

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

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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 μ M. 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 adenylyltransferase was co-expressed with *sigH* from a plasmid. Balanced expression of *sigH* and *ribF* improved accumulation of riboflavin ($19.8 \pm 0.3 \mu$ M) and allowed for its conversion to FMN ($33.1 \pm 1.8 \mu$ M) in the supernatant. While a proof-of-concept was reached, conversion was not complete and titers were not high. This study revealed that inducible and gradable overexpression of sigma factor genes is an interesting approach to switch gene expression profiles and to discover untapped potential of bacteria for chemical production.

Keywords: *Corynebacterium*, RNA polymerase sigma factor, *sigH*, *ribA*, riboflavin, FMN production

Introduction

The sigma factor is a component of RNA polymerase holoenzyme and is important to recognize the promoter sequence in transcription initiation (Vassilyev et al., 2002; Feklistov 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 (Eggeling and Bott, 2005, 2015; Burkovski, 2008; 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 and Wendisch, 2010; Schneider et al., 2012) 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 regulatory engineering, 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., 2009). 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 (Ehira et al., 2008; Cho et al., 2012; 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.

Materials and Methods

Bacterial Strains, Plasmid, and Primer

The strains, plasmids and oligonucleotides used in this work are listed in **Table 1**. 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 1**. The PCR product was inserted into BamHI-digested pEKEx3 or pVWEx1 plasmid by Gibson assembly (Gibson et al., 2009). *E. coli* DH5 α 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 μ F (van der Rest et al., 1999; Eggeling and Bott, 2005).

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 μ g/mL of spectinomycin, 25 μ g/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.

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 μ M 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

TABLE 1 | Bacterial strains, plasmids and oligonucleotides used in this study.

Bacterial strain	Relevant characteristic	Reference
<i>Escherchia coli</i>		
DH5 α	<i>F-thi-1 endA1 hsdR17(r-, m-) supE44 ΔlacU169 (Φ80lacZΔM15) recA1 gyrA96 relA1</i>	Bethesda Research Laboratories
<i>Corynebacterium glutamicum</i>		
WT	Wild type, ATCC 13032	ATCC
Plasmid	Relevant characteristic	Reference
pEKEx3	SpecR; <i>E. coli</i> - <i>C. glutamicum</i> shuttle vector for regulated gene expression (Ptac, <i>lacIq</i> , pBL1 oriVCg)	Stansen et al. (2005)
pVWEx1	KanR; <i>E. coli</i> - <i>C. glutamicum</i> shuttle vector for regulated gene expression (Ptac, <i>lacIq</i> , pCG1 oriVCg)	Peters-Wendisch et al. (2001)
pEKEx3- <i>sigA</i>	SpecR, pEKEx3 with <i>sigA</i> from <i>C. glutamicum</i> WT	This study
pEKEx3- <i>sigB</i>	SpecR, pEKEx3 with <i>sigB</i> from <i>C. glutamicum</i> WT	This study
pEKEx3- <i>sigC</i>	SpecR, pEKEx3 with <i>sigC</i> from <i>C. glutamicum</i> WT	This study
pEKEx3- <i>sigD</i>	SpecR, pEKEx3 with <i>sigD</i> from <i>C. glutamicum</i> WT	This study
pEKEx3- <i>sigE</i>	SpecR, pEKEx3 with <i>sigE</i> from <i>C. glutamicum</i> WT	This study
pEKEx3- <i>sigH</i>	SpecR, pEKEx3 with <i>sigH</i> from <i>C. glutamicum</i> WT	This study
pEKEx3- <i>sigM</i>	SpecR, pEKEx3 with <i>sigM</i> from <i>C. glutamicum</i> WT	This study
pVWEx1- <i>sigH</i>	KanR, pVWEx1 with <i>sigH</i> from <i>C. glutamicum</i> WT	This study
pEKEx3- <i>ribF</i>	SpecR, pEKEx3 with <i>ribF</i> from <i>C. glutamicum</i> WT	This study
pVWEx1- <i>ribF</i>	KanR, pVWEx1 with <i>ribF</i> from <i>C. glutamicum</i> WT	This study
Oligonucleotide	Sequence (5'-3')	Reference
<i>sigA</i> -fwd	<u>GCCTGCAGGTCGACTCTAGAG</u> <i>GAAAGGAGG</i> CCCTTCAGATG <i>GTAGAAAACAACGTAGCAAAAAAGACGGTCG</i>	This study
<i>sigA</i> -rev	<u>CGGTACCCGGGGATC</u> TTAGTCCAGGTAGTCGCGAAGGACCTG	This study
<i>sigB</i> -fwd	<u>GCCTGCAGGTCGACTCTAGAG</u> <i>GAAAGGAGG</i> CCCTTCAGATG <i>ACAGCACCGTCCACGCAG</i>	This study
<i>sigB</i> -rev	<u>CGGTACCCGGGGATC</u> TTACTGGGCGTACTCAGAAAGACGTG	This study
<i>sigC</i> -fwd	<u>GCCTGCAGGTCGACTCTAGAG</u> <i>GAAAGGAGG</i> CCCTTCAG <i>GTGAAGTCAAAGAGCGTAACGACGC</i>	This study
<i>sigC</i> -rev	<u>CGGTACCCGGGGATC</u> CTAACCTTGGGCGGATTTGCCATCTTCG	This study
<i>sigD</i> -fwd	<u>GCCTGCAGGTCGACTCTAGAG</u> <i>GAAAGGAGG</i> CCCTTCAG <i>TTGGCTGATACTGAGCGCGAGCTC</i>	This study
<i>sigD</i> -rev	<u>CGGTACCCGGGGATC</u> TTACTTGTCTCCTGCTGCTCAAGTGTGCTTC	This study
<i>sigE</i> -fwd	<u>GCCTGCAGGTCGACTCTAGAG</u> <i>GAAAGGAGG</i> CCCTTCAGATG <i>ACTTATATGAAAAAGAAGTCCCGAGATGACGCAC</i>	This study
<i>sigE</i> -rev	<u>CGGTACCCGGGGATC</u> TTAGTGGGTTGGAACCAACAAGAACTTCCTCG	This study
<i>sigH</i> -fwd	<u>GCCTGCAGGTCGACTCTAGAG</u> <i>GAAAGGAGG</i> CCCTTCAGATG <i>GGCTGAAAACCGAACCGGCAC</i>	This study
<i>sigH</i> -rev	<u>CGGTACCCGGGGATC</u> TTATGCCTCCGAATTTTTCTTCATGTCCGGGATG	This study
<i>sigM</i> -fwd	<u>GCCTGCAGGTCGACTCTAGAG</u> <i>GAAAGGAGG</i> CCCTTCAGATG <i>ACAGTACTGCCTAAAAACCATGACCTAAGC</i>	This study
<i>sigM</i> -rev	<u>CGGTACCCGGGGATC</u> TCAGTTGCTTTCGCACTGTATGGAGCC	This study
<i>ribF</i> -fwd	<u>GCCTGCAGGTCGACTCTAGAG</u> <i>GAAAGGAGG</i> CCCTTCAG <i>GTGATATTTGGAGTGGACT</i>	This study
<i>ribF</i> -rev	<u>CGGTACCCGGGGATC</u> TTAAGCGCTGGGCTGGGTGT	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.

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-5 μ (40 mm \times 4 mm), CS-Chromatographie Service GmbH, Langerwehe, Germany) and a main column (LiChrospher 100 RP18 EC-5 μ (125 mm \times 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%.

Transcriptome Analysis of *sigH* Overexpressing Strain using DNA Microarrays

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 μ M 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 upregulated in WT(pEKEEx3-*sigH*) under both 10 and 15 μM of IPTG concentration were taken into account for further analysis (p -value < 0.05 , M -value > 1).

Measurement of Glucose-6-Phosphate 1-Dehydrogenase Enzyme Activities

Enzyme activities of glucose-6-phosphate 1-dehydrogenase in *C. glutamicum* WT (pEKEEx3) and *C. glutamicum* WT(pEKEEx3-*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 MgCl_2 , and 1 mM DTT) and stored at -20°C until use. Protein concentrations were determined with the Bradford reagent using bovin 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 $6.3 \text{ mM}^{-1} \text{ cm}^{-1}$ at 340 nm was used for calculating enzyme activities. The assay contained 50 mM Tris-HCl pH 8.5, 10 mM MgCl_2 , 100 mM NADP^+ and 100 mM glucose-6-phosphate.

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%.

Results

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

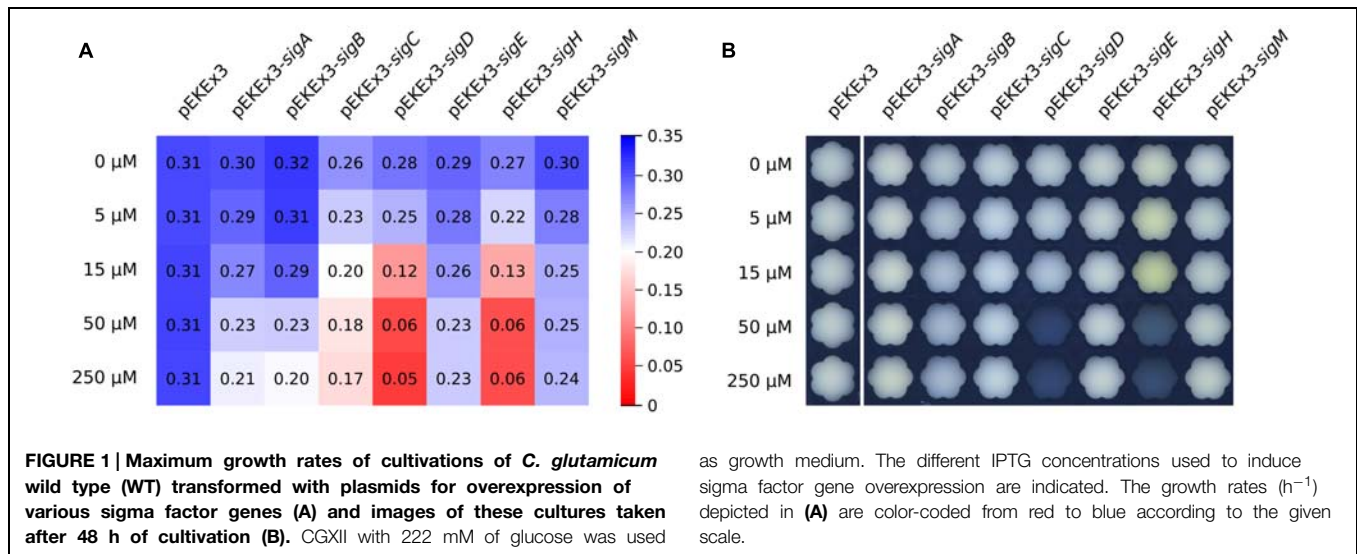
vector pEKEEx3 and transformed into *C. glutamicum* WT. Growth of these strains and of a control strain containing the empty vector pEKEEx3 was monitored in the presence of different IPTG concentrations (0, 5, 15, 50, or 250 μM) in CGXII medium containing 222 mM of glucose. Growth of the control strains 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 1A**). Interestingly, the cultures of the *sigH* transformant with up to 15 μM IPTG were colored yellow (**Figure 1B**). Therefore, the supernatants of all cultures were analyzed by recording absorbance spectra from 350 to 600 nm (**Figure 2**). 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.

Overexpression of *sigH* Resulted in Riboflavin Secretion

To verify the yellow color phenotype of WT(pEKEEx3-*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(pEKEEx3-*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(pEKEEx3-*sigH*; **Figure 3A**) and comparable absorption spectra (300–550 nm; **Figure 3B**) revealed that riboflavin was produced by *C. glutamicum* WT(pEKEEx3-*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(pEKEEx3-*sigH*) was about seven times as high as that of control strain WT(pEKEEx3), ($68.0 \pm 1.3 \mu\text{M}$ and $10.4 \pm 1.5 \mu\text{M}$, 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 4**).

Global Gene Expression Changes due to *sigH* Overexpression

To determine if *sigH* overexpression affects riboflavin biosynthesis genes, DNA microarray experiments with *C. glutamicum* WT(pEKEEx3-*sigH*) were performed and global gene expression at two different IPTG concentrations (10 and 15 μM) was compared to the control strain WT(pEKEEx3). 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 2**). 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 2**).

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.

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-fnu-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 2**), 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 ($\text{OD} \sim 10$) 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 ($32.4 \pm 1.8 \mu\text{M}$), but neither FMN nor FAD accumulated (**Figure 4**). However, when both genes were overexpressed in *C. glutamicum* WT(pEKEx3-*sigH*, pVWEx1-*ribF*) secretion of FMN ($17.0 \pm 0.6 \mu\text{M}$) in addition to riboflavin ($11.8 \pm 0.4 \mu\text{M}$) 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 ($19.8 \pm 0.3 \mu\text{M}$) and FMN ($33.1 \pm 1.8 \mu\text{M}$).

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., Fecl 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

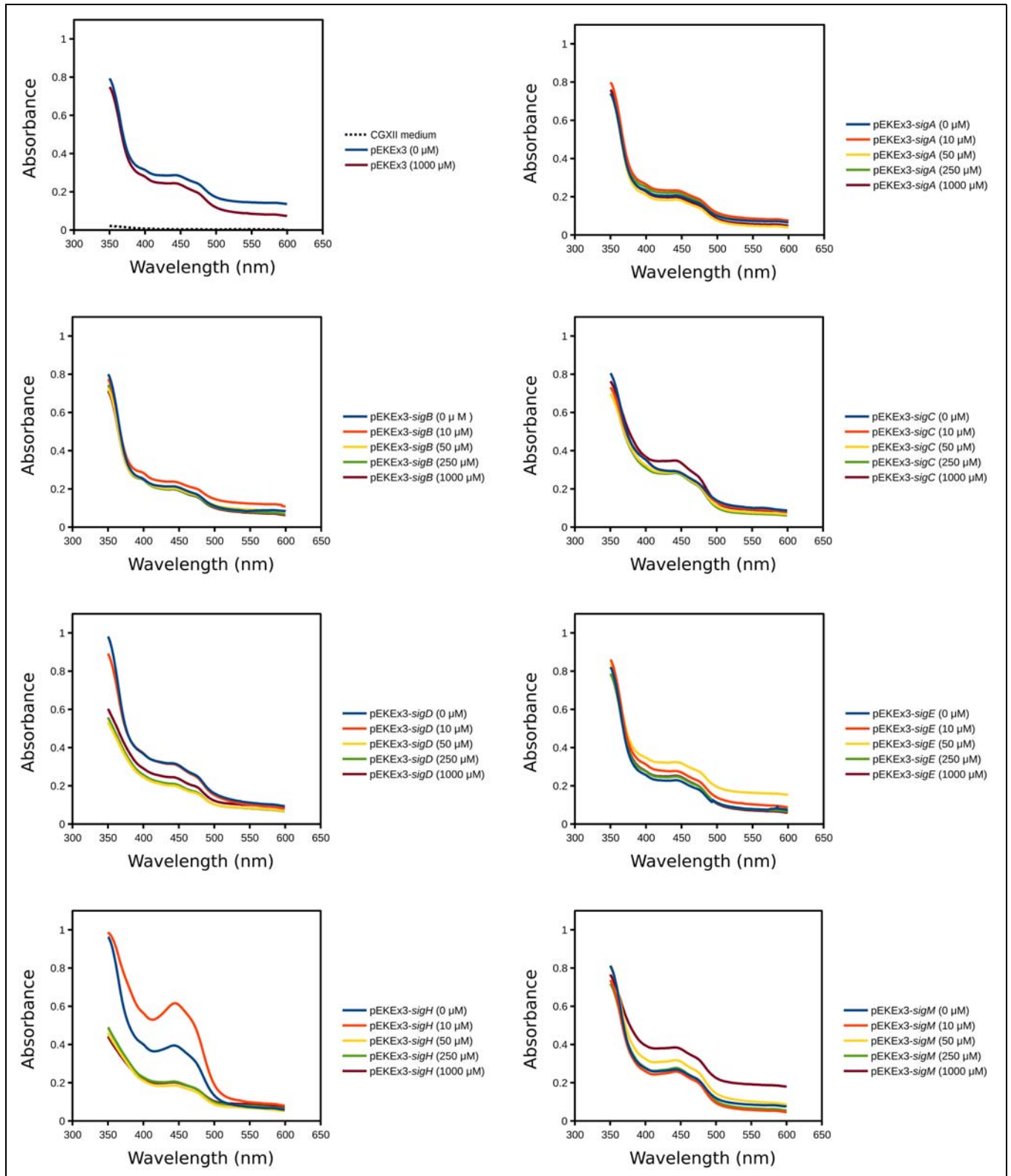
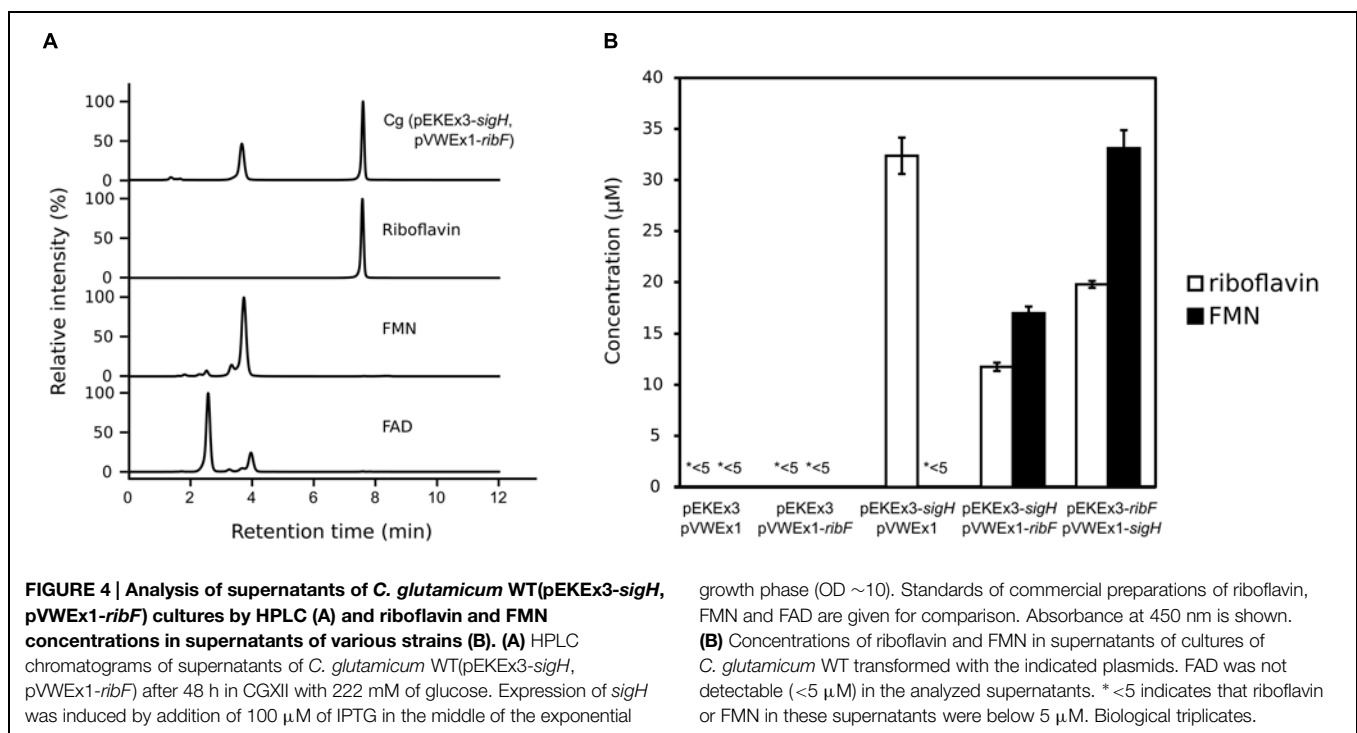
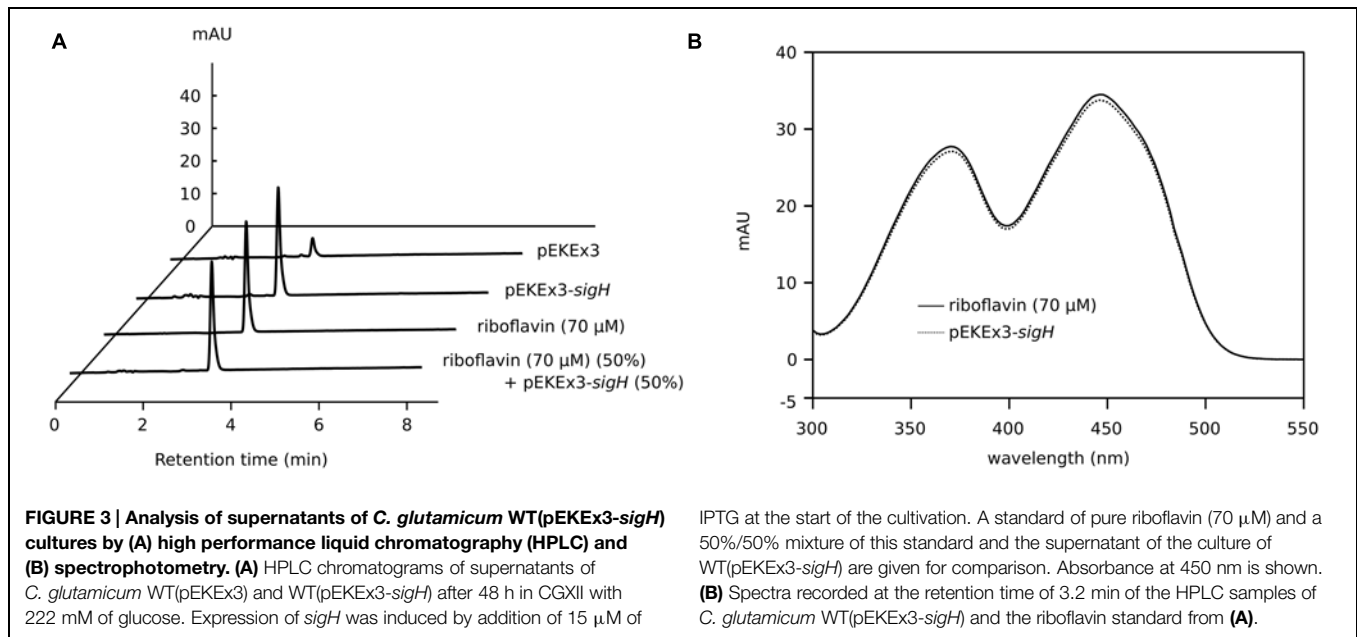


FIGURE 2 | Absorption spectra of supernatants of cultures of *C. glutamicum* WT transformed with plasmids for overexpression of various sigma factor genes. CGXII with 222 mM of glucose was used as growth medium. The IPTG concentrations and the plasmids used for overexpression of sigma factor genes are indicated. Supernatants were analysed after 48 h of cultivation.



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 1**). Overexpression

of *sigC* in glucose minimal medium with 250 μ M IPTG reduced the growth rate by about one third (**Figure 1**). The growth was severely inhibited (about half-maximal inhibition with IPTG concentrations as low as 15 μ M) 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

TABLE 2 | DNA microarray analysis of genes differentially expressed upon *sigH* overexpression.

Gene ID ^a	Gene name ^a	Function of protein ^a	M-value ^b		P-value ^c	
			10 μ M	15 μ M	10 μ M	15 μ M
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	<i>fdhD</i>	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	<i>sigH</i>	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	<i>fixA</i>	Putative electron transfer flavoprotein, beta subunit	1.1	2.0	1.7E-3	1.4E-2
cg1397	<i>trmU</i>	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	<i>ilvD</i>	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	<i>mshC</i>	Putative 1-D-myo-inositol-2-amino-2-deoxy-alpha-D-glucopyranoside-L-cysteine ligase	2.9	1.9	1.2E-4	1.5E-3
cg1764	<i>sufB</i>	FeS assembly membrane protein, SufB-family	1.2	1.0	1.8E-3	9.1E-3
cg1776	<i>tal</i>	Transaldolase	1.0	1.7	1.6E-2	1.2E-3
cg1778	<i>zwf</i>	Glucose-6-phosphate 1-dehydrogenase	1.2	2.0	4.1E-3	4.6E-3
cg1779	<i>opcA</i>	Glucose-6-phosphate 1-dehydrogenase subunit	1.6	1.2	1.5E-3	5.0E-2
cg1796	<i>ribX</i>	Conserved putative membrane protein, RibX-like	1.2	1.7	6.7E-3	3.5E-3
cg1797	<i>ribH</i>	Riboflavin synthase, beta chain	1.7	1.3	6.4E-5	1.7E-3
cg1798	<i>ribA</i>	Putative GTP cyclohydrolase II/3,4-dihydroxy-2-butanone-4-phosphatesynthase	2.0	1.0	4.6E-5	8.0E-3
cg1799	<i>ribC</i>	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	<i>mtr</i>	Putative NADPH-dependent mycothiol reductase	3.1	2.9	2.9E-8	2.2E-3
cg2206	<i>ispG</i>	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	<i>hisI</i>	Phosphoribosyl-AMP cyclohydrolase	1.2	1.1	3.1E-3	8.4E-3
cg2297	<i>hisF</i>	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	<i>lipA</i>	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	<i>clpP2</i>	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	<i>murI</i>	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	<i>msrA</i>	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	<i>trxB</i>	Thioredoxin reductase	1.8	2.2	3.1E-4	1.8E-4
cg3423	<i>trxC</i>	Thioredoxin	1.5	2.1	4.1E-4	6.8E-5
cg3424	<i>cwIM</i>	N-acetylmuramoyl-L-alanine amidase	1.3	1.7	8.2E-3	1.4E-2

^aGene ID, gene name and function of proteins are given according to CoryneRegNet (<http://coryneregnet.de/>). ^bRelative 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 or 15 μ M IPTG were added. ^cP-values were determined by Student's t-test.

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 2**) revealed that only *sigH* overexpression led to the production of a colored compound, which was identified to be riboflavin (**Figure 3**). Moreover, *sigH* overexpression slowed growth (**Figure 1**). 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 FOF1-ATP synthase operon *atpBEFHAGDC* (Barriuso-Iglesias et al., 2013), mycothiol peroxidase gene *mpx* (Si et al., 2015a), mycothiol S-conjugate amidase gene *mca* (Si et al., 2014), and methionine sulfoxide reductase A gene *msrA* (Si et al., 2015b). 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* (Ehira et al., 2009; Busche et al., 2012; 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 μ M IPTG (**Table 2**). These data generally agree with previous data on control by SigH (Ehira et al., 2009; Busche et al., 2012; 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 2**) 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 2**) 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 RNAseq-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 3**; 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 2**).

Riboflavin concentrations in supernatants of wild-type *C. glutamicum* cultures were low, but traces may be present (**Figure 3**). *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 4**). 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 μ M 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.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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