell wall integrity, even though overexpression of *sigD* did. This can be explained by involvement of other sigma factors in transcription of these genes due to redundant promoter sequences. Therefore, SigD of *M. tuberculosis* may have an unexplored role for the regulation of cell wall integrity, especially for mycomembrane, as is the case for *C. glutamicum*. In *M. tuberculosis*, the activity of SigD is regulated by the anti-sigma factor RsdA, which is encoded next to *sigD* locus (Thakur *et al.,* 2010). In *C. glutamicum*, cg0697 annotated as hypothetical gene are located downstream of *sigD*. Therefore, it may be possible that Cg0697 works as the anti-sigma factor for SigD in *C. glutamicum*.

The protein analysis by SDS-PAGE revealed that the abundance of Psp3 decreased in under *sigD* overexpression. Psp3 has 3 tandem LGFP repeat sequences at the C-terminal region. This tandem sequence is hypothesized to be important for anchoring proteins to the cell wall (Brand *et al.,* 2003; Adindla *et al.,* 2004). *C. glutamicum* ATCC13032 has six genes which products contain LGFP repeat sequence, *cop1*(cgl2875, NCgl2777), *psp1* (cg2069, NCgl1774), *psp2* (cg0905, NCgl0760), *psp3* (cg2061, NCgl1766), *psp4* (cg2805, NCgl2458) and *psp5* (cg3197, NCgl2789). Interestingly, RNA-seq analysis suggested that the expression of four genes (*psp1*, *psp2*, *psp3* and *psp4*) was weakly downregulated under *sigD* overexpression (M value, -0.95, -0.81, -0.67 and -0.45, respectively). The function of these four genes is not known yet, however, the result proposes that these gene products may play a role for the regulation of cell envelope integrity.

The protein sequence of band 5 in SDS-PAGE (**Figure 13**) was not identified by MALDI-TOF/TOF MS. This band can be PorH or PorA. PorH and PorA are relatively small proteins of 4.7 kDa and 6.2 kDa, respectively. The protein sequence of PorH and PorA contains many negatively charged amino acids, and its pI value (3.47 and 3.77 respectively) is very small compared to usual proteins (Weiller *et al.,* 2004). Therefore, the migration of this protein can be very fast in electrophoresis. PorA and PorH are corynomycolylated *in vivo* (Huc *et al.,* 2010). Therefore, the mass spectrum can be different from the expected spectrum derived from amino acid sequence,

and this makes it difficult to identify the protein sequence.

SigD overexpression led to cell aggregation, less foaming of the culture and turbidity of the supernatant, however, the precise mechanism was not elucidated in this study. In *M. tuberculosis* and *Mycobacterium smegmatis*, polysaccharides containing arabinose are suggested to be involved in the aggregation of cells via interaction with Antigen 85, which has the same function as corynomycolyl transferase (Anton *et al.,* 1996). Furthermore, nanoparticles conjugated with arabinose caused the aggregation of *M. smegmatis* cells (Jayawardana *et al.,* 2015). Therefore, excretion of polysaccharides or carbohydrate containing compounds caused by *sigD* overexpression may induce the cell aggregation in *C. glutamicum*. In *M. tuberculosis*, TDM (trehalose dimycolate) is also called “cord factor”, since it facilitates long and slender formations of cells in vitro (Saita *et al.,* 2000). The change of TDCM content in *C. glutamicum* may also influence cell contact and aggregation. In *C. glutamicum* CGL2005, various types of polysaccharides were detected extracellularly (Puech *et al.,* 2001; Kacem *et al.,* 2004). In addition, *Corynebacterium glutamicum* CCTCC M201005 was screened from soil as a producer of bioflocculant consisting of galacturonic acid as the main structural unit (He *et al.,* 2002). Therefore, it is not surprising that *C. glutamicum* ATCC13032 has a mechanism to excrete polysaccharide or carbohydrate containing compounds to supernatant under certain conditions.

Overexpression of *sigD* elucidated the regulatory architecture for the cell envelope integrity, especially for the mycomembrane. Furthermore, *sigD* overexpression changed many aspects of *C. glutamicum,* which mechanisms have not been understood yet. In this study, it was shown that overexpression of a sigma factor has potential to exploit an unknown regulatory network as well as to alter metabolic flux for accumulation of certain metabolites. TDCM was shown to induce priming and activation of macrophage *in vivo* and in vitro in a similar manner as TDM (trehalose dimycolate) from *M. tuberculosis* (Chami *et al.,* 2002). Therefore, TDCM might be relevant for medical research. However, chemical synthesis of TDCM or TDM is not easy because it requires multiple steps. *C. glutamicum* is easy to handle compared to *M. tuberculosis*, therefore, the production of TDCM with *C. glutamicum* can be interesting. On the other hand, coryneomycolate-less strain is known to excrete more L-glutamate and L-lysine (Gebhardt *et al.,* 2007) as well as take up glycerol and acetate more efficiently (Puech *et al.,* 2000). Therefore, reorganization of

mycomembrane by controlling *sigD* expression can be helpful to understand the permeability barrier of cells and construct the strain with higher or lower permeability barriers.

Disruption of cg0697 resulted in the very similar effect on phenotype as *sigD* overexpression. These data suggested that Cg0697 works as the anti-sigma factor for SigD in *C. glutamicum*. The RNA-seq analysis of the cg0697 disrupted mutant is very similar to that of *sigD* overexpressing strain. The expression of genes related to corynomycolic acid synthesis increased in the cg0697 disrupted mutant. Furthermore, some of genes which did not appear as upregulated in *sigD* overexpression were observed as upregulated in the cg0697 disruption mutant. The function of some of those genes is known or predicted. For example, PknB (Cg0057, NCgl0040) is serine/threonine protein kinase, and its overexpression resulted in a lack of apical growth and a coccoid-like morphology (Fiuza *et al.,* 2008). Furthermore, PknB is suggested to phosphorylate the cell division protein FtsZ (Schultz *et al.,* 2009). Therefore, it may be related to the regulation of cell shape and peptidoglycan synthesis. The expression of *wzx* (Cg0421, NCgl0344) was weakly upregulated under *sigD* overexpression, and significantly upregulated in cg0697 disrupted mutant. As mentioned before, Wzx is expected to be related to export polysaccharide from inner membrane to outer membrane, but its fuction is not confirmed yet by experiment in *C. glutamicum*. Rpf2 (Cg1037, NCgl0872) possesses resuscitation-promoting factor motif, and overexpression of this gene reduced the lag phase upon transfer to new medium after long storage (Hartmann *et al.,* 2004). CtcP (Cg1329, NCgl1129) is annotated as putative Cu^{2+}/Cd^{2+} transport ATPase, however, its precise function is not confirmed. UgpB (Cg1570, NCgl1331) is annotated as ABC-type sn-glycerol-3-phosphate transporter, substrate-binding lipoprotein. Although its fuction is not studied in *C. glutamicum*, it is expected to be necessary for the uptake of phosphate containing compounds such as glycerol-3-phosphate or glycerophosphocholine (Schneider, 2001; Jiang *et al.,* 2014). SufR (Cg1765, NCgl1504) is annotated as repressor for *sufR* operon (Wennerhold and Bott, 2006). InhA3 (Cg2313, NCgl2029) is annotated as myo-inositol 2-dehydrogenase and its function is not confirmed by experiment. It is expected to be related to inositol metabolism. In case of *pknB* and *wzx*, its expression is also upregulated under *sigD* overexpression and slight downregulation is observed in Δ*sigD* (**Table 7**). In order to elucidate the effect of SigD on these genes, confirmation by real-time PCR should be performed.

Cg0697 is predicted to contain the transmembrane domain (inside:1-158, transmembrane: 159-181, outside:182 to 342. The number is amino acid residue (a.a.) in protein sequence.) by TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Therefore, Cg0697 is expected to be localized in the cell membrane. Similarity of amino acid sequence was calculated between Cg0697 and RsdA (anti-sigma factor D) of *M. tuberculosis* by BLASTP (https://blast.ncbi.nlm.nih.gov/). The similarity was found between 164 a.a. to 222 a.a. of Cg0697 and 89 a.a. and 151 a.a. of RsdA of *M. tuberculosis* (Identity: 48%, Similarity: 50%, Expect: 2e-05). This region is suggested to be localized at transmembrane and outer membrane. In *M. tuberculosis*, ClpX–ClpP1–ClpP2 proteolytic complex and a site-2 protease, Rip1, are related to activation of SigD by cleavage of RsdA (Jaiswal *et al.,* 2013; Schneider *et al.,* 2014). Rip1 is the transmembrane protein and cleave transmembrane region of RsdA. Considering the conservation of transmembrane region between Cg0697 and RsdA of *M. tuberculosis*, SigD in *C. glutamicum* can be activated by a similar mechanism.

From the RNA-seq analysis, the consensus promoter sequence for SigD was predicted. Pátek and co-workers revealed that "**GTAAC** - 15 nt – CTC**GAT**" is the putative consensus promoter sequence of SigD. Genes with similar promoter sequence were confirmed to be transcribed by SigD *in vivo* and *in vitro*. Further possibility of SigD-dependent promoter can be detected by 5' RNA-seq analysis or ChIP-on-chip analysis (Busche *et al.,* 2012; Toyoda and Inui, 2015). It will show us more precise overview of regulation by SigD.

2.3 Harnessing sigma factor gene expression for production in *Corynebacterium glutamicum*: sigma factor A and carotenoids as an example

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2.3.1 Abstract

Transcription is the first step of gene expression and tightly regulated in bacteria. Sigma factors are deeply related to the transcriptional regulation due to their promoter selectivity upon transcription initiation. Therefore, overexpression of sigma factor genes is expected to perturb transcriptome profile drastically by altering the promoter selectivity of RNA polymerase holoenzyme. This perturbation may increase the metabolic flux into a certain pathway which is helpful for production.

Carotenoids are drawing attention because of their versatile applications in industry. *C. glutamicum* naturally produces the yellow C_{50} carotenoid decaprenoxanthin. Recently, it has been further metabolically engineered to produce different carotenoids such as lycopene and β-carotene. In this study, the potential of sigma factor overexpression was examined in order to enhance carotenoid productions in *Corynebacterium glutamicum*.

First, all seven endogenous sigma factor genes of *C. glutamicum* were overexpressed individually in the carotenoid producing recombinant strain. As a result, overexpression of sigma factor genes influenced carotenoid production differently. Based on the production titer, the primary sigma factor, SigA, was selected as the best target for improving carotenoid production. Accumulation of carotenoids in the *sigA* overexpressing strain was observed especially in the stationary phase of cell growth. Transcriptome analysis revealed that the expression of genes related to thiamine synthesis and aromatic compound degradation pathway were upregulated with many other genes under *sigA* overexpression. Addition of thiamine or protocatechuic acid in culture medium enhanced carotenoid production only in the *sigA* overexpressing strain. The effect of *sigA* overexpression was successfully transferred to different carotenoid producing strains.

This study indicates that the synergistic effect of differential expression of multiple genes will be helpful for carotenoid production. Therefore, overexpression of sigma factor genes is an useful approach in metabolic engineering by inducing synergistic effects of endogenous gene expression.

2.3.2 Introduction

Carotenoids are natural pigments which show various color from yellow to red depending on their chemical structures (Britton *et al.,* 2004). Because of the versatile applications, the demand of carotenoids is increasing, especially in food and feed industry (Breithaupt, 2007; Scotter, 2011). Bio-based carotenoid synthesis is favored due to the application as food and feed additives. In order to increase the production and efficiency, metabolic engineering has been applied in the natural carotenoid producers and non-carotenogenic organisms (Ausich, 2009; Ye and Bhatia, 2012). The pathways and related enzymes for carotenogenesis are well understood. First, IPP (Isopentenyl pyrophosphate) and DMAPP (Dimethylallyl pyrophosphate) are synthesized by one of the two pathways, the mevalonate pathway or the non-mevalonate pathway (also called as MEP pathway) (Chang *et al.,* 2013). IPP and its isomer DMAPP are condensed to lycopene (Moise *et al.,* 2014). Lycopene is a red C_{40} carotenoid and serves as an important intermediate for the biosynthesis of other C_{40} and C_{50} carotenoids such as β-carotene, lutein and astaxanthin (Misawa and Shimada, 1998; Heider *et al.,* 2014b).

C. glutamicum is a non pathogenic bacterium and has been used for amino acid production, especially for L-glutamate and L-lysine, in industry for several decades (Eggeling and Bott, 2005). Furthermore, its biological knowledge and tools of genetic engineering encourages the use of this bacterium for production of industry relevant chemicals (Wendisch, 2014). *C. glutamicum* naturally produces the C_{50} carotenoid decaprenoxanthin (Krubasik *et al.,* 2001). Recently, this bacterium was successfully engineered to accumulate lycopene by deletion and overexpression of several endogenous genes (Heider *et al.,* 2012, 2014c). Lycopene producing strain was further engineered to produce various species of carotenoids such as β-carotene by overexpression of exogenous genes (Heider *et al.,* 2014a, 2014c). Therefore, *C. glutamicum* is a suitable host organism for various carotenoid productions and further improvement of carotenoid production is desired.

Sigma factors are one of the components of RNA polymerase holoenzyme, and it is important for promoter recognition and transcription initiation (Gruber and Gross, 2003). Bacteria often possess multiple sigma factor genes which encode primary and alternative sigma factors (Feklístov *et al.,* 2014). Recently, several attempts have been performed to use sigma factors as a

metabolic engineering tool (Tripathi *et al.,* 2014). For example, strains with high ethanol tolerance or high capability of lycopene production were successfully isolated by the gTME (global transcription machinery engineering) approach, in which the randomly mutated sigma factors are overexpressed and the favorable strains are isolated through selection/screening (Alper and Stephanopoulos, 2007). In *Synechocystis* sp. PCC 6803, the potential of one of the alternative sigma factors was explored for the production of polyhydroxybutyrate (Osanai *et al.,* 2013). Metabolic engineering using sigma factors can alter the transcription profile globally, therefore, a synergistic effect by multiple gene expression can be expected.

C. glutamicum has seven sigma factor genes, *sigA*, *sigB*, *sigC*, *sigD*, *sigE*, *sigH* and *sigM* (Kalinowski *et al.,* 2003). Regulons of sigma factors were elucidated from several studies, expecially for SigA, SigB, SigE, SigH and SigM (Pátek and Nešvera, 2011). Recently, the regulon of SigC was elucidated (Toyoda *et al.,* 2015). Furthermore, SigD was shown to be related to the regulation of cell wall integrity (not published). In addition, it was shown that overexpression of endogenous sigma factor genes can perturb cellular phenotype differently (Taniguchi and Wendisch, 2015).

In this study, overexpression of sigma factor genes was performed in *C. glutamicum*, aiming at increasing the carotenoid production and elucidating the target for further improvement of carotenoid production.

2.3.3 <u>Material and methods</u>

2.3.3.1 Bacterial strains, plasmids and primers

The strains, plasmids used in this work are listed in **Table 9**. Plasmids for sigma factor overexpression were constructed based on pVWEx1, an IPTG inducible *Escherichia coli* – *C. glutamicum* shuttle vector (Peters-Wendisch *et al.,* 2001). Plasmid and strain construction was performed as described previously (Taniguchi and Wendisch, 2015). Briefly, the DNA fragment of a gene was amplified with respective primer pairs (Taniguchi and Wendisch, 2015), and inserted into pVWEx1 by Gibson assembly (Gibson *et al.,* 2009). *E. coli* DH5α was used for cloning. The sequence of inserted DNA fragments was confirmed to be correct. *C. glutamicum* competent cells were transformed by electroporation at 2.5 kV, 200 Ω, and 25 µF (van der Rest *et al.,* 1999; Eggeling and Bott, 2005).

Table 9. Bacterial strains, plasmids and oligonucleotides used in this study

Bacterial strain	Relevant characteristic	Reference or source
<u>*C. glutamicum*</u>		
WT	Wild-type, ATCC 13032	ATCC
LYC5	LYC3-P_{tuf}-*dxs* derivatives with insertion of *crtEBI* operon under the control of P_{tuf} promoter integrated into the *cgp2* cured region between cg1745 and cg 1753	not published
BETA3	LYC5 derivatives with insertion of *crtY* from *P. ananatis* under the control of P_{tuf} promoter integrated into the cgp2 cured region between cg1745 and cg 1753	not published
MB001Δ*crtR*	In frame deletion of prophages cgp1(cg1507-cg1524), cgp2(cg1746-cg1752) and cgp3(cg1890-cg2071), and deletion of cg0725 (*crtR*)	not published
Bacterial strain	**Relevant characteristic**	**Reference or source**
<u>*Other strains*</u>		
E. coli DH5α	F−*thi*-1 *endA*1 *hsdR*17(r−, m−) *supE*44 Δ*lacU*169 (Φ80*lacZ*ΔM15) *recA*1 *gyrA*96 *relA*1	Bethesda Research Laboratories
<u>Plasmid</u>	**Relevant characteristic**	**References**
pVWEx1	KanR; *E. coli*-*C. glutamicum* shuttle vector for regulated gene expression (Ptac, *lacIq*, pCG1 oriVCg)	(Peters-Wendisch *et al.*, 2001)
pVWEx1-*sigA*	KanR, pVWEx1 with *sigA* from *C. glutamicum* WT	this study
pVWEx1-*sigB*	KanR, pVWEx1 with *sigB* from *C. glutamicum* WT	this study
pVWEx1-*sigC*	KanR, pVWEx1 with *sigC* from *C. glutamicum* WT	this study
pVWEx1-*sigD*	KanR, pVWEx1 with *sigD* from *C. glutamicum* WT	this study
pVWEx1-*sigE*	KanR, pVWEx1 with *sigE* from *C. glutamicum* WT	this study
pVWEx1-*sigH*	KanR, pVWEx1 with *sigH* from *C. glutamicum* WT	(Taniguchi and Wendisch, 2015)
pVWEx1-*sigM*	KanR, pVWEx1 with *sigM* from *C. glutamicum* WT	this study

2.3.3.2 Medium, growth conditions and growth rate comparison

As far as not mentioned specifically, *C. glutamicum* was precultured in LB medium (Sambrook, 2001) with 56 mM of glucose overnight, washed once with CGXII medium (Eggeling and Bott, 2005) without carbon source and inoculated in CGXII with 222 mM of glucose at initial OD of 1 (λ = 600 nm). The OD was measured with UV-1202 spectrophotometer (Shimadzu, Duisburg, Germany) with suitable dilutions. When appropriate, 25 µg/mL of kanamycin and IPTG were added. Growth experiment with BioLector® cultivation system (m2pLabs, Baesweiler, Germany) was performed in 1 mL of CGXII with 222 mM of glucose using FlowerPlate® (m2pLabs, Baesweiler, Germany) at 30°C, 1,100 rpm. Growth experiment with flask was performed in 50 mL of CGXII 222 mM of glucose using 500 mL of baffled flask at 30 °C, 120 rpm. For growth rate calculation, cell growth was monitored online every 10 min for 48 hours with BioLector®. Maximum growth rate μ (h^{-1}) was calculated from 20 measuring points of arbitrary unit of the backscattering light (620 nm). Plate image was scanned with Perfection V750-M Pro scanner (Epson, Ludwigshafen am Rhein, Germany).

2.3.3.3 Carotenoid extraction and quantification

200 to 1000 µL of cell culture was centrifuged and washed once with 500 µL of CGXII without carbon source. The cell pellet was frozen and stored at -20 °C until further use. For carotenoid extraction, the pellet was suspended with 400 µL of acetone and carotenoid was extracted for 60 min at 50 °C, 1400 rpm. Supernatant was separated by centrifugation and absorption spectrum from 400 nm to 550 nm was measured by UV-1800 spectrophotometer (Shimadzu, Duisburg, Germany). The spectrum was normalized by the absorbance at wavelength of 550 nm and the maximum absorbance in the spectrum was used for each carotenoid quantification (474 nm for lycopene, 440 nm for decaprenoxanthin, 454 nm for β-carotene). Concentration of lycopene and β-carotene in the extract was determined by a standard curve prepared by sequential dilution of each carotenoid solution. The amount of carotenoid was normalized again to cell weight which was calculated based on OD of cell culture at wavelength of 600 nm (OD_{600} = 1 was converted to 0.25 mg of cells per 1 mL of cell culture).

2.3.3.4 Transcriptome analysis of the *sigA* overexpressing strain using DNA microarray

C. glutamicum strains WT(pVWEx1) and WT(pVWEx1-*sigA*) were cultured in LB medium with 56 mM of glucose and 25 µg/mL of kanamycin and inoculated in CGXII medium with 222 mM of glucose and 25 µg/mL of kanamycin for adaptation. Cells were cultured overnight and inoculated into 50 mL of CGXII medium with 222 mM of glucose, 50 µM of IPTG and 25 µg/mL of kanamycin at the initial OD of 1. Cells were harvested after 8 hours of inoculation in the early exponential growth phase (OD between 6 and 8) and after 24 hours in the stationary phase. RNA isolation was performed as described previously (Wendisch, 2003). The purified RNA was analyzed by spectrophotometer (NanoDrop) for quantity and gel electrophoresis for quality. The RNA sample was stored at -80°C until further use. cDNA synthesis as well as DNA microarray hybridization were performed as described previously (Netzer *et al.,* 2004; Polen *et al.,* 2007). Normalization and evaluation of the data were done with the software package EMMA 2 (Dondrup *et al.,* 2009). Genes which expression was upregulated or downregulated in WT(pVWEx1-*sigA*) were taken into account for further analysis (FDR < 0.05, M-value > 1 for upregulation, M-value < -1 for downregulation).

2.3.4 Results

2.3.4.1 The *sigA* overexpressing strain showed the best improvement of lycopene production

All seven sigma factor genes of *C. glutamicum* were overexpressed in *C. glutamicum* LYC5 strain individually and the influence on lycopene production was evaluated. LYC5 strain was constructed based on LYC3-P_{tuf}dxs with further integration of an extra copy of the carotenogenic operon *crtEBI* under the control of P_{tuf} promoter (Heider *et al.,* 2014c). Each of sigma factor genes was expressed from IPTG inducible plasmid, pVWEx1, with 50 µM of IPTG and the growth of each strain was monitored every 10 min with BioLector. Overexpression of each sigma factor gene influenced the color of cell culture in a different manner (**Figure 19. A**). The strain overexpressing *sigA* showed more reddish color compared to the control strain LYC5(pVWEx1). The *sigH* strain showed a distinct yellow color because of riboflavin accumulation in the supernatant as reported previously (Taniguchi and Wendisch, 2015). Overexpression of sigma factor genes also influenced the maximum growth rate differently (**Figure 19. B**). Overexpression of *sigA*, *sigD* and *sigE* led to a slight decrease of the growth rate, and overexpression of *sigB* and *sigM* did not influence the growth rate. On the other hand, overexpression of *sigC* and *sigH* inhibited the cell growth drastically. Quantification of the lycopene amount showed that *sigA* overexpression increased lycopene production more than 350% compared to the control strain (**Figure 19. C**). Overexpression of *sigC*, *sigD*, *sigH* and *sigM* showed a moderate increase of lycopene production, and overexpression of *sigB* and *sigE* resulted in decrease of the production titer. Overexpression of sigma factor genes influenced the final biomass less than 10% except for *sigH* overexpression (-20%). Overexpression of different sigma factor genes affected lycopene production differently, and *sigA* overexpression was found to be the most effective for increasing lycopene production.

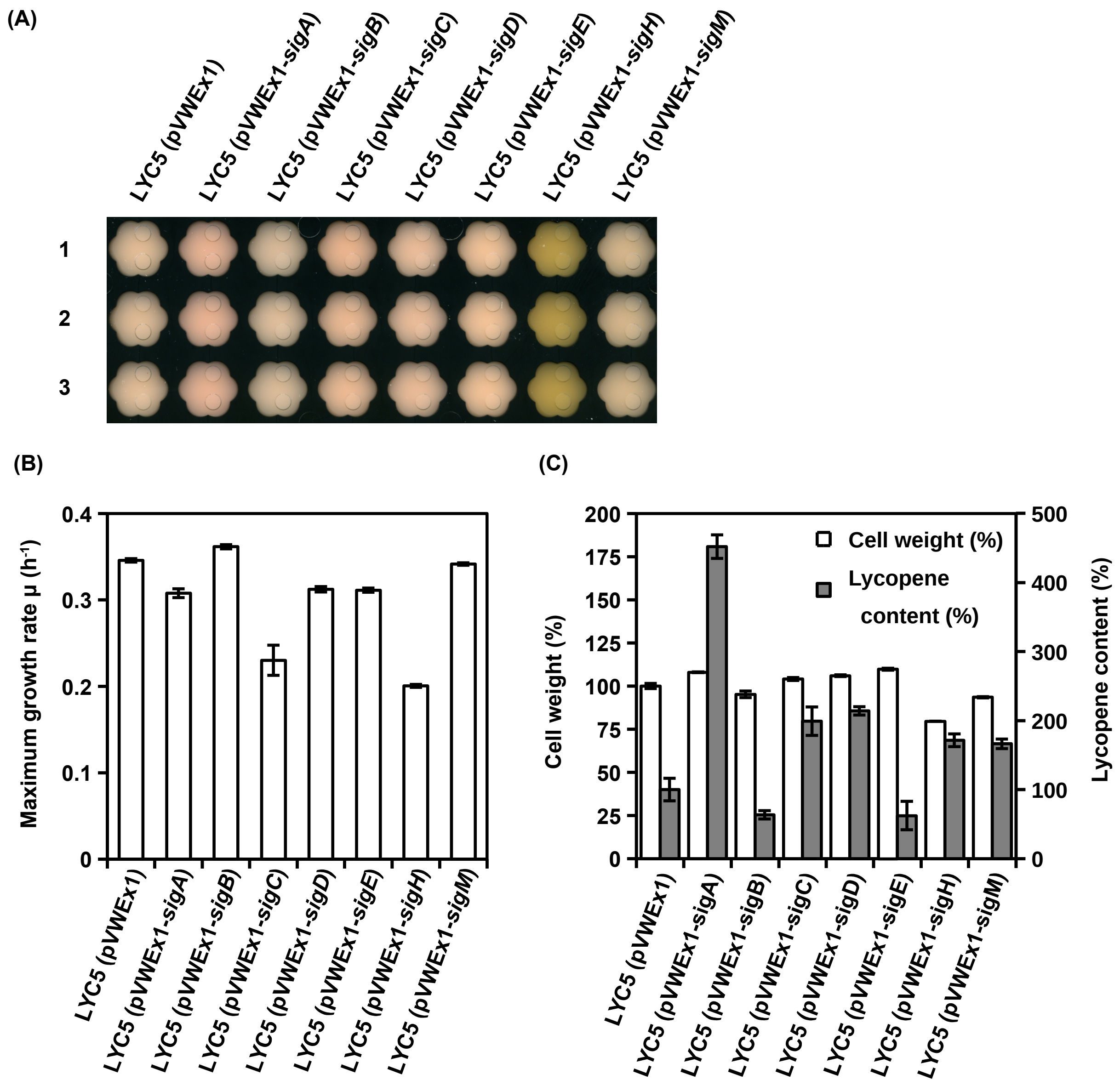


Figure 19. Overexpression of sigma factor genes in the lycopene producing strain, LYC5. (A) FlowerPlate image of the culture after 48 hours of cultivation. Strain with plasmid containing each sigma factor gene was cultivated for 48 hours in the FlowerPlate. Biological triplicates are aligned in the column (1, 2, 3). **(B) Maximum growth rate of each strain with the different sigma factor gene.** Maximum growth rate (h^{-1}) for each strain was calculated from backscatter light measured by BioLector every 10 min. **(C) Cell weight and lycopene content of each strain after 48 hours of cultivation.** Cell weight was calculated based on the optical density of cell culture at wavelength of 600 nm. Lycopene content (%) was calculated based on the optical density of carotenoid extract at wavelength of 474 nm and normalized by cell weight used for extraction. Cell weight and lycopene content were shown in percentage. The values from the control strain (LYC5(pVWEx1)) were adjusted to 100%. Error bar represents standard deviation from biological triplicates.

2.3.4.2 Overexpression of sigA improved lycopene accumulation especially in the stationary phase

SigA is the primary sigma factor in *C. glutamicum* and important for transcription of house keeping genes especially in the exponential phase (Pátek and Nešvera, 2011; Pfeifer-Sancar *et al.,* 2013). It is also known that the transcript of *sigA* is abundant in the exponential phase and decreases during transition from the exponential phase to the stationary phase (Larisch *et al.,* 2007). Therefore, in order to understand the effect of *sigA* overexpression through the growth phase, the change of lycopene content in cells was determined at different time points in 500 mL baffled flasks.

Glucose was consumed completely after 24 h, and the growth of each strain reached the stationary phase at least after 24 h (**Figure 20. A**). Interestingly, the accumulation of lycopene increased drastically after 48 h and 72 h in the *sigA* strain, but not in the control strain (**Figure 20. B**). The accumulation of lycopene in the *sigA* overexpressing strain reached 0.82 ± 0.15 mg/g CDW at 72 h, which was 770% of increase compared to the control strain at the same time point. The accumulation occurred especially in the late stationary phase when the initial carbon source of glucose was completely consumed.

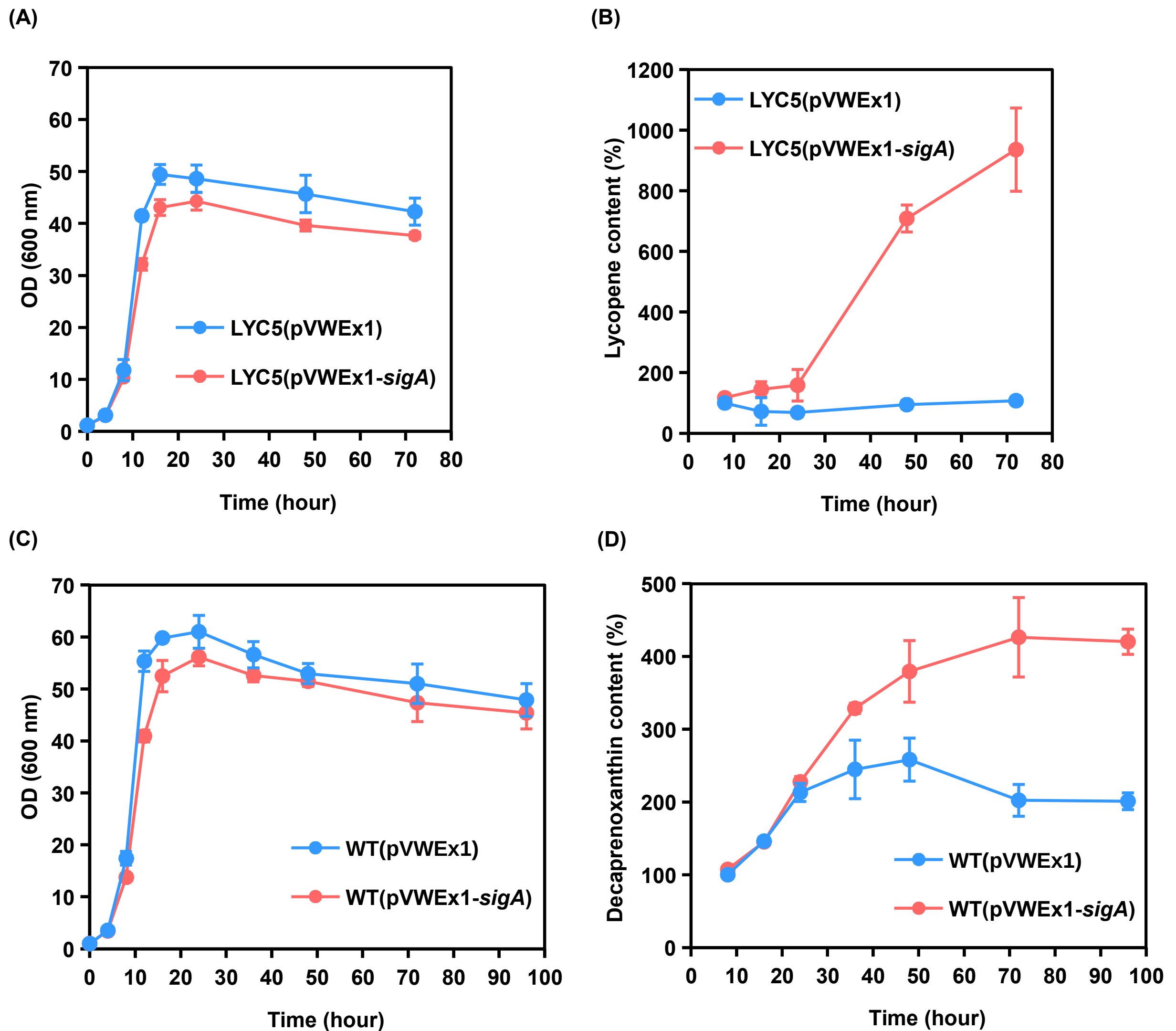


Figure 20. Accumulation of carotenoid during the growth phase. Growth profile of LYC5 (A) and WT (C) with or without *sigA* overexpression. Transition of optical density at wavelength of 600 nm was plotted over time. **Transition of lycopene content in LYC5 (B) and decaprenoxanthin content in WT (D) with or without *sigA* overexpression.** Lycopene or decaprenoxanthin content (%) was calculated based on the optical density of carotenoid extract at wavelength of 474 nm for lycopene and 440 nm for decaprenoxanthin, and normalized by cell weight used for extraction. The value from the control strain LYC5(pVWEx1) and WT(pVWEx1) at 8 h was adjusted to 100% and carotenoid content was shown in percentage. Experiment was performed in baffled flasks and error bar represents standard deviation from biological triplicates.

2.3.4.3 Overexpression of *sigA* also influenced decaprenoxanthin accumulation of the wild type strain in the stationary phase

Overexpression of *sigA* enhanced lycopene production in the metabolically engineered strain LYC5. The question arose whether overexpression of *sigA* influenced the expression of the integrated carotenogenic genes or the other endogenous genes. In order to elucidate the influence, overexpression of *sigA* was performed in the wild type strain *C. glutamicum* WT. The change of decaprenoxanthin content was determined at different time points in 500 mL baffled flasks since WT produces the C_{50} carotenoid decaprenoxanthin.

Glucose was consumed completely after 24 h for both the control and the *sigA* overexpressing strain. The growth of each strain reached the stationary phase at least after 24 h (**Figure 20. C**). Gradual accumulation of decaprenoxanthin until 24 hours were confirmed in the control strain and the *sigA* overexpressing strain (**Figure 20. D**). The control strain stopped the accumulation after 24 hours, on the other hand, the *sigA* overexpressing strain increased the accumulation up to 72 hours. Overexpression of *sigA* resulted in 100% of increase in the decaprenoxanthin production compared to the control strain at 96 h. The effect of *sigA* on carotenoid production is lower in WT than in LYC5. Therefore, overexpression of *sigA* influenced not only the integrated genes but also the other endogenous genes relevant for carotenoid production.

2.3.4.4 Global gene expression changed due to sigA overexpression

Overexpression of *sigA* increased carotenoid production in WT strain. To reveal the effect of *sigA* overexpression on the transcriptional level, differential gene expression was analyzed by DNA microarray. Considering the accumulation of carotenoids in the stationary phase, RNA was extracted at two different time points of 8 h and 24 h after inoculation. Genes with false discovery rate less than 0.05 were taken into account for further analysis, and genes with M-value higher than 1.0 or lower than -1.0 were considered to be upregulated or downregulated, respectively.

Expression of 50 and 65 genes was upregulated at 8 h and 24 h, respectively (**Table 10**, **Table 11**). The expression of *sigA* was confirmed to be upregulated at both time points. Among those 50 and 65 genes, 9 genes including *catA* (cg2636) encoding catechol 1, 2-dioxygenase and *cls* (cg3037) encoding cardiolipin synthase were upregulated at both time points. At 8 hours, genes related to thiamine synthesis (*thiOSG*) and pyridoxal phosphate synthesis (*pdxST*) were upregulated. At 24 hours, genes related to thiamine synthesis (*thiC*, *thiD1*), cell wall integrity (*cmt1*, *wzx*, *murA*, *sigE* and *lysA*) and citric acid cycle (*acn*, *gdh* and *malE*) were upregulated. On the other hand, expression of 18 and 32 genes was downregulated at 8 h and 24 h, respectively (**Table 10**, **Table 11**). Only one gene (cg0612), which is annotated as putative aldo/keto reductase, was downregulated at both time points.

Table 10. Genes differentially expressed upon *sigA* overexpression after 8 hours of cultivation

50 genes		*Up-regulated genes*		
gene ID[a]	gene name[a]	Function of protein[a]	M-value[b]	FDR[c]
cg0096		Conserved hypothetical protein	1.1	3.5E-04
cg0107		Putative secreted protein	1.0	2.0E-06
cg0291		Putative dioxygenase	1.1	1.7E-03
cg0808	***wbpC***	Conserved putative membrane protein	1.2	1.5E-05
cg0898	***pdxS***	pyridoxal 5'-phosphate (PLP) synthase subunit	1.4	2.3E-06
cg0899	***pdxT***	pyridoxal 5'-phosphate (PLP) synthase subunit, glutamine amidotransferase	1.0	4.8E-04
cg0998		Trypsin-like serine protease	1.0	1.3E-03
cg1088		ABC-type putative multidrug transporter, ATPase and permease subunit	1.2	2.3E-05
cg1095		Hypothetical protein	1.4	1.2E-04
cg1096		Hypothetical protein	1.0	3.3E-04
cg1109		Hypothetical protein	1.1	4.9E-06
cg1139		Allophanate hydrolase subunit 2	1.1	6.6E-05
cg1140		Allophanate hydrolase subunit 1	1.0	1.2E-05
cg1142		Putative Mn2+ transporter, metal ion (Mn2+-iron) transporter (Nramp) family	1.3	1.9E-06
cg1147	***ssuI***	NAD(P)H-dependent FMN reductase	1.2	5.2E-03
cg1210		Putative membrane protein	1.4	3.4E-05
cg1227		Putative membrane protein	1.4	1.6E-08
cg1230		Conserved hypothetical protein	1.1	4.9E-06
cg1231	***chaA***	Putative secondary Na+/Ca2+ antiporter, Ca2+:cation antiporter (CaCA) Family	1.1	1.1E-05
cg1232		Conserved hypothetical protein, LmbE-family	1.2	3.4E-03
cg1294		Putative esterase, alpha-beta hydrolase superfamily	1.0	7.4E-05
cg1391		Conserved hypothetical protein, related to capsule biosynthesis enzymes	1.3	7.0E-07
cg1513	***tnp23a(ISCg23a)***	Transposase, putative pseudogene	1.1	8.9E-07
cg1552	***qorR***	transcriptional repressor of quinone oxidoreductase qor2	1.0	9.1E-03
cg1902		Putative secreted protein	1.1	1.8E-03
cg1907		Putative phosphopantothenoylcysteine synthetase/decarboxylase	1.2	1.0E-08
cg1908		Hypothetical protein	1.5	1.0E-06
cg1909		Hypothetical protein	2.3	4.0E-06
cg2030		Hypothetical protein	1.2	2.0E-02
cg2036		Putative secreted protein	1.4	5.3E-04
cg2046		Hypothetical protein	1.6	5.2E-05
cg2053		Putative membrane protein	1.5	4.9E-06
cg2070	***'int2***	Putative phage integrase (C-terminal fragment)	1.2	8.6E-06
cg2092	***sigA***	RNA polymerase sigma factor rpoD (Sigma-A)	3.8	6.4E-11
cg2237	***thiO***	Putative D-amino acid dehydrogenase, small subunit	1.2	6.6E-05
cg2238	***thiS***	Sulfur transfer protein involved in thiamine biosynthesis, ThiS-like	1.2	1.4E-06
cg2239	***thiG***	Thiamine biosynthesis protein, ThiG-like	1.1	2.2E-09

Table 10. continues from the previous page

cg2340		ABC-type putative amino acid transporter, substrate-binding lipoprotein	1.2	1.0E-03
cg2341		Putative Co/Zn/Cd cation transporter	1.0	4.1E-04
cg2438		Hypothetical protein	1.7	7.0E-06
cg2636	***catA***	Catechol 1,2-dioxygenase	1.2	1.0E-08
cg2836	***sucD***	Succinate--CoA ligase (ADP-forming), alpha subunit	1.0	6.1E-08
cg3037	***cls***	Cardiolipin synthase	1.2	1.5E-06
cg3269		Putative membrane protein, putative pseudogene	1.1	7.8E-04
cg3270		Putative membrane protein, putative pseudogene (N-terminal fragment)	1.1	3.9E-04
cg3280		Putative secreted protein	1.2	5.1E-04
cg3330		Putative secreted protein	1.3	3.6E-03
cg3368		ABC-type putative multidrug transporter, permease subunit	1.1	8.1E-04
cg3408		Hypothetical protein	1.1	1.5E-03
cg4006		Hypothetical protein	1.3	1.4E-02

18 genes		***Down regulated genes***		
gene ID[a]	**gene name**[a]	**Function of protein**[a]	**M-value**[b]	**FDR**[c]
cg0091		Putative D-isomer specific 2-hydroxyacid dehydrogenase	-1.1	4.5E-05
cg0131		Putative aldo-keto reductase	-1.2	6.9E-05
cg0195		Putative membrane protein	-1.1	5.8E-04
cg0612	***dkg***	Putative aldo/keto reductase, related to diketogulonate reductase	-1.2	5.6E-06
cg0828		Putative dihydrofolate reductase	-1.0	1.3E-04
cg0975		Putative chorismate mutase	-1.2	9.4E-05
cg1068		Putative oxidoreductase	-1.1	2.6E-04
cg1418		ABC-type putative iron-siderophore transporter, substrate-binding lipoprotein	-1.0	5.4E-03
cg1702		Hypothetical protein	-1.2	1.6E-05
cg1918		Putative secreted protein	-1.1	6.4E-06
cg2156		Hypothetical protein	-1.0	3.9E-03
cg2478		Putative penicillin binding protein	-1.0	1.6E-02
cg2546		Putative secondary C4-dicarboxylate transporter, tripartite ATP-independent transporter (TRAP-T) family	-1.0	5.4E-03
cg2838		Putative dithiol-disulfide isomerase	-2.2	9.4E-07
cg3107	***adhA***	Alcohol dehydrogenase	-1.0	2.5E-05
cg3129		ABC-type transporter, ATPase subunit	-1.6	5.4E-05
cg3155	***dcd***	dCTP deaminase	-1.0	5.2E-06
cg3405		NADPH:quinone reductase Zn-dependent oxidoreductase	-1.1	1.4E-02

[a]Gene ID, gene name and function of proteins are given according to CoryneRegNet (http://coryneregnet.compbio.sdu.dk/v6/index.html) and from previous studies. [b]Relative RNA amount of *sigA* overexpressing strain against the control strain with the empty vector was shown as log 2 values (M-values). [c]FDR represents false discover y rate. 50 µM IPTG were added from the beginning of the cultivation.

Table 11. Genes differentially expressed upon *sigA* overexpression after 24 hours of cultivation

65 genes		*Up-regulated genes*		
gene ID[a]	**gene name[a]**	**Function of protein[a]**	**M-value[b]**	**FDR[c]**
cg0171		Putative secreted protein	1.5	2.9E-03
cg0311		Putative secreted protein	1.2	3.6E-04
cg0413	***cmt1***	Trehalose corynomycolyl transferase	1.4	1.4E-02
cg0421	***wzx***	Putative PST O-antigen protein, multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) translocase	1.1	2.3E-03
cg0422	***murA***	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	1.3	2.9E-03
cg0495		Putative phosphatase	2.9	3.7E-05
cg0659		Putative acetyltransferase, GNAT-family	1.7	2.7E-03
cg0683		Putative permease	1.0	6.6E-03
cg0690	***groES***	10kDa chaperonin	3.0	4.0E-05
cg0693	***'groEL***	60kDa chaperonin, putative pseudogene (C-terminal fragment)	2.9	2.3E-03
cg0753		Putative secreted protein	1.6	2.4E-02
cg0998		Trypsin-like serine protease	3.1	1.1E-03
cg1070		Conserved hypothetical protein	1.8	1.2E-03
cg1096		Hypothetical protein	1.3	3.6E-02
cg1121		Permease, MFS-type	1.0	2.6E-02
cg1271	***sigE***	RNA polymerase sigma factor, ECF-family	2.0	2.2E-05
cg1277		Conserved putative membrane protein	1.2	1.4E-02
cg1334	***lysA***	Diaminopimelate decarboxylase	5.9	2.7E-08
cg1383		ABC-type putative molybdenum transporter, ATPase subunit	1.0	5.2E-03
cg1466		Putative secreted protein	1.1	3.2E-03
cg1476	***thiC***	Thiamine biosynthesis protein ThiC	1.7	3.4E-02
cg1514		Secreted protein	1.2	1.7E-06
cg1654	***thiD1***	Phosphomethylpyrimidine kinase/thiamine-phosphate diphosphorylase	1.7	1.6E-03
cg1685	***tatX***	Putative twin arginine targeting (Tat) Preprotein translocase subunit	1.2	6.3E-04
cg1734	***hemH***	Ferrochelatase	1.3	1.9E-02
cg1737	***acn***	Aconitate hydratase	1.5	1.1E-03
cg1821		Conserved hypothetical protein	1.7	2.5E-03
cg1859		Putative secreted protein	2.1	1.9E-03
cg1903		ABC-type transporter, ATPase subunit	2.1	1.0E-03
cg1904		ABC-type transporter, permease subunit	1.4	4.0E-03
cg1905		Hypothetical protein	2.5	8.2E-04
cg1907		Putative phosphopantothenoylcysteine synthetase/decarboxylase	2.8	4.5E-03
cg1930		Putative secreted hydrolase	2.1	1.0E-04
cg1931		Putative secreted protein	1.8	6.9E-05
cg1966		Hypothetical protein	1.2	2.1E-04
cg2030		Hypothetical protein	1.2	1.7E-02
cg2034		Hypothetical protein	1.3	3.8E-03
cg2037		Conserved hypothetical protein	1.2	1.7E-04
cg2040		Putative transcriptional regulator, HTH_3-family	1.5	6.5E-03
cg2052		Putative secreted protein	1.1	4.6E-02
cg2071	***int2'***	Putative phage Integrase (N-terminal fragment)	1.6	2.1E-02
cg2092	***sigA***	RNA polymerase sigma factor *rpoD* (Sigma-A)	5.4	3.4E-06
cg2114	***lexA***	Putative transcriptional regulator, LexA-family	1.3	1.4E-03
cg2280	***gdh***	Glutamate dehydrogenase (NADP(+))	2.1	2.5E-03
cg2306		Conserved hypothetical protein	1.6	3.7E-03
cg2308		Putative secreted protein	2.7	4.6E-02
cg2339		Putative secondary chloramphenicol transporter, drug/metabolite transporter (DMT) superfamily	1.6	1.8E-02
cg2340		ABC-type putative amino acid transporter, substrate-binding lipoprotein	1.2	1.7E-02
cg2341		Putative Co/Zn/Cd cation transporter	1.3	1.2E-03

Table 11. continues from the previous page

cg2378	***mraZ***	Putative MraZ protein	1.5	4.4E-03
cg2565		Hypothetical protein	1.1	4.9E-02
cg2572		Conserved hypothetical protein	1.2	2.3E-06
cg2617	***vanB***	Vanillate O-demethylase oxidoreductase	5.2	2.7E-05
cg2636	***catA***	Catechol 1,2-dioxygenase	1.1	1.2E-02
cg2704		ABC-type putative sugar transporter, permease subunit	2.7	1.9E-03
cg2707		Conserved hypothetical protein	1.0	2.3E-03
cg3011	***groEL***	Chaperonin Cpn60 (60Kd subunit)	3.2	5.4E-05
cg3022		Conserved hypothetical protein	1.3	3.8E-05
cg3037	***cls***	Cardiolipin synthase	4.6	1.1E-05
cg3225		Putative serine/threonine-specific protein phosphatase	1.9	9.2E-05
cg3282		Putative Cu2+ transporting P-type ATPase	1.2	2.7E-02
cg3283		Hypothetical protein	1.7	9.6E-07
cg3335	***malE***	Malate dehydrogenase (oxaloacetate-decarboxylating) (NADP(+))	1.2	5.0E-02
cg3367		ABC-type putative multidrug transporter, ATPase subunit	1.2	2.9E-03
cg4005		Putative secreted protein	2.1	1.4E-02
32 genes		***Down regulated genes***		
gene ID[a]	**gene name[a]**	**Function of protein[a]**	**M-value[b]**	**FDR[c]**
cg0047		Conserved hypothetical protein	-1.6	8.8E-05
cg0120		Putative hydrolase	-1.8	1.4E-04
cg0129	***putA***	Proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase	-1.7	2.5E-05
cg0242		Hypothetical protein	-1.9	4.4E-06
cg0260	***moaC***	Molybdopterin cofactor synthesis protein C	-1.4	6.4E-04
cg0282		CsbD family protein probably involved in stress response	-1.9	5.7E-06
cg0337	***whiB4***	Putative transcriptional regulator, WhiB-family	-1.1	2.3E-06
cg0562	***nusG***	Transcription antitermination protein NusG	-1.1	1.3E-02
cg0564	***rplA***	50S ribosomal protein L1	-1.1	2.2E-03
cg0612	***dkg***	Putative aldo/keto reductase, related to diketogulonate reductase	-1.2	6.2E-04
cg0780		Membrane protein, ribonuclease BN-like family	-1.5	8.2E-05
cg0797	***prpB1***	Putative (methyl)isocitrate lyase	-1.5	1.9E-04
cg0798	***prpC1***	Putative (methyl)citrate synthase	-1.5	2.7E-05
cg1106		conserved hypothetical protein	-2.0	2.8E-03
cg1236	***tpx***	Thiol peroxidase	-1.9	1.9E-03
cg1409	***pfkA***	6-Phosphofructokinase	-1.6	9.3E-04
cg1512		Hypothetical protein	-2.6	3.2E-03
cg1642		Siderophore-interacting protein	-2.4	2.4E-03
cg2191		Conserved hypothetical protein	-1.3	3.3E-03
cg2250		Putative secreted protein	-1.4	1.4E-03
cg2320		Putative transcriptional regulator, ArsR-family	-1.6	4.7E-03
cg2411		Conserved hypothetical protein, HesB/YadR/YfhF family	-1.1	2.3E-02
cg2451		Conserved hypothetical protein	-2.2	2.7E-05
cg2510	***bex***	Putative Bex protein, GTP-binding protein ERA-like	-1.3	1.0E-03
cg2554	***rbsK2***	Ribokinase	-1.0	6.9E-05
cg2591	***dkgA***	Putative 2,5-diketo-D-gluconic acid reductase	-2.1	4.8E-04
cg2782	***ftn***	Ferritin-like protein	-2.4	1.9E-03
cg2833	***cysK***	Cysteine synthase	-1.4	1.2E-05
cg2891	***pqo***	Pyruvate:quinone oxidoreductase	-1.4	3.8E-06
cg2957		Conserved hypothetical protein	-1.1	5.7E-03
cg3264		Conserved hypothetical protein	-1.8	9.9E-05
cg3299	***trxB1***	Thioredoxin (TRX)	-1.8	2.2E-05

[a]Gene ID, gene name and function of proteins are given according to CoryneRegNet (http://coryneregnet.compbio.sdu.dk/v6/index.html) and from previous studies. [b]Relative RNA amount of *sigA* overexpressing strain against the control strain with the empty vector was shown as log 2 values (M-values). [c] FDR represents false discover y rate. 50 µM IPTG were added from the beginning of the cultivation.

2.3.4.5 Addition of thiamine/PCA improved carotenoid production in the *sigA* overexpressing strain

The DNA microarray analysis revealed that expression of several thiamine synthesis genes was upregulated either at 8 h or 24 h. In addition, expression of *catA* was upregulated both at 8 h and 24 h. Thiamine is necessary as a cofactor for DXP synthase (Dxs), which catalyzes the first step of isopentenyl diphosphate (IPP) synthesis in the MEP pathway (Vranová *et al.,* 2013). Furthermore, the reaction catalyzed by Dxs is also a part of thiamine synthesis (Begley *et al.,* 1999). On the other hand, *catA* encodes catechol 1,2-dioxygenase and cleaves the ring structure of aromatic compounds such as catechol (Liu and Liu, 2004). Even though CatA in *C. glutamicum* cannot cleave PCA (protocatechuic acid) (Liu and Liu, 2004), the increase of its expression under the presence of PCA was suggested (Unthan *et al.,* 2014). In order to understand upregulation of thiamine synthesis genes and *catA*, thiamine (10 µg/L) or 10 times the amount of PCA (300 mg/L) , which is regularly supplemented in CGXII medium, was added to the medium and the decaprenoxanthin production was compared between the control strain and the *sigA* overexpressing strain with or without supplement after 48 hours of cultivation in BioLector.

The supplementation did not influence the final biomass of all strains (**Figure 21. A**), and decaprenoxanthin production in the control strain (**Figure 21. B**). In contrast, the supplementation of thiamine as well as the excess amount of PCA enhanced the accumulation of decaprenoxanthin production under *sigA* overexpression compared to the condition without supplementation (+10% and +39%, respectively). Therefore, thiamine and PCA are supportive for carotenoid production only in the *sigA* overexpressing strain but not in the control strain.

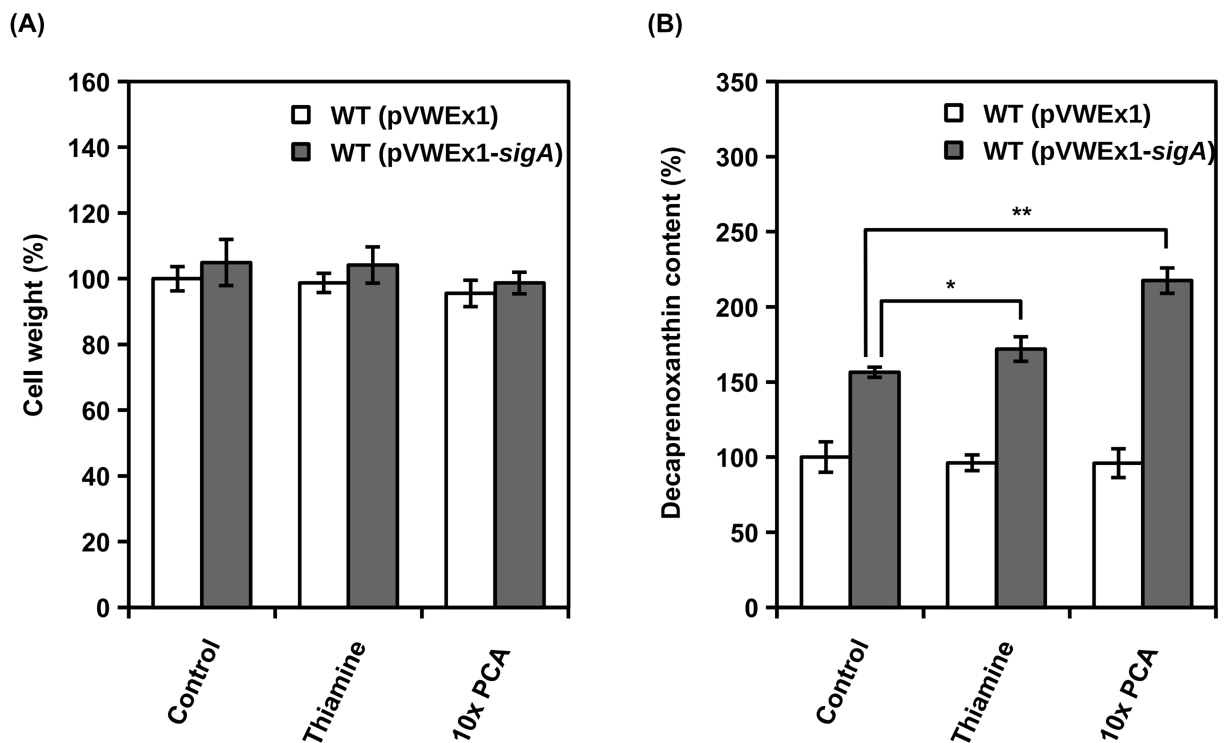


Figure 21. Effect of thiamine or excess protocatechuic acid on decaprenoxanthin production. (A) Cell weight after 48 h. Cell weight was calculated based on the optical density of cell culture at wavelength of 600 nm. Control is cultivation with CGXII. Thiamine or 10 x PCA represent cultivation with CGXII with 10 µg/L of thiamine or 300 mg/L of PCA. The value of WT(pVWEx1) in the control medium was adjusted to 100%. **(B)** Decaprenoxanthin content after 48 h. Decaprenoxanthin content (%) was calculated based on the optical density of carotenoid extract at wavelength of 440 nm and normalized by cell weight used for extraction. The value of WT(pVWEx1) in the control medium was adjusted to 100%, and decaprenoxanthin content was shown on percentage. Statistic significance was calculated with paired Student t-test (two-tailed). * and ** represents p-value less than 0.05 and 0.01, respectively. The experiment was performed in a FlowerPlate and error bar represents standard deviation from biological triplicates.

2.3.4.6 The effect of *sigA* overexpression was transferable to the β-carotene producing strain

In the previous work, *C. glutamicum* was engineered to produce β-carotene, which is one of the most important carotenoids in industry (Heider *et al.,* 2014a). Overexpression of *sigA* increased production of lycopene in LYC5 and decaprenoxanthin in WT, therefore, it was applied for β-carotene producing strain BETA3. BETA3 was constructed based on LYC5 and contains further genomic integration of $crtY_{p.a.}$ from *Pantoea ananatis* under the control of P_{tuf} promoter. The experiment was performed in 500 mL baffled flasks.

Glucose was completely consumed after 24 h and the influence of *sigA* overexpression on final biomass was small for all samples (<±12%) (**Figure 22**). Overexpression of *sigA* enhanced β-carotene accumulation more than 140% after 24 h and more than 330% after 48 h compared to the control strain at each time point. The titer of β-carotene in the *sigA* overexpressing strain after 48 h was 11.9 ± 1.5 mg/g CDW. The accumulation of β-carotene in the control strain did not change significantly between after 24 h and 48 h, on the other hand, the accumulation under *sigA* overexpression increased from 24 h to 48 h as was observed in LYC5 and WT.

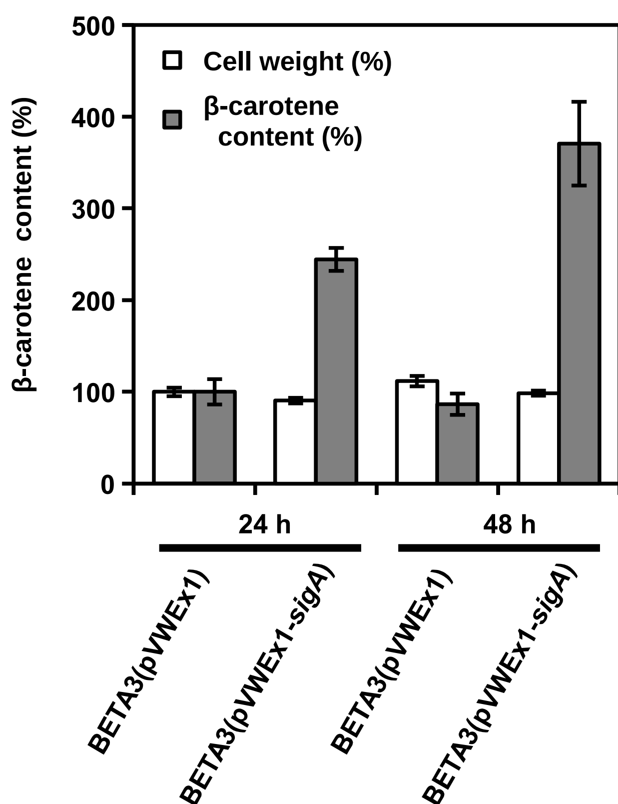


Figure 22. Effect of *sigA* overexpression on the β-carotene producing strain (BETA3). β-carotene content (%) was calculated based on the optical density of carotenoid extract at wavelength of 454 nm and normalized by cell weight used for extraction. 24 h and 48 h represent each sampling time point. The values from the control strain, BETA3(pVWEx1), at 24 h were adjusted to 100%, and β-carotene content was shown on percentage. Experiment was performed in baffled flasks and error bar represents standard deviation from biological triplicates.

2.3.4.7 The effect of *sigA* overexpression is additive to the effect of the derepression of crt operon

C. glutamicum possesses the *crt* operon for carotenoid synthesis (cg0723 – cg0717) (Heider *et al.,* 2012). In our group, it was recently shown that Cg0725 (named as CrtR) works as a repressor for this operon and the deletion of cg0725 improved decaprenoxanthin production in *C. glutamicum* (data not published). Therefore, the effect of *sigA* overexpression along with the deletion of *crtR* was tested in MB001Δ*crtR* strain. MB001Δ*crtR* was constructed based on the prophage cured strain MB001 (Baumgart *et al.,* 2013) with further deletion of *crtR*. The experiment was performed in FlowerPlate and the sample was taken at 48 h.

Deletion of *crtR* led to the yellow cell culture, but the effect of *sigA* overexpression was not clearly visible in both of WT and MB001Δ*crtR* (**Figure 23. A**). The quantification of decaprenoxanthin revealed that MB001Δ*crtR* accumulated decaprenoxanthin more than 440% compared to WT(pVWEx1). Overexpression of *sigA* enhanced the accumulation of decaprenoxanthin for 120% in WT and for 190% in MB001 compared to the empty vector strain, respectively (**Figure 23. B**). No significant difference was observed for the final biomass. This result showed that the effect of *sigA* overexpression is additive to the effect of *crtR* deletion.

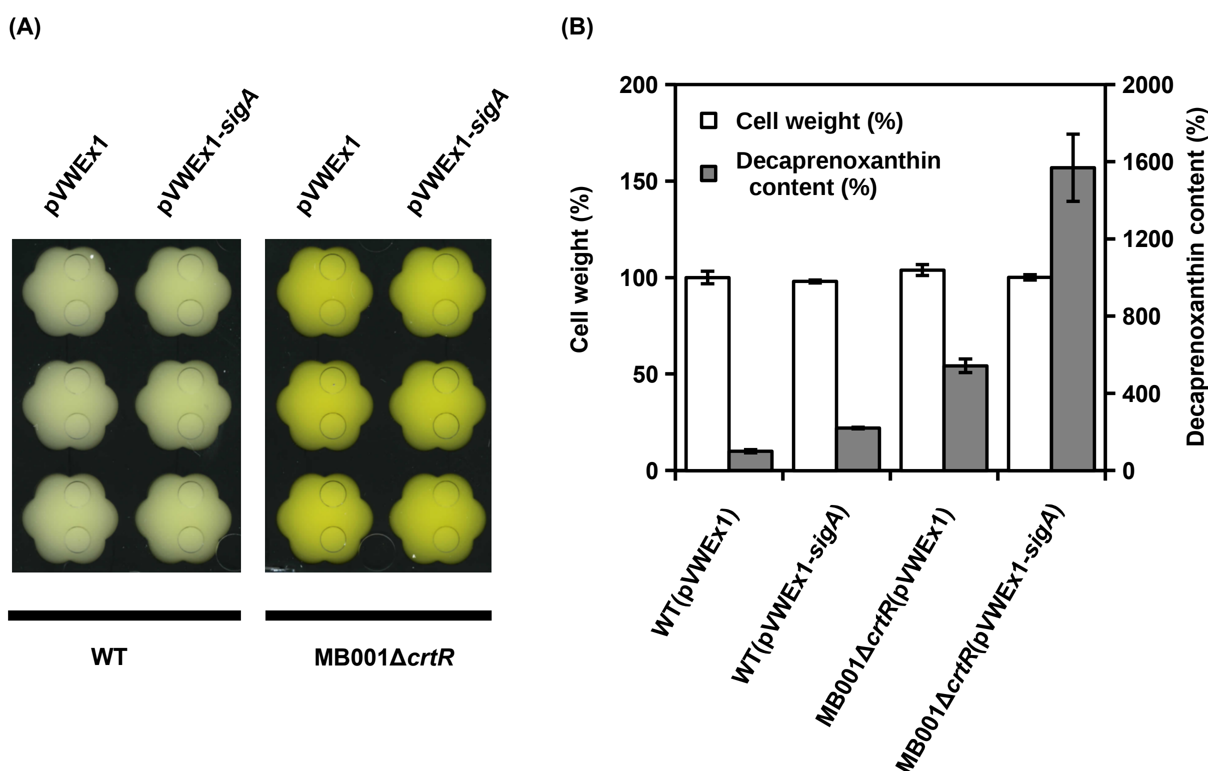


Figure 23. Effect of *sigA* overexpression on decaprenoxanthin producing strains (WT and MB001Δ*crtR*). (A) FlowerPlate image of culture after 48 hours of cultivation. Biological triplicates are aligned in the column. **(B) Decaprenoxanthin content after 48 h.** Decaprenoxanthin content (%) was calculated based on the optical density and normalized by cell weight. The values of WT(pVWEx1) were adjusted to 100%. Decaprenoxanthin content was shown on percentage. Experiment was performed in FlowerPlate and error bar represents standard deviation from biological triplicates.

2.3.5 Discussion

In this study, the endogenous sigma factor genes were overexpressed, aiming to increase carotenoid production, and *sigA* was selected as the most effective sigma factor gene. Interestingly, the accumulation of carotenoids in *sigA* overexpressing strains occurred especially in the stationary phase. Transcriptome analysis revealed that overexpression of *sigA* upregulated the expression of various genes including genes encoding enzymes for thiamine synthesis and for aromatic compound degradation. Supplementation of thiamine or the excess amount of protocatechuic acid showed a positive effect for carotenoid production only when *sigA* was overexpressed. *sigA* overexpression enhanced carotenoid production in different background strains and its effect was accumulative to derepression of carotenogenic operon caused by deletion of the repressor gene *crtR*.

Overexpression of different sigma factor genes showed different effects on lycopene accumulation. Whereas overexpression of *sigA*, *sigC*, *sigD*, *sigH* and *sigM* increased the accumulation, overexpression of *sigB* and *sigE* decreased the lycopene accumulation. This difference is assumed to be caused by distinct function of each sigma factor on cellular regulation. For example, SigA is a primary sigma factor and is responsible for the transcription of more than thousands of genes (Pfeifer-Sancar *et al.,* 2013), which are often under the control of other transcriptional regulators (Busche *et al.,* 2012). SigC regulates the balance of the quinol oxidative pathway (Toyoda and Inui, 2016). The relation of SigD to the cell wall integrity, especially for mycolate membrane, is implied by our study (data not published). SigH is responsible for the oxidative stress response including redox homeostasis (Busche *et al.,* 2012), and SigM is also related to thiol-oxidative stress conditions (Nakunst *et al.,* 2007). On the other hand, SigB modulates global gene expression during the transition from the exponential growth phase to the stationary phase (Larisch *et al.,* 2007), and regulates glucose metabolism especially under oxygen deprived condition (Ehira *et al.,* 2008). SigE is involved in the response to cell surface stress (Park *et al.,* 2008). These results indicate that differential expression of multiple genes caused by sigma factor overexpression can influence the accumulation of lycopene in a different manner and to a different extent. In nature, carotenoids are suggested to work as stabilizer for phospholipid bilayer and as a scavenger for reactive oxygen species under oxidative stress (Moise *et al.,* 2014).

Therefore, the different mechanisms may exist for induction of carotenoid production For example, the light induced carotenogenesis is known in several organisms such as *Streptomyces setonii* (Kato *et al.,* 1995), *Myxococcus xanthus* (Gorham *et al.,* 1996) and *Streptomyces coelicolor* (Takano *et al.,* 2005). Up to now, the regulation and meaning of carotenoid production in *C. glutamicum* is not fully understood, however, overexpression of different sigma factor genes could influence the carotenoid accumulation by stimulating the response mechanism equipped by nature.

Transcriptome analysis in the wild type strain unveiled the effect of *sigA* overexpression on transcriptome profile. Expression of genes directly related to carotenogenesis were neither significantly upregulated nor downregulated by *sigA* overexpression. The expression of *catA* was upregulated both at 8 h and 24 h (M-value, 1.2 and 1.1, respectively). CatA is an iron containing enzyme and its transcription is directly repressed by iron homeostasis regulator RipA (Wennerhold *et al.,* 2005). RipA also represses the transcription of *acn* encoding aconitase (Wennerhold and Bott, 2006), which expression level was upregulated at 24 h under *sigA* overexpression (M value, 1.5). Furthermore, the expression of *hemH* related to heme synthesis was upregulated (M value, 1.3) at 24 h. On the other hand, the expression of *ftn* annotated as ferritin like iron storage protein was downregulated (M value, -2.4) after 24h. These results suggested that overexpression of *sigA* may affect iron homeostasis especially in the stationary phase. The expression of *cls* was upregulated both at 8 h and 24 h (M-value, 1.2 and 4.6, respectively). *cls* encodes cardiolipin synthetase, and its inactivation led to change the lipid composition in cellular membrane and increase temperature sensitivity (Nampoothiri *et al.,* 2002). Furthermore, expression of *sigE*, which is related to cell surface response, was upregulated after 24 h (M-value, 2.0). These results imply the effect on cellular membrane by *sigA* overexpression, especially in the stationary phase.

The accumulation of carotenoids increased under *sigA* overexpression especially in the stationary phase in LYC5 and WT. During the transition from the exponential phase to the stationary phase, the transcription level of *sigA* is known to decrease, on the other hand, the transcription level of *sigB* increases during the transition in *C. glutamicum* (Larisch *et al.,* 2007). Therefore, it is considered that SigA is a main sigma factor in the exponential phase, and its role is taken over by SigB or other sigma factors during the transition and in the stationary phase. *pfkA* and *pqo* were shown to be upregulated during the transition and in the stationary phase (Ehira *et*

al., 2008). However, the expression of these genes was downregulated at 24 h under *sigA* overexpression (M-value, 1.6 and 1.4, respectively). These results suggest that unusual SigA abundance in the stationary phase caused by artificial overexpression may alter the tendency of promoter selectivity of RNA polymerase and induce the perturbation of transcription profile which can affect lycopene accumulation in the stationary phase.

Some of the genes related to thiamine synthesis were shown to be upregulated at 8 h or 24 h under *sigA* overexpression. The influence of thiamine on carotenoid production was reported for different *Corynebacterium* species such as *Corynebacterium poinsettiae* (Starr and Saperstein, 1953) or *Corynebacterium michiganense* (Saperstein *et al.,* 1954). In the present study, addition of thiamine enhanced lycopene production only in the *sigA* overexpressing strain, but not in the control strain. This fact indicates that the availability of thiamine was somehow limited under *sigA* overexpression. The synthesis of carotenoid starts from condensation of glyceraldehyde 3-phosphate and pyruvate by Dxs (Begley *et al.,* 1999). This reaction is considered to be one of the rate-limiting steps of the MEP pathway (Estévez *et al.,* 2001, Heider *et al.,* 2014). Dxs requires thiamine pyrophosphate as cofactor, and the product, 1-deoxy-D-xylulose-5-phosphate (DXP) is an intermediate also for thiamine synthesis and pyridoxal synthesis (Sprenger *et al.,* 1997). Interestingly, expression of pyridoxal 5'-phosphate synthase genes (*pdxST*) was upregulated at 8 h under *sigA* overexpression (M-value, 1.4 and 1.0, respectively). This information implies the effect of *sigA* overexpression on cofactor synthesis and flux balancing for carotenogenesis.

PCA is considered as iron chelator (Liebl *et al.,* 1989) and *C. glutamicum* can utilize PCA as carbon source (Merkens *et al.,* 2005). The presence of PCA in the medium is shown to accelerate the growth of *C. glutamicum* by changing the expression profile (Unthan *et al.,* 2014). In the present study, higher amounts of PCA in the medium did not influence the final biomass significantly. Even though PCA is shown to be consumed in the exponential phase (Unthan *et al.,* 2014), the accumulation of carotenoid occurred in the stationary phase only in the *sigA* overexpressing strain. In *C. glutamicum*, PCA is degraded to acetyl-CoA and succinyl-CoA via aromatic compound degradation pathway, which is called β-ketoadipate pathway (Brinkrolf *et al.,* 2006). The genes related to the β-ketoadipate pathway were not significantly upregulated except for *catA* and *vanB*, which are involved in the branched .β-ketoadipate pathway. Therefore, high

amounts of PCA may work as a factor to change the balance of metabolites rather than as a simple carbon source, and it enhances carotenoid production synergistically with genes upregulated under *sigA* overexpression, considering that high amounts of PCA was effective to improve carotenoid production only for the *sigA* overexpressing strain.

Overexpression of *sigA* could enhance carotenoid production in different background strains, such as the wild type strain (WT), metabolically engineered strains (LYC5, BETA3) and the derepressed strain (MB001Δ*crtR*). The effect of *sigA* overexpression in WT was smaller than in LYC5, BETA3 and MB001Δ*crtR*. LYC5 and BETA3 contain additional carotenogenic genes under the control of P_{tuf}, which is used as constitutive promoter in *C. glutamicum* (Pátek *et al.,* 2013). MB001Δ*crtR* lacks the repressor for carotenogenic *crt* operon. Those different regulations on *crt* operon in each strain may cause different strength of effect of sigma factor gene overexpression on carotenoid production. From transcriptome analysis of WT, overexpression of *sigA* did not change the expression level of carotenogenic genes. Therefore, the effect of *sigA* overexpression cannot be explained simply by upregulation of carotenogenic genes.

Since carotenoids are promising compounds in industry, metabolic engineering has been applied intensively to construct better production strains. For example, the shot gun method (Kang *et al.,* 2005), the multi dimensional gene target search (Jin and Stephanopoulos, 2007) and the global transcription machinery engineering (gTME) (Alper and Stephanopoulos, 2007) were performed in *E. coli*, and disclosed the high complexity in metabolic landscapes for carotenoid production. Interestingly, overexpression of *rpoS*, which is similar to *sigB* in *C. glutamicum*, increased the lycopene production (Kang *et al.,* 2005; Jin and Stephanopoulos, 2007). This effect is suggested to be caused by altering cellular oxidative status and preventing degradation of lycopene (Bongers *et al.,* 2015). In *C. glutamicum*, overexpression of *sigB* led to decrease of lycopene production, however, sigma factors related to oxidative stress such as SigH and SigM contributed to an increase of lycopene production. Unexpectedly, overexpression of SigA, which is the primary sigma factor in *C. glutamicum*, led to the best result. Therefore, different mechanisms for cellular regulation may influence carotenoid productions in different organisms.

In this study, metabolic engineering based on sigma factor overexpression was successfully applied for carotenoid production. It is not clearly elucidated how overexpression of

sigA increases carotenoid production, and further studies are necessary to understand the mechanism. During the evolution, bacteria have optimized the balance of gene expression under the specific regulation architecture. Therefore, overexpression of sigma factor genes may exploit the unexpected synergistic effect of multiple gene expression, and helpful to understand the unrevealed regulatory mechanism in bacteria. In this study, sigma factors were successfully used to change the metabolic flux globally by tuning the expression of a sets of genes and to improve carotenoid production. This method can be applied for production of other compounds and for other bacteria which sigma factor genes are already known.

3 Discussion

In this study, the potential of sigma factor overexpression for strain development was explored. The artificial overexpression of sigma factor genes successfully perturbed the transcriptome profile, and the perturbation resulted in the physiological changes of cells under normal condition in the laboratory. When properly chosen, the effect of sigma factor overexpression was able to be used for carotenoid productions. This study showed that the strain development using sigma factor can be a powerful tool for strain development.

3.1 *sigH* overexpression on production: riboflavin, FMN and further

As a start , the effect of sigma factor overexpression on the cell behaviors and the metabolite accumulation were investigated. The experiment revealed that overexpression of sigma factor genes do have an influence on the cell behaviors and different sigma factor influenced the cell behaviors differently in *C. glutamicum*. Overexpression of *sigH* induced the apparent phenotype of riboflavin accumulation.

In *C. glutamicum*, the strain development for riboflavin production has not been performed to the author's knowledge. In *Corynebacterium ammoniagenes*, which is a close related organism to *C. glutamicum*, the riboflavin producing strain was constructed by overexpression of the *rib* operon. (Koizumi *et al.,* 2000). In *B. subtilis*, overexpression of several target genes identified that the *rib* operon and the pentose phosphate pathway are targets for improvement of riboflavin production, and the overproducing strain for riboflavin was constructed based on the rational approach (Hümbelin *et al.,* 1999; Duan *et al.,* 2010). In the present study, overexpressing only one gene of *sigH* led to the accumulation of riboflavin, upregulating the expression of genes in *rib* operon as well as *zwf* and *opcA* encoding for enzyme of the pentose phosphate pathway, which were identified as targets for riboflavin production in *B. subtilis* from several studies (**Figure 24**). This result showed that overexpression of factor genes switched on the part of the endogenous pathways artificially without precise prediction Therefore, overexpression of sigma factor genes is an interesting tool to explore the hidden potential of host organisms by activating a set of genes which products work in a collaborative manner.

Overexpression of sigma factor genes is expected to influence the promoter selectivity of RNA polymerase holoenzyme and perturb the transcription of other genes through the sigma factor

competition. This effect is also expected for transcription of genes from plasmid. In this study, overexpression of another gene *ribF* in addition to *sigH* overexpression resulted in the production of FMN from riboflavin, which was accumulated due to *sigH* overexpression. This showed that expression of other genes is also possible in the sigma factor overexpressing strains for further strain development.

Overexpression of *sigH* upregulated the expression of genes encoding enzyme for the pentose phosphate pathway (PPP), which is important for NADPH regeneration. Since the increase of NADPH supply is helpful for lysine production, overexpression of *sigH* was performed in the lysine producing strain *C. glutamicum* DM1729 (Becker *et al.,* 2011), aiming to explore the further potential use of *sigH* overexpression. Overexpression of *sigH* led to slight increase of lysine production, however, overexpression of *sigH* decreased the cell growth drastically (**Figure S1**). Therefore, *sigH* overexpression is not a good strategy for lysine production. This result indicates that the exact effect of overexpression of sigma factor genes is difficult to prediction since overexpression of sigma factor genes influences transcriptome profile globally. Thus, in order to maximize the potential use of sigma factor genes, all the sigma factor genes should be tested first and the most potent sigma factor gene should be selected for a certain purpose .

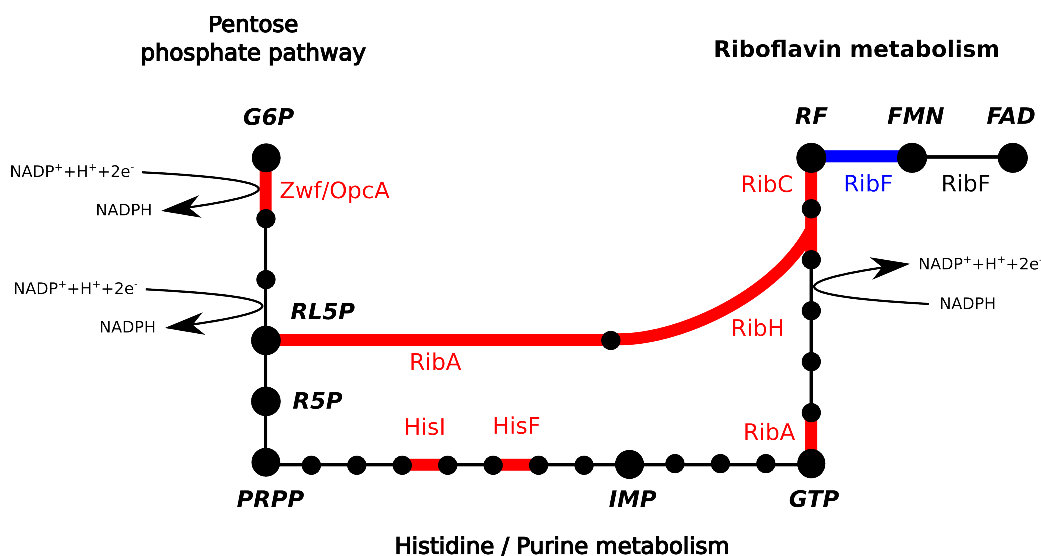


Figure 24 Riboflavin synthesis pathway and proteins encoded by genes induced under *sigH* overexpression. Riboflavin synthesis and metabolism pathway is shown with glucose 6-phosphate (G6P) as starting compound. All the black points represent the intermediates and big black points are indicated with the name of compound. The names of enzymes are shown in red character and the catalyzing reactions are shown in red lines, only when the expression of corresponding genes were confirmed to be upregulated under *sigH* overexpression by transcriptome analysis. NADPH regeneration and consumption in the reaction is also shown. The reactions from riboflavin (RF) to FAD, which RibF catalyzes, are also shown here. The reaction from riboflavin to flavin mononucleotide (FMN), which mainly took place in the *ribF* overexpressing experiment is shown in a blue line. **G6P**: glucose 6-phosphate, **RL5P**: ribulose 5-phosphate, **R5P**: ribose 5-phosphate, **PRPP**: phosphoribosyl pyrophosphate, **IMP**: inosine monophosphate, **GTP**: guanosine triphosphate, **RF**: riboflavin, **FMN**: flavin mononucleotide, **FAD**: flavin adenine dinucleotide

3.2 Overexpression of a sigma factor gene *sigD* for biological understanding

Overexpression of *sigD* deepened the biological knowledge such as the regulation of cell envelope integrity and regulation of SigD by the anti-sigma factor (**Figure 25**). Therefore, overexpression of sigma factor genes was shown to be an useful tool also for understanding the biological mechanisms. In addition, transcriptome analysis suggested a list of unannotated genes which may be involved in cell envelope integrity such as genes encoding glycosyltransferases or membrane proteins. This information will be helpful for further biological understanding.

Even thought it is difficult to predict the overall effect of overexpression of sigma factor genes, *sigD* overexpression can be utilized for perturbing the cell envelope integrity and the permeability barrier. Cell envelope integrity is important for permeability barrier of cells, and increasing of permeability is known to increase the resistance to surface stress or high temperatures (Bayan *et al.,* 2003). In addition, decreasing the permeability barrier is known to increase excretion of amino acids or uptake of nutrients (Hashimoto *et al.,* 2006).

Under *sigD* overexpression, the accumulation of polysaccharides or carbohydrate containing compounds was assumed to cause less foaming culture and cell aggregation. Since foaming during the cultivation causes problems in the fermenter, antifoams are often added to bioprocesses (Routledge, 2012). Foaming also inhibits efficient oxygen exchange in the culture and atmosphere, therefore, it may be an interesting topic how overexpression of *sigD* can lead to anti foaming phenotypes. In order to understand the mechanism, metabolome analysis such as LC-MS/MS may be helpful for identifying the compounds and understanding of mechanisms. Th less foaming of cultures can be also the consequences of mixed effects. In such a case, deletion of genes in a pathway such as for corynomycolate synthesis or peptidoglycan synthesis under *sigD* overexpression and evaluation of the extent of anti forming will be helpful to understand and identify the relevant compounds .

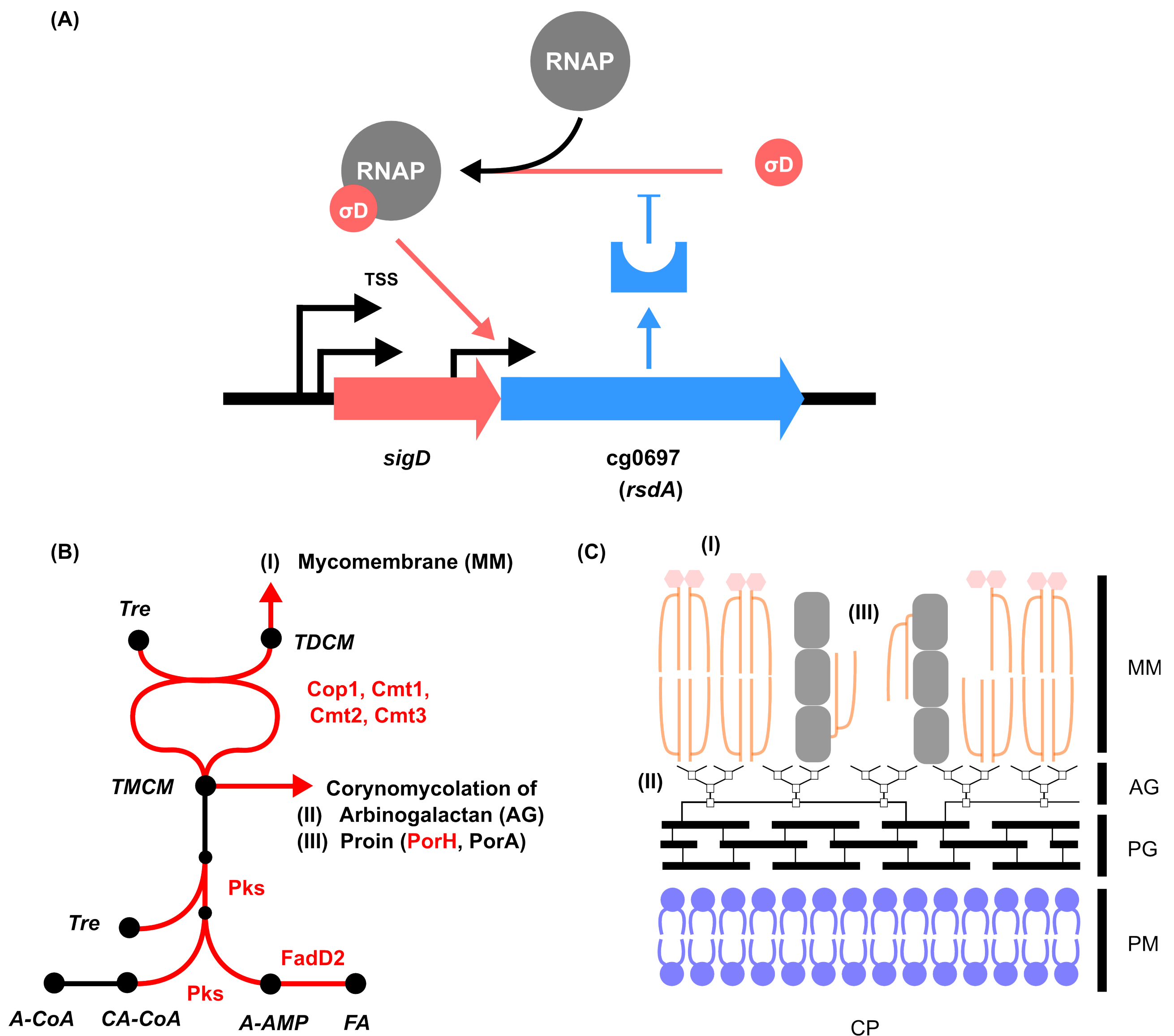


Figure 25. Regulation of SigD by anti-sigma factor and effect of overexpression of *sigD* on cell envelope. (A) RNA polymerase (**RNAP**) containing SigD (**σD**) initiates transcription from the promoter in the *sigD* ORF. As a result, anti-sigma factor for SigD (Cg0697, RsdA) is induced and inhibits the binding of SigD to RNA polymerase core enzyme. **(B)** The synthesis pathway of trehalose dicorynomycolate (**TDCM**) and trehalose monocorynomycolate (**TMCM**) are shown with acyl-CoA (**A-CoA**) and fatty acid (**FA**) as starting compounds. All the black points represent the intermediates and big black points are indicated with the name of compounds. The names of enzymes are shown in red letters and the catalyzing reactions are shown in red lines, only when the expression of corresponding genes were confirmed to be upregulated under *sigD* overexpression by transcriptome analysis. The destinations of TDCM and TMCM are shown as (I) Mycomembrane, (II) Arabinogalactan and (III) Porin. **(C)** Localization of corynomycolic acid is illustrated. TDCM (two red carbohydrate heads and two orange corynomycolate tails) and TMCM (one carbohydrate head and two corynomycolate tail) are localized in mycomembrane (**MM**). Arabinogalactan (**AG**) and porin subunits (gray) are corynomycolated (orange) by TMCM. **Tre**: trehalose, **TDCM**: trehalose dicorynomycolate, **TMCM**: trehalose monocorynomycolate, **A-CoA**: acyl-CoA, **CA-CoA**: carboxylated acyl-CoA, **A-AMP**: acyl-AMP, **FA**: fatty acid, **MM**: mycomembrane, **AG**: arabinogalactan, **PG**: peptidoglycan, **PM**: periplasmic membrane, **CP**: cytoplasmic membrane.

3.3 Strain development using sigma factors: Carotenoids as targets

Using the lycopene producing strain, overexpression of sigma factor genes and the following screening was shown to be an effective approach for the strain development. Furthermore, overexpression of *sigA* was successfully applied for different carotenoids production in the wild type strain and the metabolically engineered platform strains. As a result, the production of lycopene and β -carotene was increased up to 0.82 ± 0.15 mg/g CDW (+770% of increase) and 11.9 ± 1.5 mg/g CDW (+330% of increase), respectively.

Interestingly, the effect of *sigA* overexpression on the carotenoid accumulation was observed especially in the late stationary phase. This effect is hypothesized to be caused by disturbing the sigma factor competition (Mauri and Klumpp, 2014). More precisely, overexpression of *sigA* may maintain the protein concentration of SigA high in the stationary phase, and the unusual abundance of SigA in the stationary phase may change the transcriptome profile through the sigma factor competition between SigA and SigB or other alternative sigma factors. This hypothesis is supported by the low transcriptional level of *pfkA* and *pqo* in the stationary phase under *sigA* overexpression, which expression increases during the transition and in the stationary phase under the usual condition (Ehira *et al.,* 2008). This hypothesis can be confirmed by the deletion of *sigB* and the monitoring of carotenoid accumulation over time, since the deletion of *sigB* will change the transcription profile as *sigA* overexpression does by increasing the relative ratio of SigA over other sigma factors.

The excess overexpression of *sigA* retarded the cell growth, but did not increase carotenoid production (**Figure S2**). This indicates that the appropriate level of SigA is also important for the optimal cell growth, even though SigA is known to be the primary sigma factor and responsible for the transcription of most of the housekeeping genes (Pátek *et al*., 2003). In *E. coli* and *B. subtilis*, the function of SigA is regulated by 6S RNA in the stationary phase (Cavanagh and Wassarman, 2014), however, the regulation of SigA in *C. glutamicum* was not yet elucidated. Finding and deleting SigA inhibitors such as 6S RNA of *C. glutamicum* can be also a target of strain development for carotenoid production in the future.

At the stationary phase, *sigA* overexpression highly upregulated the expression of *lysA* encoding diaminopimelate dehydrogenase, which is responsible for the last step of lysine

biosynthesis in *C. glutamicum* (Table 11) (Becker *et al.,* 2011). However, *sigA* overexpression showed no significant influence on lysine production in the lysine producing strain GRLP (Unthan *et al.,* 2015) (**Figure S3**). The reaction of LysA is known to be not the limiting step for lysine production and other limiting steps are known in the upstream of lysine biosynthesis such as aspartokinase LysC (Becker *et al.,* 2011). Since the expression of *lysA* is known to be SigA-dependent (Pátek *et al.,* 2003), there may be other reason for *lysA* overexpression in the stationary phase under *sigA* overexpression. Since the substrate of LysA is diaminopimelate, which is also the precursor for the component of peptidoglycan (Pavelka and Jacobs, 1996), overexpression of *sigA* may influence the cell wall synthesis and the production of carotenoids indirectly. Overexpression or deletion of *lysA* in the carotenoid producing strains and the following quantification of carotenoid production may elucidate the biological meaning of *lysA* upregulation at the transcriptional level under *sigA* overexpression.

The precise mechanism how SigA influences the carotenoid accumulation is still unknown. Deletion and overexpression of genes, which were highly expressed under *sigA* overexpression, may elucidate the direct effect of *sigA* overexpression. However, the effect of *sigA* overexpression on carotenoid production is assumed to be caused by many effects, since SigA serves as a primary sigma factor. Therefore, the approach such as ^{13}C metabolic flux analysis may be helpful to identify a reasonable explanation and potential targets for further strain development of carotenoid production.

Along with *sigA* overexpression in the carotenoid producing strains, overexpression of *sigD* was also investigated due to high accumulation of lycopene from BioLector experiment (**Figure 19**). The effect of *sigD* overexpression on carotenoid production was detected in the strains LYC5 or BETA3, which contains the extra carotenogenic genes, but neither the wild type strain, WT, nor the derepression strain, MB001Δ*crtR* (**Figure S4**). Interestingly, overexpression of *sigD* enhanced the accumulation of carotenoids in the first 24 hours, however further enhancement was not observed after 24 hours in LYC5 or BETA3, which is very different from the accumulation pattern of carotenoids under *sigA* overexpression. Therefore, *sigD* overexpression is assumed to influence different regulatory networks or metabolic pathways compared to *sigA* overexpression. The effect of *sigD* overexpression on transcriptome is not determined in the carotenoid producing strains, and

the transcriptome analysis of *sigD* overexpression in the wild type did not show upregulation of gene expression which is considered to be related directly to carotenoid production (**Table 6**). Furthermore, the possible target cannot be found from the comparison between transcriptome data of *sigA* overexpression and *sigD* overexpression Considering that overexpression of different sigma factor genes affects the lycopene production in a different manner (**Figure 19**), the unknown regulatory mechanisms seems to exist in carotenoid production in *C. glutamicum*. These result strongly support the idea that overexpressing sigma factors is helpful to investigate targets in the complex cellular mechanisms.

Based on the results, overexpression of sigma factors is shown as an effective tool to perturb regulatory networks and develop strains for a desired purpose by harnessing unknown effects in the endogenous pathways.

3.4 The effect of overexpression of different sigma factor genes

Overexpression of each sigma factor gene was performed in the wild type strain and the lycopene producing strain in this study. The effect of overexpressing sigma factor genes differed depending on the gene expressed. Therefore, it is strongly suggested that different sigma factors are responsible for transcription of different sets of genes in *C. glutamicum*. Therefore, the strain development using sigma factor can be a powerful tool for perturbing regulatory networks and boosting some of the endogenous metabolic pathways. Overexpression of *sigD* and *sigH* decreased growth rate drastically under high IPTG concentrations in the wild type strain and the lycopene producing strain. Because no remarkable overlap of genes up or downregulated under *sigD* or *sigH* overexpression was confirmed, the precise mechanism of growth inhibition by overexpression of *sigD* and *sigH* is still unknown. Considering that the drastic growth decrease occurs only in some strains such as the *sigC*, *sigD* and *sigH* transformants, those drastic growth decrease occurs not simply because of the stress due to protein expression. For the growth inhibition, two reasons can be hypothesized. The first reason is the lack of essential components and the second reason is the accumulation of toxic components. For the lack of components, overexpression of a certain sigma factor can disturb the sigma factor competition in cell and interfere with transcription of housekeeping genes. Those perturbations inhibit the cell growth by decreasing the synthesis of essential components at the level of protein and metabolites. For the

toxicity, the toxic components can be accumulated due to the effect of sigma factor overexpression and may hinder the cell growth. These two effects can also influence the growth at the same time. Even though elucidating the reason is not very easy, omics analysis such as proteomics and metabolomics analysis in addition to transcriptomic analysis may be helpful to understand the global effect of each sigma factor.

The function of the ECF type sigma factor is often regulated by anti-sigma factors (Paget, 2015). The function of SigD, SigE and SigH is inhibited by anti-sigma factor RsdA, CseE and RshA, respectively. On the other hand, no anti-sigma factors are known for SigC and SigM. The experiment of *sigD* and *sigH* overexpression confirmed that overexpression of sigma factor genes is valid for perturbing the transcriptome profile even with the existence of anti-sigma factors for some cases.

The effect of overexpression of *sigE* and *sigM* showed less influence on the cell growth compared to other alternative sigma factors. With the assistance from the group of Dr. Miroslav Pátek, overexpression of *sigE* was confirmed to enhance the *in vivo* transcription from the promoter of *cseE*, which is considered to be SigE-dependent, using two plasmid system experiment (**Figure S5. A**). Therefore, overexpression of *sigE* is expected to perturb transcription profile even with existence of anti-sigma factor CseE. Also with the assistance from the group of Dr. Miroslav Pátek, the effect of *sigM* overexpression was investigated on the transcription from the promoter of *trxB*1 encoding thioredoxin, which is considered to be SigM-dependent (Nakunst *et al.,* 2007), however, the expression of the reporter protein gene *gfpuv* did not change under *sigM* overexpression (**Figure S5. B**). Considering that the effect of *sigM* overexpression for lycopene production appeared in the lycopene producing strain, the transcription uder the control of SigM may be regulated by other mechanisms such as requirement of transcription factors. The different effects caused by each sigma factor also indicate that *C. glutamicum* utilizes different sigma factors for specific conditions under different regulations.

3.5 Strain development by overexpression of sigma factor genes

C. glutamicum has been engineered for many decades for different purposes, therefore, various platform strains are available in many groups. The strain development using sigma factor can be easily applied to those platform strains to increase the production by perturbing the endogenous

pathways and discover a new target for further metabolic engineering. There are several advantages for this approach.

First, overexpression of sigma factor genes can upregulate the expression of many genes at the same time by utilizing the endogenous regulatory architectures, and may switch on a desired pathway artificially. For example, overexpression of *sigH* induced the expression of several genes which products are involved in the disulfide stress response (Chapter. 2.1.4.3, Busche *et al.,* 2012; Toyoda *et al.,* 2015). On the other hand, overexpression of *sigD* induced the expression of several genes which products are involved in the regulation of the cell envelope integrity (Chapter. 2.2.4.4). Some of those upregulation of a specific pathway by overexpression of sigma factor genes can be helpful in terms of alteration of metabolic flux, energy balancing and cofactor production.

Secondly, selection of the potent sigma factor gene after overexpression is applicable for any platform strain. This step only requires simple overexpression of sigma factor genes. In this study, the successful application was shown for increasing the lycopene production (Chapter. 2.3.4.1).

Thirdly, different IPTG concentrations can control the level of perturbation in the tested strain and optimize the effect of sigma factor expression for production and cell growth (Figure S2). Furthermore, the arrest of growth by high concentration of IPTG may be helpful to switch the growth phase and production phase during fermentation.

Even though the application of this approach is not yet examined for other production strains except the carotenoid producing strains, the existence of seven sigma factor genes and the turnability of the expression levels by different IPTG concentrations increase the possibility that this approach is successfully transferred to other production strains. Furthermore, the number of conditions to be tested is manageable because of seven sigma factor genes and several IPTG concentrations. This makes it easier to transfer this approach to other strains. Therefore, the strain development using sigma factors in a different platform strain is an interesting approach.

3.6 Potential use of sigma factors and more in the future

The stress response of organisms has been defined by the physical condition such as heat shock or addition of detergents (Requena, 2012). However, these responses are often the consequence of several cellular response mechanisms under different regulations. Therefore, it may be

important to define the unit of response mechanisms which are under the control of the same regulators. Genomic loci and promoter sequences were optimized during evolution along with the regulation of sigma factors, therefore, a set of proteins under the control of the same sigma factor are expected not to have irrelevant functions each other, but to have linked functions for a certain purpose. These feature of sigma factor suggest that elucidating the regulatory network by sigma factor may reveal the strategies of adaptation which are rarely recognized under laboratory conditions (Cho *et al.,* 2014). The classification of response mechanisms based on sigma factor may be also helpful to construct the desired strain rationally.

Overexpression of sigma factors can be used for understanding the biological mechanism in bacteria. For elucidating the regulation by a sigma factor, the effect of deleting a sigma factor gene has been mainly tested. However, the effect of deletion can be canceled by transcription from other sigma factors due to a substantial number of overlapping promoters (Cho *et al.,* 2014). From this perspective, overexpression of sigma factors can be more advantageous since sigma factors mainly work as activators for transcription and their global influence can be revealed by upregulated expression of genes in transcriptome analysis (Bro and Nielsen, 2004). Furthermore, the effect of sigma factors for cell behavior can be evaluated with proteomics and metabolomics. The information derived from those analysis can be used to identify a target for further strain development.

For strain development using sigma factor, random mutagenesis of sigma factor genes can be also an interesting tool for perturbing regulator network in bacteria (Alper and Stephanopoulos, 2007). Actually, the gTME approach has achieved a substantial number of successes especially for increasing the tolerance to chemicals, pH or temperature (Tripathi *et al.,* 2014) (**Table 1**). Until now, the primary sigma factor is targeted for random mutagenesis in the most studies of gTME approach, however, it is also interesting to mutate alternative sigma factor genes since alternative sigma factors are more responsible for specific metabolic pathways. The alteration of promoter selectivity of alternative sigma factors may change the expression balance of genes in a regulon as well as the member of genes in a regulon. Furthermore, the gTME approach has been successfully applied especially for increasing the tolerance of bacteria, which is known to be difficult only by the rational approach due to complex mechanisms (Woolston *et al*., 2013). This seems to be mainly because the selection based on tolerance and cell growth is the easiest way to isolate the desired strain. Combining the gTME approach an the efficient high throughput screening method may

prove the applicability of the gTME approach for production.

In addition to sigma factors, there are also many other regulatory factors which can be used for strain development and biological understanding by perturbing cellular mechanisms. For example, transcriptional repressors/activators can be good targets since deletion/overexpression of those regulators can change the transcription profiles in cells. Random mutagenesis of the components of RNA polymerase such as α-subunit can be also an interesting target, changing the binding affinity of RNA polymerase to DNA strands (Klein-Marcuschamer *et al.,* 2009). In addition, the transcription termination factor, Rho, is an interesting target to perturb transcript profile not at the transcription initiation but at the transcription termination, since Rho factors promote transcription termination at the factor dependent termination site as well as perturb coupling of translation and transcription (Boudvillain *et al.,* 2013). Furthermore, metabolic engineering with the sigma factor and other factors at the same time can be also an interesting approach to reprogram cells. For example, deletion or overexpression of transcription factor along with sigma factor overexpression may enhance the upregulation of gene sets or cancel the undesired upregulation of gene sets caused by sigma factor. Those possibility was not tested in this study and are needed to be confirmed further with experiments. Metabolic engineering with sigma factors and those regulatory factors is still challenging because of its unpredictability and uncertainty, however, such an approach may add the new layer of metabolic engineering in the future.

4 Supplemental Figures

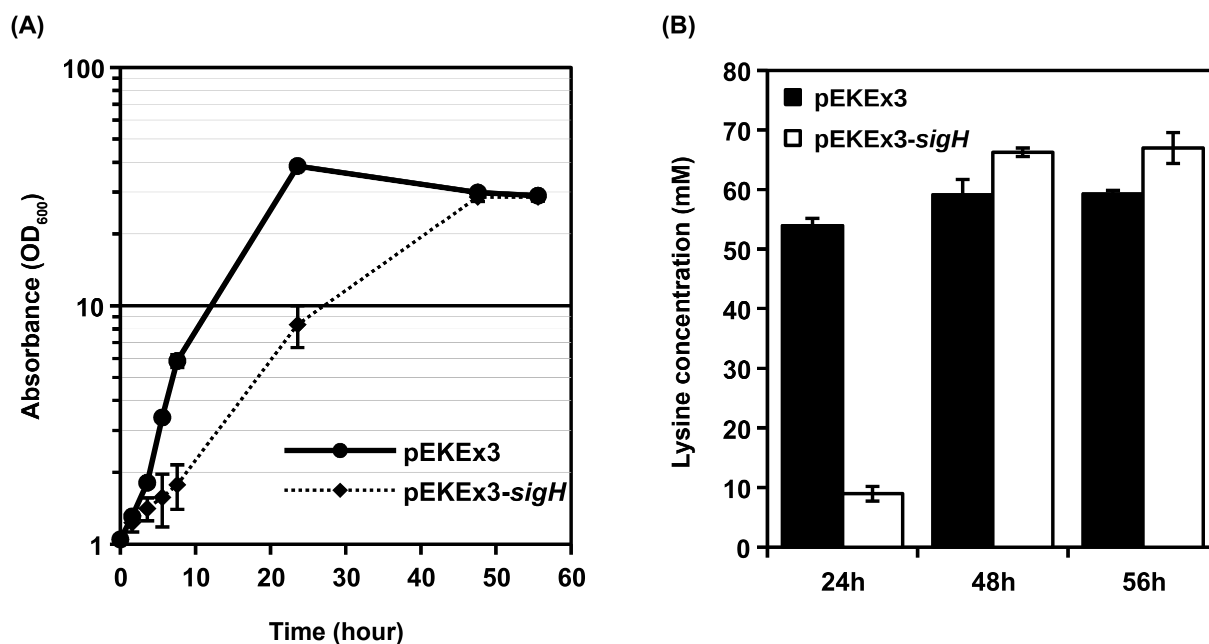


Figure S1. Effect of *sigH* overexpression on lysine production in *C. glutamicum* DM1729 strain. (A) Growth profile of DM1729(pEKEx3) and DM1729(pEKEx3-*sigH*). (B) Lysine production of DM1729(pEKEx3) and DM1729(pEKEx3-*sigH*) at different time points. The recombinant strain DM1729 was used as the parental strain (Becker *et al.,* 2011). Growth and lysine production were compared between the control strain, DM1729(pEKEx3), and the *sigH* overexpressing strain, DM1729(pEKEx3-*sigH*). Experiments were performed in 50 mL of CGXII medium with 222 mM of glucose, 15 µM of IPTG and 100 µg/mL of spectinomycin using 500 mL baffled flasks at 30 °C, 120 rpm. Inoculation of cells and absorbance measurement were performed as mentioned in section "2.1.3.2 Medium and growth conditions". Concentration of lysine was measured as described previously with dilution (Georgi *et al.,* 2005). For the quantification of lysine concentration, samples were taken at 24 hours, 48 hours, 56 hours after inoculation. Experiment was performed with biological triplicates and the error bars represent standard deviation from triplicates.

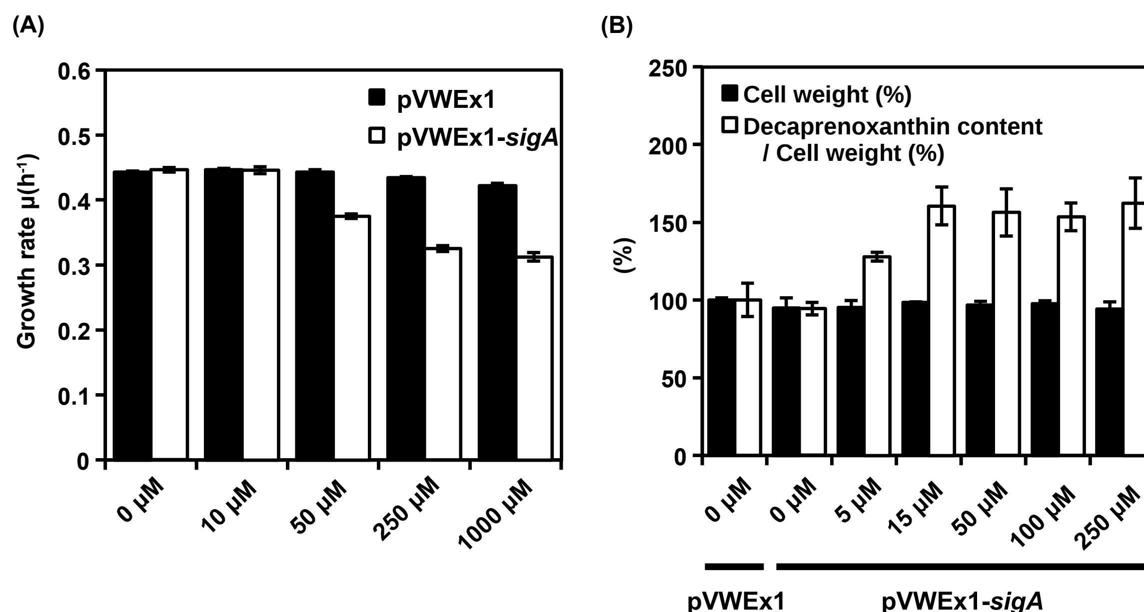


Figure S2. Effect of *sigA* overexpression on decaprenoxanthin production in *C. glutamicum* WT strain. (A) The maximum growth rate of WT(pVWEx1) and WT(pVWEx1-*sigA*). (B) Decaprenoxanthin production of WT(pVWEx1) and WT(pVWEx1-*sigA*) at different time points. The wild type strain WT was used as the parental strain. The maximum growth rate and decaprenoxanthin production were compared between the control strain, WT(pVWEx1), and the *sigA* overexpressing strain, WT(pVWEx1-*sigA*), under different IPTG concentrations. Experiments were performed using BioLector in CGXII medium with 222 mM of glucose, 25 µg/mL of kanamycin and different concentrations of IPTG. Inoculation of cells and the growth rate calculation were performed as mentioned in the section “2.3.3.2 Medium, growth conditions and growth rate comparison”. Comparison of decaprenoxanthin production was performed as mentioned in the section “2.3.3.3 Carotenoid extraction and quantification”. Experiment was performed with biological triplicates and the error bars represent standard deviation from triplicates.

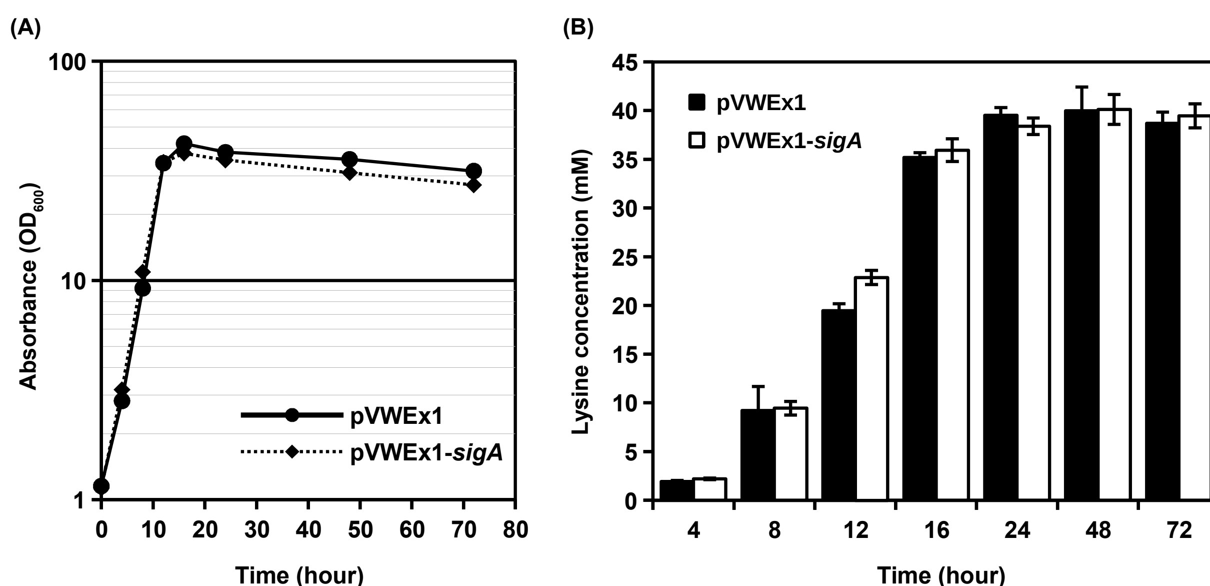
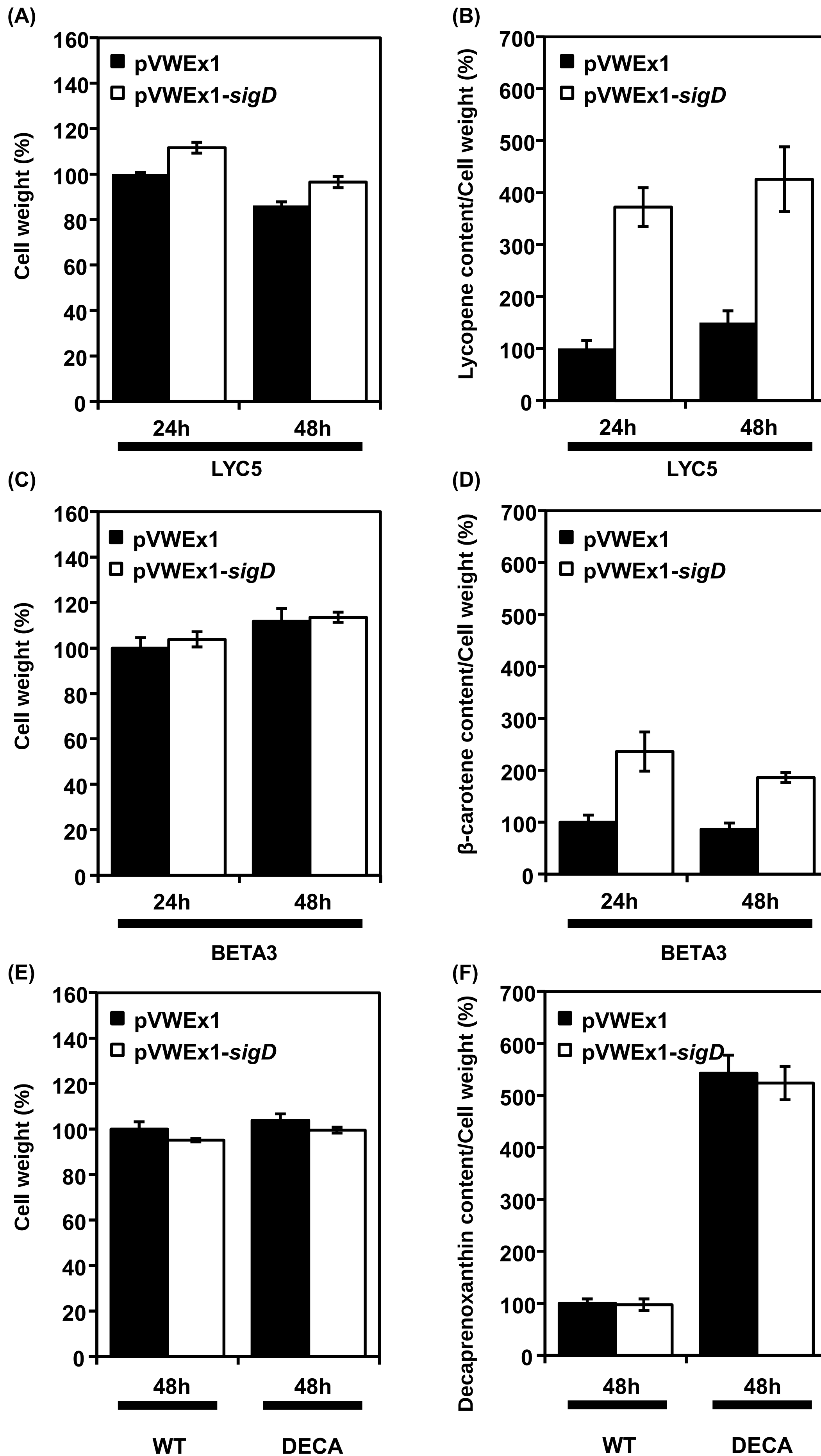


Figure S3. Effect of *sigA* overexpression on lysine production in *C. glutamicum* GRLP strain. (A) Growth profile of GRLP(pVWEx1) and GRLP(pVWEx1-*sigA*). (B) Lysine production of GRLP (pVWEx1) and GRLP(pVWEx1-*sigA*) at different time points. The recombinant strain GRLP was used as the parental strain (Unthan *et al.,* 2015). Growth and lysine production were compared between the control strain, GRLP(pVWEx1), and the *sigA* overexpressing strain, GRLP(pVWEx1-*sigA*). Experiments were performed in 50 mL of CGXII medium with 222 mM of glucose, 50 µM of IPTG and 25 µg/mL of kanamycin using 500 mL baffled flasks at 30 °C, 120 rpm. Inoculation of cells and absorbance measurement were performed as mentioned in section "2.3.3.2 Medium, growth conditions and growth rate comparison". Concentration of lysine was measured as described previously with dilution (Georgi *et al.,* 2005). Experiment was performed with biological triplicates and the error bars represent standard deviation from triplicates.

Figure S4. Effect of *sigD* overexpression on lycopene production in LYC5, β-carotene production in BETA3, and decaprenoxanthin production in WT and MB001Δ*crtR*. Cell weight of LYC5(pVWEx1) and LYC5(pVWEx1-*sigD*) **(A)** BETA3(pVWEx1) and BETA3(pVWEx1-*sigD*) **(C)** WT(pVWEx1) and WT(pVWEx1-*sigD*) or MB001Δ*crtR*(pVWEx1) and MB001Δ*crtR*(pVWEx1-*sigD*) **(E)** Lycopene production of LYC5(pVWEx1) and LYC5(pVWEx1-*sigD*) **(B)** β-carotene production of BETA3(pVWEx1) and BETA3(pVWEx1-*sigD*) **(D)** decaprenoxanthin production of WT(pVWEx1) and WT(pVWEx1-*sigD*) or MB001Δ*crtR*(pVWEx1) and MB001Δ*crtR*(pVWEx1-*sigD*) **(F)** The maximum growth rate and carotenoid production were compared between the control strain and the *sigD* overexpressing strain. Experiments were performed in CGXII medium with 222 mM of glucose, 25 µg/mL of kanamycin and different concentration of IPTG using 500 mL baffled flasks for LYC5 and BETA3 and BioLector for WT and MB001Δ*crtR*. Samples were taken at 24 h and 48 h for LYC5 and BETA3 and only 48 h for WT and MB001Δ*crtR* after inoculation. Inoculation of cells was performed as mentioned in the section "2.3.3.2 Medium, growth conditions and growth rate comparison". Calculation of cell weight and comparison of carotenoid production were performed as mention in the section "2.3.3.3 Carotenoid extraction and quantification". Experiment was performed with biological triplicates and the error bars represent standard deviation from triplicates.


(A)
160
140
120
100
80
60
40
20
0
Cell weight (%)
pVWEx1
pVWEx1-sigD
24h
48h
LYC5
(B)
700
600
500
400
300
200
100
0
Lycopene content/Cell weight (%)
pVWEx1
pVWEx1-sigD
24h
48h
LYC5
(C)
Cell weight (%)
pVWEx1
pVWEx1-sigD
24h
48h
BETA3
(D)
β-carotene content/Cell weight (%)
pVWEx1
pVWEx1-sigD
24h
48h
BETA3
(E)
Cell weight (%)
pVWEx1
pVWEx1-sigD
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WT
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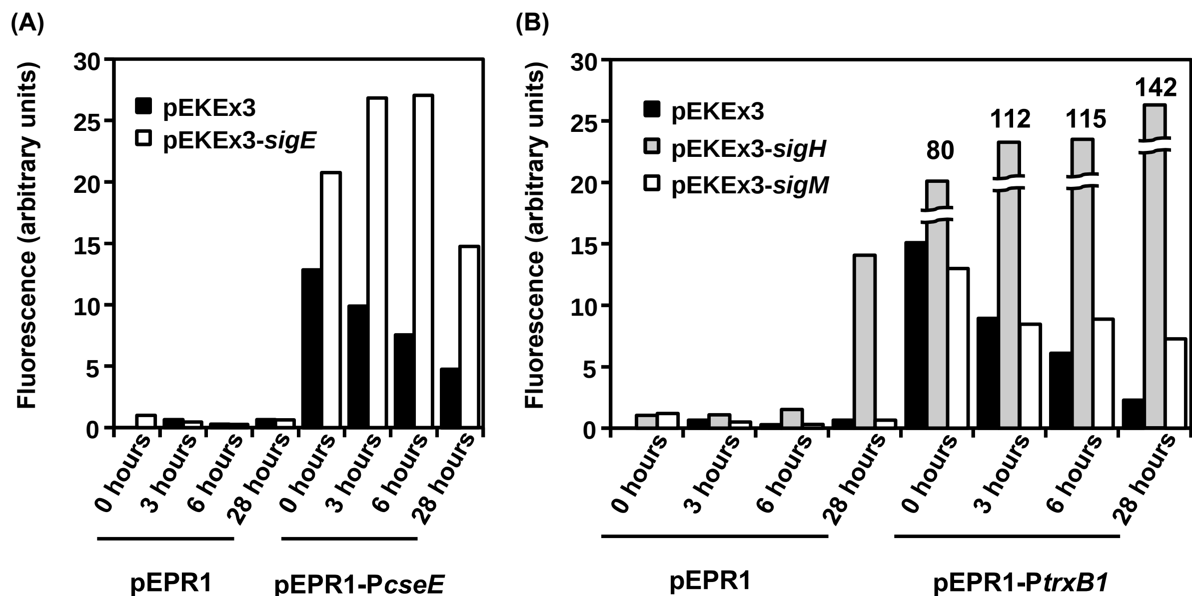

Figure S5. Quantification of sigma factor activity *in vivo* with GFPuv reporter assay. (A) Reporter assay of promoter activity of *cseE* under *sigE* overexpression. (B) Reporter assay of promoter activity of *trxB*1 under *sigH* or *sigM* overexpression. Plasmids, pEPR1, pEPR1-P*cseE* and pEPR1-P*trxB*1, were kindly provided by Dr. Miroslav Pátek (Knoppová *et al.,* 2007). Strains containing the pEKEx3 based plasmid and the pEPR1 based plasmid were constructed based on *C. glutamicum* WT strain as the parental strain. Experiments were performed in 50 mL of CGXII medium with 222 mM of glucose, 100 µg/mL of spectinomycin and 25 µg/mL of kanamycin using 500 mL baffled flasks at 30 °C, 120 rpm. Inoculation of cells and absorbance measurement were performed as mentioned in section "2.1.3.2 Medium and growth conditions". 1 mM of IPTG was added 3 hours after inoculation, and samples were taken 0 h, 3 h, 6 h and 28 h. Cells were resuspended with 250 µL of PBS and disrupted by beat beating for 30 sec. After centrifugation, supernatant was carefully transferred to a new tube. Protein concentration of the supernatant was measured by Bradford assay. Fluorescence of supernatant was measured with fluorophotometer (excitation 395 nm, emission 520 nm). Fluorescence intensity was normalized with protein concentration. Combination of two plasmids was shown. Fluorescence intensity of the strain containing pEKEx3-*sigH* and pEPR1-P*trxB*1 was rescaled, and the value was presented on the bar. Experiment was performed without replicates. Fluorescence intensity of the strain containing pEKEx3 and pEPR1 at 0 h was not measured because the sample was lost during the experiment.

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Mit freundlichen Grüßen

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