Assignment of sigma factors of RNA polymerase to promoters in Corynebacterium glutamicum

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Abstract

Corynebacterium glutamicum is an important industrial producer of various amino acids and other metabolites. The C. glutamicum genome encodes seven sigma subunits (factors) of RNA polymerase: the primary sigma factor SigA (σA), the primary-like σB and five alternative sigma factors (σC, σD, σE, σH and σM). We have developed in vitro and in vivo methods to assign particular sigma factors to individual promoters of different classes. In vitro transcription assays and measurements of promoter activity using the overexpression of a single sigma factor gene and the transcriptional fusion of the promoter to the gfpuv reporter gene enabled us to reliably define the sigma factor dependency of promoters. To document the strengths of these methods, we tested examples of respective promoters for each C. glutamicum sigma factor. Promoters of the rshA (anti-sigma for σH) and trxB1 (thioredoxin) genes were found to be σH-dependent, whereas the promoter of the sigB gene (sigma factor σB) was σE- and σH-dependent. It was confirmed that the promoter of the cg2556 gene (iron-regulated membrane protein) is σC-dependent as suggested recently by other authors. The promoter of the cmt1 (trehalose corynemycolyl transferase) was found to be clearly σD-dependent. No σM-dependent promoter was identified. The typical housekeeping promoter P2sigA (sigma factor σA) was proven to be σA-dependent but also recognized by σB. Similarly, the promoter of fba (fructose-1,6-bisphosphate aldolase) was confirmed to be σA-dependent but also functional with σA. The study provided demonstrations of the broad applicability of the developed methods and produced original data on the analyzed promoters.

Keywords: Corynebacterium glutamicum, Promoter, Sigma factor, In vitro transcription, RNA polymerase

Introduction

The multisubunit RNA polymerase (RNAP) holoenzyme in bacteria consists of core enzyme (2α, β, β′ and ω subunits) and a dissociable σ subunit (σ factor) that recognizes specific promoter sequences. Sigma factors are thus key regulatory elements that control different classes of promoters and activate expression of the respective groups of genes (regulons or sigmulons). Bacterial cells adapt in this way to changes in nutritional and environmental conditions. Bacteria typically possess a primary σ factor that is responsible for the transcription of housekeeping genes and a variable number of alternative σ factors that enable the cell to cope with various environmental stimuli. Since the activities of the different holo-RNAPs and the respective promoters orchestrate the cell metabolism in complex responses to various nutrition, growth and stress conditions, engineering σ factors has recently become an important tool in biotechnology and synthetic biology, particularly for the development of synthetic transcriptional control (Rhodius et al. 2013; Tripathi et al. 2014).

Corynebacterium glutamicum is a Gram-positive non-pathogenic soil bacterium used particularly for the industrial production of l-amino acids. The existing large toolbox for the genetic and metabolic engineering of C. glutamicum (Nešvera and Pátek 2011) enabled...
the construction of *C. glutamicum* producers of amino acids, carboxylic acids, alcohols, amines, polymers and biofuels as well as the use of alternative carbon sources like organic acids, pentoses, glycerol, starch and cellulose (Becker and Wittmann 2012). The *C. glutamicum* genome encodes seven sigma subunits of RNAP: the primary sigma factor σ^A^, the alternative primary-like σ^B^ and five other alternative σ factors with extracytoplasmic functions (ECF) (σ^C^, σ^D^, σ^E^, σ^H^ and σ^M^) (for a review, see Pátek and Nešvera 2011).

*Corynebacterium glutamicum* σ^A^ is an essential primary σ factor that directs the transcription of the majority of genes expressed during exponential growth which are termed “housekeeping” or “vegetative”. The promoters are usually considered housekeeping (σ^A^-dependent) if their −35 and −10 promoter sequences match the generally accepted consensus of housekeeping promoters. The consensus sequence of σ^A^-dependent promoters (−35 t/tgna and −10 TA/nnT) was deduced from a large number of defined promoters (Pátek and Nešvera 2011; Pfeifer-Sancar et al. 2013) which are believed to be σ^A^-dependent.

σ^A^ is a non-essential primary-like σ factor that is present in *C. glutamicum* cells in the highest levels at the transition and in the early stationary phase (Larisch et al. 2007). It is involved in responses to various stresses such as acid and heat stress and oxygen deprivation (EHira et al. 2008; Halgasova et al. 2002). In addition to its involvement in stress-protective functions, σ^A^ drives the transcription of the genes active in glucose utilization during exponential growth (EHira et al. 2008). σ^A^ can thus be considered to be a σ factor for slow growth and general stress conditions and another σ factor that recognizes some housekeeping promoters in the exponential growth phase. Only a few σ^A^-dependent promoters (13 in the review of Pátek and Nešvera 2011) have been localized and their key sequences were found to be essentially indistinguishable from the consensus sequences of σ^A^-dependent promoters.

σ^H^ is the most studied *C. glutamicum* ECF sigma factor, which controls a transcriptional regulatory network enabling the *C. glutamicum* cell to respond to temperature, oxidative and growth-phase induced stresses (Busche et al. 2012; EHira et al. 2009; Toyoda and Inui 2016b; Toyoda et al. 2015). The consensus sequence −35 g/tGGAAt and −10 t/cGTTgaa was defined (Busche et al. 2012; EHira et al. 2009) based on the 45 proposed σ^H^-regulated promoters.

*Corynebacterium glutamicum* σ^E^ was found to be involved in heat and cell surface stress response (Park et al. 2008). We have recently shown that the promoters P1clgR, P2dnaK and P2dnaJ2 are recognized by both σ^E^ and σ^H^ (Silar et al. 2016). This indicates that there is a certain overlap in promoter recognition specificity for σ^H^ and σ^E^. The consensus sequence of σ^E^-specific promoters has not been determined yet.

σ^E^ regulon that is induced in response to defects in aerobic respiration has been recently described (Toyoda and Inui 2016a). Eight σ^E^-specific promoters were found, and their consensus sequence was defined as −35 GGAAt and −10 CGACT.

A group of genes involved in oxidative stress response was found to be σ^M^-dependent (Nakunst et al. 2007). Some of these genes were found to be σ^H^,-dependent in another study (EHira et al. 2009). It therefore needs to be elucidated whether σ^H^ and σ^M^ are members of a regulatory cascade or if their recognition specificities overlap. No σ^D^,-dependent genes and σ^D^,-specific promoters have been described yet.

It was found that the overexpression of the *C. glutamicum* sigH gene resulted in enhanced riboflavin biosynthesis and its excretion to the medium (Taniguchi and Wendisch 2015; Toyoda et al. 2015). Further advances in the use of σ factor manipulations are still hampered in *C. glutamicum* by limited knowledge of the mechanisms of σ factor regulations, as well as by current lack of reliable methods for identifying the target promoters for various σ factors.

Knowledge of the recognition specificity of σ factors and assignment of σ factors to particular promoters is necessary to modulate the effects of sigma factors on the production of specific metabolites. In addition to manipulating sigma factors, the construction of artificial promoters recognized by specific σ factors is a promising strategy for modulating gene expression and improving the production of selected metabolites (Pátek et al. 2013). A self-inducible σ^B^,-dependent *C. glutamicum* promoter has recently been developed which can be useful for the production of metabolites in the stationary growth phase (Kim et al. 2016).

Multiple promoters upstream of many bacterial genes pose another challenge to their classification. The genes have frequently two or more promoters, which can overlap. Two overlapping promoters can be controlled by different sigma factors. Moreover, some promoters are recognized by two or even more sigma factors. As a result, determining which regulon the gene belongs to may be difficult. Overlapping σ-factor binding sites were detected frequently in *Escherichia coli*: e.g. 38 genes were assigned to 4 different sigma factors and 2 genes were even assigned to 6 sigma factors (Cho et al. 2014).

In this study, we used an in vitro transcription system and in vivo methods (overexpression of sigma genes to drive transcription from the promoters transcriptionally fused to the gfpuv reporter, use of sig-deletion strains) to reliably determine which sigma factors control
transcription driven by individual tested promoters in \textit{C. glutamicum}. We analyzed both housekeeping \( \sigma \) factors and ECF \( \sigma \) factors involved in stress responses. Overexpression of the \( \text{sig} \) genes encoding ECF \( \sigma \) factors usually results in the stronger expression of the \( \text{sig} \) factor-specific genes, even in the absence of the respective stress signal. This is advantageous particularly when the conditions under which the respective sigma factor is active are not known. The consistency of results achieved by the in vivo and in vitro techniques provided reliable promoter classification as well as new data on the analyzed promoters.

**Materials and methods**

**Bacterial strains, plasmids, oligonucleotide primers and growth conditions**

The bacterial strains and plasmids used are listed in Table 1. The oligonucleotide primers are listed in Additional file 1: Table S1. \textit{E. coli} DH5\( \alpha \) was used for cloning purposes. Wild-type (WT) \textit{C. glutamicum} ATCC 13032 and its deletion derivatives \textit{C. glutamicum} \( \Delta \text{sigB} \), \( \Delta \text{sigE} \), \( \Delta \text{sigH} \) and \( \Delta \text{sigM} \) were used as hosts for testing the activities of promoters cloned in the promoter-test vector pEPR1. \textit{E. coli} was cultivated aerobically in 500-ml flasks containing 80 ml of 2xYT medium (Sambrook and Russel 2001) on a rotary shaker at 150 rpm and 37 \( ^\circ \text{C} \). \textit{C. glutamicum} was cultivated in 500-ml flasks with 80 ml of complete 2xYT medium (Sambrook and Russel 2001) or in minimal CGXII medium (Keilhauer et al. 1993) with protocatechuic acid at a concentration of 0.03 g/l on a rotary shaker at 150 rpm and 30 \( ^\circ \text{C} \). Kanamycin (30 \( \mu \text{g/ml} \)), tetracycline (10 \( \mu \text{g/ml} \)), spectinomycin (200 \( \mu \text{g/ml} \)) or ampicillin (100 \( \mu \text{g/ml} \)) was added to the selective media when appropriate.

**DNA manipulations**

DNA isolation, PCR, transformation of \textit{E. coli}, DNA cloning and DNA analysis were performed using standard methods (Sambrook and Russel 2001). Genomic DNA from \textit{C. glutamicum} was isolated as described (Eikmanns et al. 1994). \textit{C. glutamicum} cells were transformed by electroporation (van der Rest et al. 1999).

**Table 1** Strains and plasmids used in this study

| Strains | Relevant characteristics | Source/reference/application |
|---------|--------------------------|------------------------------|
| \textit{E. coli} DH5\( \alpha \) | Cloning host | Hanahan (1985) |
| \textit{C. glutamicum} | ATCC 13032, wild type | ATCC |
| \( \Delta \text{sigB} \) | Deletion in \text{sigB} | Larisch et al. (2007) |
| \( \Delta \text{sigE} \) | Deletion in \text{sigE} | Park et al. (2008) |
| \( \Delta \text{sigH} \) | Deletion in \text{sigH} | Zemanová et al. (2008) |
| \( \Delta \text{sigM} \) | Deletion in \text{sigM} | Nakunst et al. (2007) |
| \textbf{Plasmids} | | |
| pEC-XT99A | \textit{E. coli}–\textit{C. glutamicum} expression vector, Tc\( ^\text{R} \), IPTG-inducible trc promoter | Kirchner and Tauch (2003) |
| pEKEx3 | \textit{E. coli}–\textit{C. glutamicum} expression vector, Sp\( ^\text{R} \), IPTG-inducible tac promoter | Hoffelder et al. (2010) |
| pEPR1 | \textit{E. coli}–\textit{C. glutamicum} promoter-test vector, Km\( ^\text{R} \), promoterless gfpuv as a reporter | Knoppová et al. (2007) |
| pEPR-P2sigA | pEPR1 with P2sigA | This work |
| pEPR-Pfba | pEPR1 with Pfba | This work |
| pEPR-Pcg2556 | pEPR1 with Pcg2556 | This work |
| pEPR-Pcrm1 | pEPR1 with Pcrm1 | This work |
| pEPR-PsigB | pEPR1 with PsigB | This work |
| pEPR-PrshA | pEPR1 with PrshA | This work |
| pEPR-PtnxB1 | pEPR1 with PtnxB1 | This work |
| pRLG770 | \textit{E. coli} vector for in vitro transcription, \textit{mmB} terminator, Ap\( ^\text{R} \) | Ross et al. (1990) |
| pRLG770P2sigA | pRLG770 with P2sigA | This work |
| pRLG770Pfba | pRLG770 with Pfba | This work |
| pRLG770Pcg2556 | pRLG770 with Pcg2556 | This work |
| pRLG770Pcrm1 | pRLG770 with Pcrm1 | This work |
| pRLG770PsigB | pRLG770 with PsigB | This work |
| pRLG770PrshA | pRLG770 with PrshA | This work |
| pRLG770PtnxB1 | pRLG770 with PtnxB1 | This work |

\text{IPTG} isopropyl-\( \beta \)-thiogalactopyranoside, Tc\( ^\text{R} \) tetracycline resistance, Sp\( ^\text{R} \) spectinomycin resistance, Km\( ^\text{R} \) kanamycin resistance, Ap\( ^\text{R} \) ampicillin resistance
Construction of the two-plasmid system for assignment of sigma factors to promoters in vivo

We constructed a system for the in vivo identification of *C. glutamicum* promoters recognized by RNAP containing a particular sigma factor, which is based on the two-plasmid *C. glutamicum* strains similar to that developed for the identification of σ^E^−dependent promoters in *E. coli* (Rezuchova and Kormanec 2001). Promoters carried on the BamHI–PstI DNA fragments (amplified using PCR, oligonucleotide primers listed in Additional file 1: Table S1 and *C. glutamicum* chromosome as a template) were cloned in the promoter-test vector pEPR1 containing the promoterless gfp reporter gene (Knoppová et al. 2007). The genes encoding seven different sigma factors were cloned under the P{trc} promoter inducible with isopropyl-β-d-1-thiogalactopyranoside (IPTG) in the expression vector pEC-XT99A (Kirchner and Tauch 2003). The sequences of all inserts were checked by sequencing. Analogous constructions carrying the σ^E^ genes under P{trc} in the expression vector pEKEx3 (Hoffelder et al. 2010) were kindly supplied by Taniguchi and Wendisch (2015). There was negligible gene expression from pEC-XT99A without IPTG when we tested the model expression of the gfpuv gene, whereas the expression of the gfpuv gene from pEKEx3 without IPTG addition was 20–30% of the maximum after IPTG induction (data not shown). *C. glutamicum* strains harboring both the pEPR1 + promoter and pEC-XT99A (or pEKEx3) + σ^E^ gene were obtained by successive transformation. The presence of correct plasmids in two-plasmid strains was checked by restriction enzyme analysis and PCR.

### In vivo promoter activity measurements

To evaluate the effect of σ^E^ gene overexpression on the activity of a particular promoter, the assay was performed as follows: The two-plasmid strain carrying the σ^E^ gene in the expression vector and a promoter in the promoter test vector pEPR1 was cultivated aerobically in 80 ml 2xYT medium at 30 °C. IPTG (1 mM) was added to the culture when OD_{600} reached 1 to overexpress a particular σ^E^ gene. Samples of the culture were then taken at various time points (mostly 0, 3, 6 and 24 h). The cells were washed with phosphate-buffered saline pH 8.0 (PBS) (Sambrook and Russel 2001) with 1 mM phenylmethylsulfonyl fluoride and 2 mM 2-mercaptoethanol, concentrated in 0.5 ml of cell suspension to a final OD_{600} = 24 in PBS and disrupted with a FastPrep homogenizer (MP Biomedical) (3 × 60 s at speed 6 m/s with Lysing Matrix B in 2-ml tubes). After centrifugation (20 min at 15,000×g) the fluorescence of the cell-free extract was measured with a Saphire2 microplate spectrophotometer (Tecan; excitation wavelength, 397 nm; emission wavelength 509 nm). Protein concentration in the extract was determined by Bradford assay and promoter activity was expressed in arbitrary units/mg protein. Cells harboring the pEPR1 construct and the expression vector without a σ^E^ gene were used as a control.

### In vitro transcription assay

The multiple-round in vitro transcription assay was in principle performed using the recently described system (Holátko et al. 2012). Both the RNAP core and sigma factors from *C. glutamicum* were isolated as described previously, using the *C. glutamicum* rpoC-H8 strain producing the RNAP core and *E. coli* BL21 (DE3) with pET-22b(+) constructs producing individual *C. glutamicum* σ factors (Holátko et al. 2012; Šilár et al. 2016). The reconstituted holo-RNAP was prepared by mixing the RNAP core (100 nM) and the respective sigma factor in a molar ratio of 1:30 and incubating for 10 min at 37 °C. The transcription mixture contained ATP, CTP and GTP (200 μM each), 10 μM UTP and 3 μM α^{32}P-UTP. The reaction was run for 10 min at 37 °C. All promoter fragments (approx. 70 nt) were cloned in the vector pRLG770 (using EcoRI and HindIII sites) in such a way that a 150-nt transcript (terminated at the rrtB terminator) was produced by the in vitro transcription (Holátko et al. 2012). The produced radiolabeled transcripts were separated by electrophoresis on 5.5% polyacrylamide gels (PAGE) with 7 M urea. The transcripts in dried gels were detected by exposure to phosphorimaging screen (6–24 h), followed by scanning with a Molecular Imager FX (BIO-RAD). Electrophoresis gel data from the imaging systems were visualized and analyzed with Quantity One 1-D analysis software. All assays were performed at least three times and consistent results were obtained. Representative results are shown.

### Tryptic digestion of in-gel proteins and liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis

The proteins extracted from the *C. glutamicum* cells disrupted by sonication were run in SDS-PAGE gels. The gel slice containing a bend corresponding to 25 kDa protein was digested with trypsin (100 ng Promega) overnight in a cleavage buffer containing 25 mM 4-ethylmorpholine acetate, and the resulting peptides were subjected to an LC–MS/MS analysis using collision-induced fragmentation in a Synapt G2Si mass spectrometer (Waters) coupled to an ACQUITY UPLC M-class system (HSS T3 1.8 μm column, 75 μm × 150 mm). For protein identification, the tandem mass spectra were searched against the NCBI bacterial database using an in-house Mascot search engine.

### Results

σ^{H^1}-dependent promoters: PrshA and PtxB1

σ^{H^1} is the most studied ECF sigma factor, which forms a transcriptional regulatory network enabling the C.
glutamicum cell to respond to temperature, oxidative and growth-phase induced stresses (Busche et al. 2012; Ehira et al. 2009; Toyoda and Inui 2016b; Toyoda et al. 2015). Due to its role in the expression of the genes encoding regulatory proteins and prominent position in the regulatory network, it is a likely candidate for a global regulator in C. glutamicum (Schröder and Tauch 2010). The rshA gene that encodes the anti-σH factor was localized immediately downstream of the sigH gene and forms an operon with sigH (Busche et al. 2012). The rshA gene is transcribed from PrshA (internal to sigH) in addition to the PsigH promoters found upstream of the operon (Busche et al. 2012). PrshA sequence elements −35 GGAAAGA and −10 GTTAAA (Fig. 1a) conform to the consensus of σH-dependent promoters, however the expression of rshA was not found to be up-regulated in C. glutamicum by the microarray analysis (Ehira et al. 2009). In vitro transcription assays resulted in a single specific band produced by RNAP with σH (Fig. 1b). The in vivo analysis with pEC-XT99A and pEPR1 carrying the sig genes and PrshA, respectively, in the C. glutamicum ΔsigH strain unequivocally demonstrated that PrshA is controlled by σH (Fig. 1c). Very similar results were obtained when the C. glutamicum WT was used (data not shown).

The promoter of the trxB1 gene was first described as σM-dependent (Nakunst et al. 2007) but as σH-dependent in another study (Busche et al. 2012). The trxB1 gene encodes a disulfide oxido-reductase that catalyzes a wide spectrum of redox reactions in the cell and is involved in oxidative stress response. A transcriptional start site of trxB1 was determined by RACE (Nakunst et al. 2007) and later confirmed by RNA sequencing (Pfeifer-Sancar et al. 2013). A single promoter of the gene with the key promoter elements −35 GGAA and −10 GTTGGT (Fig. 2a) was thus localized. The core conserved sequence motifs −35 GGAA and −10 GTT match the proposed consensus sequences of both σM-specific (Nakunst et al. 2007) and σH-specific (Busche et al. 2012) promoters. The in vitro transcription assay showed that only σH recognized PtrxB1 (Fig. 2b).

In vivo analysis using the two-plasmid system in the C. glutamicum ΔsigH strain also clearly indicated that it was only the overexpression of σH that triggered a sharp increase in the PtrxB1 activity (Fig. 2c). Very similar results were obtained when C. glutamicum WT was used (data not shown). To detect whether some other stress sigma factor at least weakly contributes to PtrxB1 activity in vivo, the effects of deletions in the genes sigH, sigM and sigE on the PtrxB1 activity during growth were tested. As shown in Fig. 2d, the reporter fluorescence measured with strains carrying only pEPR1-PtrxB1 remained at the level of the control (WT) in the ΔsigM and ΔsigE strains, whereas it was approximately 14-fold lower in the ΔsigH strain and essentially at the same level as that obtained with the strain only carrying the empty vector pEPR1. We therefore concluded that the PtrxB1 promoter is σH-specific.

**A σE- and σH-dependent promoter: PsigB**

Next, we tested the effect of the sigE gene deletion on the transcriptional activity of the cg1266 gene. This gene was
suggested to be σE-dependent (Park et al. 2008) but we did not detect any potential σE-dependent promoter (data not shown). Based on the similarity of the promoter region of the C. glutamicum sigB gene to the Mycobacterium tuberculosis sigB promoter (controlled by σE and σH) it was suggested that the C. glutamicum sigB may be under the control of σE (Halgasova et al. 2002). The sigB gene exhibited a higher level of transcription when sigH was overexpressed and was therefore considered to be σH-dependent (Ehira et al. 2009). The PsigB sequence elements −35 GGAA and −10 GTT conform to the consensus of σH-dependent promoters, however, the σH dependence was not proved by microarrays when ΔsigH (Ehira et al. 2009) or ΔrshA strains (Busche et al. 2012) were used. This gave us a hint that still another sigma factor is involved in sigB transcription. We carried out the in vitro transcription assays with all seven C. glutamicum sigma factors. Bands were detected when RNAP + σE or σH were applied (Fig. 3b). Specific band intensities based on three in vitro assays were quantified using Quantity One 1-D software, which showed that approximately 5.5-fold more transcript was produced with RNAP + σE than with RNAP + σH. The results of in vivo analysis using the two-plasmid system in the C. glutamicum WT suggested that the transcription from PsigB is induced by the overexpression of σH and significantly less by the overexpression of σE (data not shown). To test whether RNAP + σE can initiate transcription from PsigB in vivo more efficiently when σH does not compete with σE, we used the two-plasmid assay in the ΔsigH strain with all seven sigma factors. As shown in Fig. 3c, the overexpression of both σH and σE increased expression of the gfpuv reporter gene from PsigB. These results clearly demonstrated that RNAP + σE can drive transcription from PsigB but σH competes with σE for RNAP or binding to the promoter.

A σC-dependent promoter: Pcg2556

Expression of the genes regulated by σC has recently been described (Toyoda and Inui 2016a). We selected the
promoter of the *C. glutamicum* ATCC 13032 cg2556 gene encoding an uncharacterized iron-regulated membrane protein (corresponding to the cgR_2208 gene in the sequence of *C. glutamicum* R; Toyoda and Inui 2016a) to test sigma dependency of its promoter. In vitro transcription assays confirmed that *cg2556* transcription is σC-specific (Fig. 4b). The in vivo two-plasmid assay produced the same result (Fig. 4c).

A σD-dependent promoter: *P*<sub>cmt1</sub>

RNA sequencing studies suggested that there is a group of genes which are σD-dependent (Busche and Kalinowski, unpublished). We selected the promoter of *cmt1* (encoding trehalose corynomycolyl transferase) to test its σD dependency. In vitro transcription assays only provided a specific band when RNAP + σD was used (Fig. 5b). The in vivo two-plasmid assay produced the same result (Fig. 4c).

The quest for a σM-dependent promoter

The sigma factor σM was found to be involved in oxidative stress response in *C. glutamicum*. The σM-dependent transcription of 23 genes was suggested by using microarray analyses of the *C. glutamicum* WT and ΔsigM strain (Nakunst et al. 2007). Promoter sequences of four of these genes (*PtrxB, PtrxC, PtrxB1* and *PsufR*) were localized by determination of the respective transcriptional start sites (TSSs). Three of the genes (*trxB, trxC* and *sigR*) were found to be σH-dependent by Ehira et al. (2009). We analyzed all these four promoters using both in vivo and in vitro techniques. In all cases only σH-dependent transcription was detected (data not shown). We were thus unable to confirm the σM dependency of any of these promoters. The failure to detect an in vitro transcript with RNAP + σM might be due to the limitations of the technique used. In contrast to all other σ factors purified for the in vitro assays, most of the σM protein was detected in the insoluble fraction after its isolation from *E. coli* extracts using affinity chromatography (data not shown). Various modifications of the protocol did not improve the ratio of the soluble/insoluble fraction. The σM protein was therefore denatured and renatured. Since no σM-specific promoter was detected by the in vitro assay, there is a possibility that the renatured σM was not functional in vitro. Another reason for failing to prove σM-dependent transcription using the in vitro transcription may be missing activators. To test whether σM is present in the *C. glutamicum* ΔsigM cells carrying the pEC-XT99A vector with cloned sigM after IPTG induction in the in vivo assay, we analyzed the proteins of the cell extract by LC–MS/MS. Among other
proteins the presence of the $\sigma^M$ protein was confirmed by the identification of four peptides which covered 22% of the $\sigma^M$ protein sequence (Additional file 1: Table S2). Thus, the failure to demonstrate $\sigma^M$ dependency for any of these promoters in vivo was not due to the absence of $\sigma^M$ expression. The elucidation of $\sigma^M$ function and finding $\sigma^M$-controlled promoters needs further investigation.

**A $\sigma^A$-dependent promoter: P2$\sigma^A$**

It is difficult to reliably prove that a promoter is $\sigma^A$-specific in *C. glutamicum* since $\sigma^A$ is present in the cell during all growth phases and under most conditions, and deletion of the $\sigma^A$ gene would be lethal. The promoters are usually considered “vegetative” or “housekeeping” if their $-35$ and $-10$ promoter element sequences...
match the generally accepted consensus of housekeeping promoters and the respective genes are expressed during exponential growth under optimal conditions. We selected the P2sigA promoter of the sigA gene encoding the primary sigma factor to test whether the designed methods can prove its assumed σA dependency. P2sigA was localized by the determination of sigA TSS (Halgasova et al. 2001). According to the transcriptional profile of the sigA gene (expressed mainly in the exponential growth phase; Larisch et al. 2007) it is supposed to be σA-dependent. The sequences of the key promoter elements, −35 GTGACA and −10 TATAAT (Fig. 6a), are closely similar to the defined consensus sequence of the σA-dependent promoters in C. glutamicum (Pátek and Nešvera 2011; Pfeifer-Sancar et al. 2013) and σ70-dependent promoters in E. coli (Lisser and Margalit 1993). The in vitro transcription assays showed that RNAP + σA, but also RNAP + σB provide specific transcripts with the P2sigA promoter as a template (Fig. 6b). The values of fluorescence obtained from the two-plasmid system in C. glutamicum WT using pECXT-99A with sigA or sigB were lower than those obtained with other sigma factors and the control (empty pECXT-99A) as well. We also observed this phenomenon for other σA- and σD-dependent promoters (data not shown). To see the effect of σ factor gene overexpression more clearly, the fluorescence intensity values were expressed as differences between the fluorescence at the sampling time (3, 6, 24 h) and the fluorescence at time 0 (Fig. 6c). Only the overexpression of sigA (from pEC-XT99A) resulted in an increase in P2sigA promoter activity in both the exponential and stationary phase, whereas the overexpression of other sigma factors did not change its activity, or even decreased it (σH) (Fig. 6c). This effect may be due to the competition of σH with σA for RNAP. Similarly, the low activity of P2sigA when σB was overexpressed could be the consequence of the competition of σA with σB for RNAP or binding the respective holoenzyme (RNAP + σA or RNAP + σB) to the promoter. We conclude that P2sigA is predominantly σA-dependent and may probably also be active with σB in vivo under specific conditions (e.g. stress and the stationary phase).

A σA-dependent promoter: Pfba

The fba gene (encoding fructose 1,6-bisphosphate aldolase) was found to be downregulated in the sigB deletion strain both under conditions of oxygen deprivation and during aerobic cultivation (Ehira et al. 2008). The gene is involved in glucose metabolism and is mostly expressed during exponential growth whereas its expression decreases in the transition phase. The sequences of the key promoter elements (Ehira et al. 2008), −35 CGACAA and −10 CATAAT (Fig. 7a) are very similar to those of the proposed consensus of σA-specific promoters (Pátek and Nešvera 2011; Pfeifer-Sancar et al. 2013). In vitro transcription assays showed that both σA and σB with RNAP produce specific signals with Pfba (Fig. 7b). In vivo analysis using the two-plasmid system in the C. glutamicum WT strain with the expression vector pEC-XT99A carrying cloned siggenes did not provide an increase in promoter activity with any σ factor (data not shown). However, an alternative system

![Fig 6 Assignment of sigma factors to the P2sigA promoter.](image_url)
utilizing the expression vector pEKEx3 with cloned sig genes and pEPR1-Pfba proved that Pfba is recognized by σB under the conditions used (Fig. 7c). A slight increase was also observed with σE. Since we found that the promoter of the sigB gene is transcribed by RNAP + σE, this effect may be explained by the indirect effect of sigE overexpression. Activity of Pfba was further tested in the single-plasmid strains C. glutamicum WT and ΔsigB carrying pEPR1-Pfba. The activity of Pfba was significantly lower in the ΔsigB strain than in WT, but was still higher than the activity exhibited by the ΔsigB strain carrying an empty vector pEPR1 (Fig. 7d). The observed substantial residual activity of Pfba in C. glutamicum ΔsigB is in agreement with the recognition of this promoter by σA proved in vitro. These results suggest that transcription from Pfba is mainly driven by RNAP + σB even during exponential growth, and σA may substitute for σB under some specific conditions.

**Discussion**

Expression of sigma factor genes in bacteria is organized into cascades or networks (Qiu et al. 2013; Cho et al. 2014). Therefore, it is sometimes difficult to distinguish between the direct and indirect effects of the overexpression or deletion of sig genes in studies of the dependence of promoters on sigma factors. The genome-level in vivo approaches to analyzing σ-specific regulons using microarrays or ChIP-chip techniques may therefore be negatively affected by these regulatory interactions.

Thus the results of in vivo techniques may be overshadowed by secondary effects caused by the cascade or network nature of σ regulation, the competition of σ factors for RNAP and promoters, or the activities of transcriptional regulators. In contrast, the in vitro reaction, in which the DNA template is transcribed from a single promoter by a purified RNAP core with a single σ factor, can avoid such interactions. An in vitro transcription system which mimics many features of in vivo transcription thus provides results that are free of indirect effects. On the other hand, the in vitro transcription may produce some artifacts, since some promoters may require activators or appropriate DNA conformation (superhelicity) or other type of physiological control for their natural activity.

To compensate for the drawbacks of each of these approaches, both in vivo and in vitro methods should be
applied for the analysis of sigma factor–promoter interactions. By combining the results of in vitro and in vivo experiments, one can achieve an unambiguous assignment of sigma factors to promoters.

The main aim of this work was to integrate the results of our newly developed in vitro and in vivo techniques so that we can reliably classify individual promoters according to their σ factor dependency. This approach proved to be useful, and we were able to find a representative example of a promoter for every sigma factor with the exception of σM. The achieved results show that the system can produce data that is almost free of secondary effects. We were also able to convincingly document the recognition overlap of two σ factors at a single promoter (σA/σB, σE/σI).

The rshA gene, which is located immediately downstream of sigH, encodes the anti-σH factor. The gene is transcribed together with the sigH from a σA-dependent promoters and separately as a monocistronic rshA transcript from PrshA which was proposed to be σH-dependent (Busche et al. 2012). This arrangement probably ensures the rapid shutdown of the σH-dependent stress response as soon as the stress conditions are over. We have now confirmed by both in vivo and in vitro techniques that PrshA is a σH-specific promoter (Fig. 1). Similarly, PtrxB1 was shown to be a σI-specific promoter (Fig. 2). Neither the in vitro nor in vivo assay generated a signal with any other sigma factor. The PtxB1 promoter activity measurements using the ΔsigH, ΔsigM and ΔsigE strains showed that sigH deletion completely eliminated its activity, whereas the sigM and sigE deletions did not change or even increased its activity (Fig. 2d). The activities of PtxB, PtxC and PsufR that were also predicted to be σM-dependent in a study based on a sigM deletion strain (Nakunst et al. 2007) but σH-dependent according to the disruption or overexpression of sigH (Ehira et al. 2009) were completely eliminated in ΔsigH as well (data not shown). The in vivo two-plasmid system also showed that PtxB, PsufR and PtxC are σH-specific, although the presence of σM in the C. glutamicum cells after expression was proved by mass spectrometry.

We have recently shown that the promoters P1clgR, P2dnaK and P2dnaJ2 are recognized by both σE and σH (Šilar et al. 2016). To date, no exclusively σI-specific C. glutamicum promoter has been reported. PsigB was also found to be controlled by σE and σH in this study, just like the M. tuberculosis sigB promoter (Rodrique et al. 2006). This is in agreement with suggestions that C. glutamicum σI plays a role as a general stress response σ factor and as a back-up housekeeping σ for stress conditions (Halgasova et al. 2002; Larisch et al. 2007).

The σC-dependent promoters seem to be very specific and are probably recognized exclusively by σC, although their consensus sequence elements −35 GGGAACT and −10 CGACTA (Toyoda and Inui 2016a) contain the same −35 tetramer GGA as σH-dependent promoters. Both in vitro and in vivo methods clearly confirmed the assignment of σC to Peg2556. Similarly, the two methods coincidently indicated that the Pcmt1 promoter is σC-specific. The consensus sequence of σI-specific promoters is currently being explored (Busche and Kalinowski, unpublished data).

In contrast to stress promoters, σA- and σB-dependent promoters exhibited relatively high expression in all growth phases even without the overexpression of a sig gene. The cell levels of σA and σB are apparently high enough to drive expression from the tested promoters. Moreover, as was shown for the σI-dependent promoters Ppgo (Šilar et al. 2016) and Pfbα, σA is able to partially substitute for missing σI in ΔsigB strain. The ability of σA and σB to recognize Pfbα was confirmed by the in vitro transcription assays. This interchangeability of σA and σB was also shown for the typical housekeeping promoters Pper (Šilar et al. 2016) and P2sigA (Fig. 6b). This is not surprising since the amino acid sequences of σA and σB in protein regions 2.4 and 4.2 which recognize the −10 and −35 promoter motifs respectively are highly similar, and promoter consensus sequences of σA and σB could not be distinguished. These findings for C. glutamicum are analogous to those for E. coli σ70 and σ3 (Typas et al. 2007).

Detailed knowledge of functions of σ factors is a prerequisite for their engineering aimed at modulation of transcriptional regulatory network and, consequently, strain improvement for biotechnological purposes (Tripathi et al. 2014). Mutagenesis of σ factors or other transcriptional regulators and screening the mutants for their ability to reprogram cellular metabolism and regulation to the desired phenotype is a basis of newly developed global transcriptional machinery engineering method (for a review, see Tyo et al. 2007; Lanza and Alper 2011; Liu and Jiang 2015). For the first time, this approach was used for improvement of ethanol tolerance and production in Saccharomyces cerevisiae by mutagenesis of transcription factor Spt15p (Alper et al. 2006). Random mutagenesis of E. coli primary σ70 factor was found to result in global perturbations of the transcriptome and the mutants exhibiting ethanol tolerance, increased lycopene production and multiple tolerance phenotypes, respectively, were obtained (Alper and Stephanooulos 2007). Screening the library of E. coli σ70 factor-mutants under cyclohexane pressure resulted in obtaining the strains highly tolerant to this solvent (Zhang et al. 2015). E. coli strains accumulating hyaluronic acid effectively were obtained also by screening the σ5-mutants (Yu et al. 2008).
The use of two alternative vectors (pEC-XT99A and pEKEEx3) for overexpressing sig genes gave essentially the same results, which widens the choice for in vivo promoter analysis. A comparison of the results obtained with both in vivo and in vitro approaches proved to be useful for the unequivocal assignment of a sigma factor to a single promoter. Combining the advantages of in vivo and in vitro techniques can minimize the drawbacks of the techniques as stand-alone approaches and finally provide reliable sigma factor–promoter assignment.

Additional file

Abbreviations

ECF: extracytoplasmic function; IPTG: isopropyl-β-D-1-thiogalactopyranoside; LC–MS/MS: liquid chromatography-tandem mass spectrometry; OD₆₀₀: optical density at 600 nm; PBS: phosphate-buffered saline; RNAS: RNA polymerase; SD: standard deviation; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; TSS: transcriptional start site; WT: wild-type.

Authors' contributions

HD developed the two-plasmid system and carried out most of the in vivo experiments using the two-plasmid system, TB analyzed some promoters and constructed several strains, JK led the experiments in Bielefeld and edited the manuscript and MP designed the study, coordinated the work and wrote the draft of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The data supporting the findings and conclusions of this study are included in the main manuscript and in the additional supporting materials.

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