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# Use of In Vitro Transcription System for Analysis of *Corynebacterium glutamicum* Promoters Recognized by Two Sigma Factors

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**Abstract** Promoter activities in *Corynebacterium glutamicum* strains with deletions of genes encoding sigma factors of RNA polymerase suggested that transcription from some promoters is controlled by two sigma factors. To prove that different sigma factors are involved in the recognition of selected *Corynebacterium glutamicum* promoters, in vitro transcription system was applied. It was found that a typical housekeeping promoter P*per* interacts with the alternative sigma factor $\sigma^B$ in addition to the primary sigma factor $\sigma^A$. On the other way round, the $\sigma^B$-dependent promoter of the *pqo* gene that is expressed mainly in the stationary growth phase was active also with $\sigma^A$. Some promoters of genes involved in stress responses (P1*clgR*, P2*dnaK*, and P2*dnaJ2*) were found to be recognized by two stress-responding sigma factors, $\sigma^H$ and $\sigma^E$. In vitro transcription system thus proved to be a useful direct technique for demonstrating the overlap of different sigma factors in recognition of individual promoters in *C. glutamicum*.

## Introduction

Sigma factors are dissociable subunits of RNA polymerase (RNAP) holoenzymes which are responsible for the recognition of specific promoter DNA sequences and initiation of transcription. Almost all bacterial species contain several sigma factors which alternatively associate with the RNAP core. The individual types of RNAP holoenzymes containing different sigma factors recognize distinct classes of promoters differing in target (consensus) sequences. Sigma factors thus function as positively acting global regulators of transcription. In addition to an essential primary sigma factor responsible for transcription of majority of genes (mostly housekeeping genes), a variable number of non-essential alternative sigma factors, recognizing promoters of genes responsible for specific cell functions (e.g., in stress response or in entry into and in the stationary growth phase), are encoded by most bacteria.

In spite of the fact that the recognition of promoter sequences by a sigma factor is a highly specific process, an overlap of two or more sigma factors in recognizing a single promoter was described in bacteria. In *Escherichia coli*, functional overlap between primary sigma factor $\sigma^{70}$ and heat-shock sigma factor $\sigma^{32}$ [26] or general stress-responding sigma factor $\sigma^{38}$ [14] was observed. Overlapping specificity of *Bacillus subtilis* alternative stress-responding sigma factors $\sigma^X$ and $\sigma^M$ [11] or $\sigma^X$ and $\sigma^W$ [18] was also found, and the respective dual promoters (i.e., recognized alternatively by two sigma factors) were described. In *Mycobacterium tuberculosis*, recognition of one promoter (of the *sigB* gene encoding an alternative sigma factor) even by three different stress-responding sigma factors ($\sigma^E$, $\sigma^H$, and $\sigma^L$) was proved [3]. Overlapping sigma factor specificity thus seems to be a common regulatory strategy in bacteria.

*Corynebacterium glutamicum*, a Gram-positive actinobacterium frequently used for the production of amino acids, encodes seven sigma factors: essential primary sigma factor $\sigma^A$, alternative primary-like $\sigma^B$, and five other alternative sigma factors ($\sigma^C$, $\sigma^D$, $\sigma^E$, $\sigma^H$, and $\sigma^M$),

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classified into Group 4 [or extracytoplasmic function (ECF)] sigma factors [16]. Analysis of *C. glutamicum* promoter sequences revealed high similarity between −10 motifs of promoters of housekeeping genes (supposed to be recognized by $\sigma^A$) and promoters recognized by $\sigma^B$ [4, 10, 16, 17] which suggests possible overlapping promoter specificity of $\sigma^A$ and $\sigma^B$. The data on core elements (−35 and −10 motifs) of *C. glutamicum* promoters recognized by ECF sigma factors are still very limited [16, 17]. Only the high similarity between core elements (−35 and −10) of *C. glutamicum* promoters recognized by $\sigma^H$ [2, 5] or $\sigma^M$ [12] was found so far. However, some *C. glutamicum* promoters were defined as $\sigma^M$-dependent by some authors [12] whereas as $\sigma^H$-dependent by others [5]. Very recently, a consensus sequence of several promoters of $\sigma^C$-controlled genes was deduced [24]. We report here the evidence of overlap of *C. glutamicum* sigma factors $\sigma^A$ and $\sigma^B$, or $\sigma^H$ and $\sigma^E$ in recognizing specific promoters, obtained by use of *C. glutamicum* in vitro transcription system.

## Materials and Methods

### Bacterial Strains, Plasmids, Primers, and Growth Conditions

*E. coli* DH5α [7] and *E. coli* BL21 (DE3) [23] were used as hosts for cloning and for induced overexpression of the genes encoding sigma factors, respectively. Wild-type (WT) *C. glutamicum* ATCC 13032 and its deletion derivatives *C. glutamicum* Δ*sigB* [10], Δ*sigH* [27], and Δ*sigE* [15] were used as hosts for testing the activities of promoters cloned in promoter-test vector pEPR1. *C. glutamicum rpoC*-H8 [8] was used for isolation of RNAP core. Plasmids used are listed in Table 1. Oligonucleotides used are listed in the Supplementary material (Table S1). *E. coli* and *C. glutamicum* were cultivated in 2xTY medium [20] at 37 and 30 °C, respectively. Ampicillin (100 μg/ml) or kanamycin (30 μg/ml) was added to the selective media.

### DNA Techniques

DNA isolation, PCR, transformation of *E. coli*, DNA cloning, and DNA analysis were performed using standard methods [20]. *C. glutamicum* cells were transformed by electroporation [25].

### Isolation and Purification of *C. glutamicum* RNAP Core and Sigma Factors

RNAP core subunits (with $His_8$-tagged RpoC) were isolated from *C. glutamicum* strain *rpoC*-H8 [8]. $His_6$-tagged sigma factors were isolated from *E. coli* BL21 (DE3) carrying plasmids pET-22b(+) with the respective *C. glutamicum sig* genes (Table 1) according to Holátko et al. [8]. TALON Resin (Clontech) was used (instead of Ni–NTA Agarose) for protein isolation by affinity chromatography. Isolated proteins were purified by gel filtration.

### Construction of Promoter-Carrying DNA Templates for In Vitro Transcription

Fragments carrying *C. glutamicum* promoters were amplified by PCR using oligonucleotide primers listed in the Supplementary material (Table S1). The fragment with promoter P*per* (96 bp) [13] was amplified using the plasmid pGA1 from *C. glutamicum* LP-6 as a template resulting in the construct pRLG770Pper. The fragment with promoter P2*dnaK* (109 bp) [1] was amplified using *C. glutamicum* ATCC13032 genome DNA as a template to provide the construct pRLG770P2dnaK. Promoter fragments P*pqo*, P1*clgR*, and P2*dnaJ2* were prepared by dimerization of oligonucleotides listed in Table S1 to provide the constructs pRLG770Ppqo, pRLG770P2dnaJ2, and pRLG770P1clgR, respectively. The PCR fragments were cloned into the EcoRI-HindIII sites of the vector pRLG770 in all cases. All promoter fragments were cloned to form transcripts of the same size (∼150 nt). As templates for in vitro transcription assay, PCR fragments amplified from pRLG770Px constructs, using the primer pair 30F–CM3 were applied. The PCR fragments were purified by phenol extraction and concentrated with Amicon Ultra Centrifugation Filter Ultracell 30 K.

### In Vitro Transcription Assay

The multiple-round in vitro transcription assay was done according to Holátko et al. [8] with the following modifications: The RNAP core (100 nM) was mixed with the respective σ factor in a molar ratio of 1:15 ($\sigma^A$) or 1:30 ($\sigma^B$, $\sigma^D$, $\sigma^E$, $\sigma^H$, and $\sigma^M$). The holo-RNAP was assembled for 10 min at 37 °C. The transcription mixture was incubated for 15 min at 37 °C. The transcripts labeled with $\alpha^{32}$P-UTP were separated in 5.5 % polyacrylamide gel. In vitro transcription assays were done repeatedly for each promoter with different preparations of RNAP core and sigma factors with essentially the same results. Representative figures are shown.

### GFP Fluorescence Intensity Measurements

The promoter activities were determined using a transcriptional fusion of the respective promoter regions with the *gfp*uv reporter gene in the promoter-test vector pEPR1

**Table 1** Plasmids used

| Plasmid | Relevant characteristics | Source/reference |
|---|---|---|
| pEPR1 | *E. coli-C. glutamicum* promoter-test vector, $Km^R$, promoterless *gfp*uv as a reporter | [9] |
| pRLG770 | *E. coli* vector, *rrnB* terminator, $Ap^R$, used for in vitro transcription analysis, | [19] |
| pET-22b(+) | *E. coli* expression vector, T7 promoter-driven system, His-tag coding sequence, $Ap^R$ | Novagen |
| pEPRPpqo | 59-bp insert with P*pqo* in pEPR1 | This work |
| pEPRP1clgR | 82-bp insert with P1*clgR* in pEPR1 | This work |
| pEPRP2dnaK | 259-bp insert with P2*dnaK* in pEPR1 | This work |
| pEPRP2dnaJ2 | 136-bp insert with P2*dnaJ2* in pEPR1 | This work |
| pRLG770Pper | 96-bp insert with P*per* in pRLG770 | This work |
| pRLG770Ppqo | 59-bp insert with P*pqo* in pRLG770 | This work |
| pRLG770P1clgR | 59-bp insert with P1*clgR* in pRLG770 | This work |
| pRLG770P2dnaK | 109-bp insert with P2*dnaK* in pRLG770 | [8] |
| pRLG770P2dnaJ2 | 51-bp insert with P2*dnaJ2* in pRLG770 | This work |
| pET-22b(+)*sigA* | pET-22b(+) with *sigA* | [8] |
| pET-22b(+)*sigH* | pET-22b(+) with *sigH* | [8] |
| pET-22b(+)*sigB* | pET-22b(+) with *sigB* | This work |
| pET-22b(+)*sigD* | pET-22b(+) with *sigD* | This work |
| pET-22b(+)*sigE* | pET-22b(+) with *sigE* | This work |
| pET-22b(+)*sigM* | pET-22b(+) with *sigM* | This work |

[9]. The cells were washed with PBS buffer with phenylmethylsulfonyl fluoride (0.1 mM) and disrupted using FastPrep homogenizer (MP Biomedicals, USA). The fluorescence of the cell extract was measured with a Saphire2 spectrophotometer (Tecan, USA) (excitation wavelength, 397 nm; emission wavelength, 509 nm). Protein concentration was determined by Bradford assay. Fluorescence intensity was expressed in arbitrary units per mg of proteins (AU/mg protein). Extracts from cells harboring the empty promoter-test vector pEPR1 were used as control to determine background fluorescence.

## Results

### Effects of Deletions of the *sig* Genes on the Activity of *C. glutamicum* Promoters

In previous studies of *C. glutamicum* sigma factors, their role in cell physiology and their regulons, $\sigma^B$ [4, 10], and $\sigma^H$ [2, 5] were analyzed in greatest detail. Involvement of $\sigma^E$ in response to cell surface stresses (but not its regulon) was also reported [15]. To reveal the role of individual sigma factors in recognizing promoters of the selected *C. glutamicum* genes, we have therefore decided to study the effects of deletions of the *sigB*, *sigH*, and *sigE* gene, respectively, on the activity of these promoters. Out of the promoters previously found to be dependent on the primary-like alternative sigma factor $\sigma^B$, we have chosen the promoter of the *pqo* gene encoding pyruvate:quinone oxidoreductase [21] (P*pqo*), expressed mainly in the stationary growth phase [4]. To test the effects of deletions of stress-responding ECF sigma factors, we have chosen promoters of the *dnaK-grpE-dnaJ-hspR* operon encoding proteins involved in heat-shock response [1], the *dnaJ2* gene encoding a chaperone protein [2] and the *clgR* gene encoding a transcriptional regulator [6]. It was previously found that all these genes are expressed from two promoters: a housekeeping one (i.e., most probably $\sigma^A$-dependent) and a $\sigma^H$-dependent one. For transcriptional fusions with the promoterless *gfp*uv reporter gene of the promoter-test vector pEPR1, we used DNA fragments only containing active $\sigma^H$-dependent promoters P2*dnaK*, P2*dnaJ2*, and P1*clgR*, respectively. The activities of the promoters were assayed as green fluorescence intensity of the reporter.

As shown in Fig. 1, the activity of the P*pqo* promoter decreased significantly in the *C. glutamicum* Δ*sigB* strain compared to that in *C. glutamicum* WT. This result is in agreement with the previous finding that this promoter is $\sigma^B$ dependent [4]. The observed substantial residual activity of P*pqo* in *C. glutamicum* Δ*sigB* suggests that this promoter is also recognized by another sigma factor (most probably by $\sigma^A$).

Out of the promoters recognized by an ECF sigma factor, activities of $\sigma^H$-dependent promoters P1*clgR*,

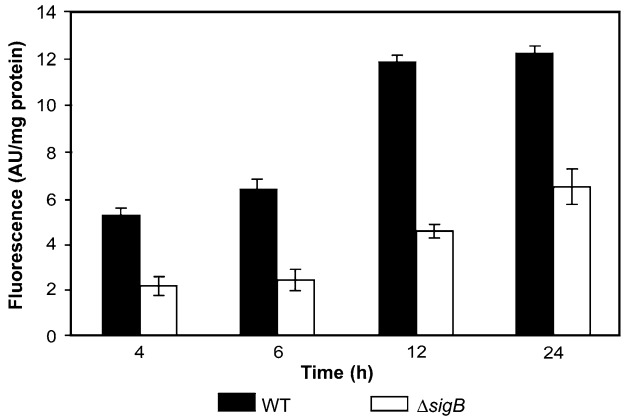


**Fig. 1** Activity of P*pqo* promoter in *C. glutamicum* WT and *C. glutamicum* Δ*sigB* strains. *AU* arbitrary units. The value of background fluorescence intensity (cells with promoterless vector pEPR1) was subtracted in all cases. Standard deviations of three measurements are depicted by *error bars*

P2*dnaK*, and P2*dnaJ2* were measured in the wild-type strain and in mutants having deletion of the *sigH* or *sigE* gene. The P1*clgR* promoter is a $\sigma^H$-dependent promoter of the *C. glutamicum clgR* gene encoding a transcriptional regulator [6]. (The housekeeping P2*clgR* promoter closely adjacent to P1*clgR* [6] was inactivated in the pEPRP1clgR plasmid by mutation of its −10 hexamer as shown in the Supplementary material). As shown in Fig. 2a, the activity of P1*clgR* decreased in *C. glutamicum* Δ*sigH* compared to that in *C. glutamicum* WT. This result confirmed the $\sigma^H$-dependency of this promoter [6]. However, its observed high residual activity in *C. glutamicum* Δ*sigH* suggested that P1*clgR* is recognized also by another (most probably an ECF) sigma factor.

As shown in Fig. 2b, activity of another known $\sigma^H$-dependent P2*dnaK* promoter was found to decrease in *C. glutamicum* Δ*sigH* only in early exponential growth phase. To test if the observed high residual activity of P2*dnaK* in *C. glutamicum* Δ*sigH* is due to the recognition of this promoter by another ECF sigma factor, the activity of this promoter was also measured in *C. glutamicum* Δ*sigE*. However, no decrease of P2*dnaK* activity was observed during the growth of this strain (P2*dnaK* activity in this strain even increased in the later growth stages). Deletion of the *sigH* gene was shown to have no effect on the activity of another known $\sigma^H$-dependent promoter P2*dnaJ2*, whereas deletion of *sigE* showed even positive effect on the activity of this promoter (Fig. 2c). The effect of deletions of both *sigE* and *sigH* in the same strain could not be tested since all attempts to construct *C. glutamicum* Δ*sigE* Δ*sigH* failed, probably due to a lethal effect of the double deletion.

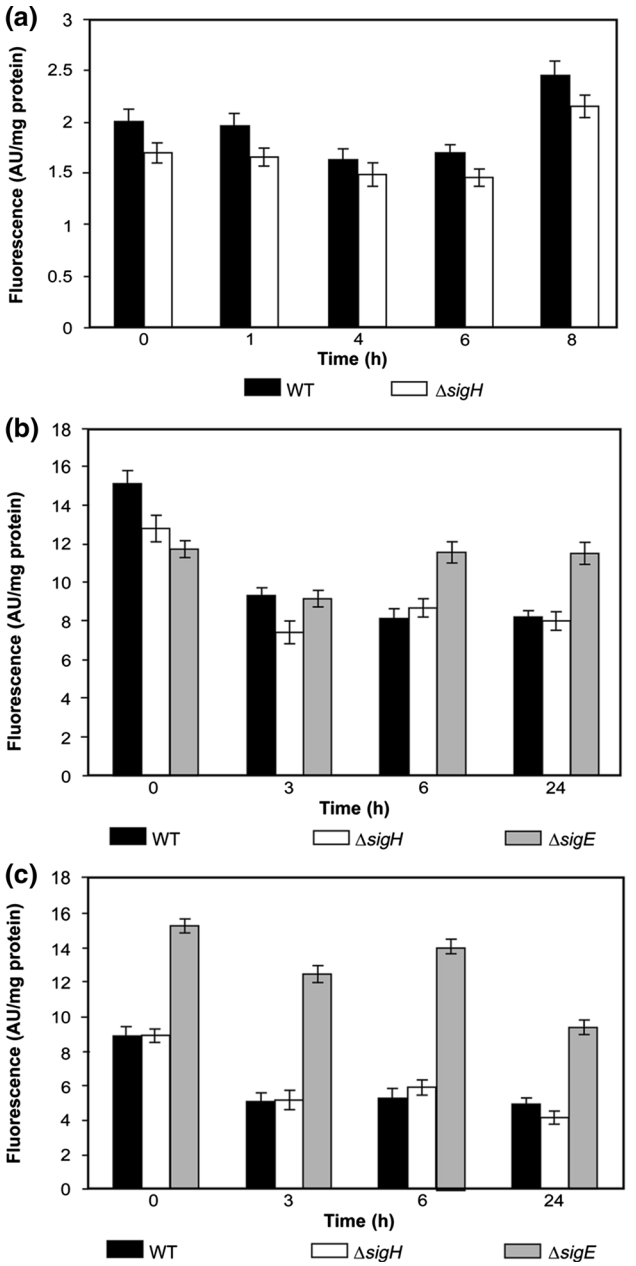


**Fig. 2** Activity of selected *C. glutamicum* promoters in *C. glutamicum* WT, *C. glutamicum* Δ*sigB* or Δ*sigE* mutants. **a** Promoter P1*clgR*; **b** Promoter P2*dnaK*; **c** Promoter P2*dnaJ2*. *AU* arbitrary units. The value of background fluorescence intensity was subtracted in all cases. Standard deviations of three measurements are depicted by *error bars*

The results of measuring activities of selected promoters in the *C. glutamicum* mutants with deletions in the genes encoding sigma factors suggested recognition of some promoters by more than one sigma factor. To prove directly the recognition of a specific promoter by different sigma factors (overlapping specificity of sigma factors), in vitro transcription assays were performed.

### Overlapping Specificity of *C. glutamicum* $\sigma^A$ and $\sigma^B$ in Promoter Recognition Proved by In Vitro Transcription

To test if the *C. glutamicum* primary sigma factor $\sigma^A$ and primary-like $\sigma^B$ overlap in recognizing promoter sequences of housekeeping genes, the multiple-round in vitro assay, using reconstituted *C. glutamicum* holoenzymes RNAP + $\sigma^A$ or RNAP + $\sigma^B$ and DNA template containing the P*per* promoter, was performed. The very strong P*per* promoter (originally present on *C. glutamicum* pGA1 plasmid [13]) contains −35 and −10 hexamers (TAGAAT and TATAAT, respectively [13]) which are highly similar to the *C. glutamicum* consensus sequences of housekeeping promoters [16]. The −10 hexamer within the promoter is even identical with the consensus sequences of *E. coli* and *B. subtilis* housekeeping promoters. As shown in Fig. 3a, use of both reconstituted RNAP + $\sigma^A$ and RNAP + $\sigma^B$ resulted in strong signals with the P*per* template. On the other hand, no signals were obtained when RNAP core only or RNAP holoenzymes with ECF sigma factors $\sigma^D$, $\sigma^E$, $\sigma^H$, and $\sigma^M$ were used for in vitro assay. The results of in vitro assay thus proved that a typical *C. glutamicum* housekeeping promoter is recognized by the primary sigma factor $\sigma^A$ but also by primary-like $\sigma^B$.

Promoter of the *C. glutamicum pqo* gene encoding pyruvate:quinone oxidoreductase [21] was previously proved to be controlled by $\sigma^B$ [4]. As shown in Fig. 1, we have confirmed $\sigma^B$-dependency of this promoter. However, strong residual activity of P*pqo* in *C. glutamicum* Δ*sigB* suggested that this promoter is recognized by still another sigma factor (most probably by $\sigma^A$) as well. Since deletion of the essential *C. glutamicum sigA* gene is lethal, the use of in vitro assay is the only way to prove directly the recognition of a promoter by $\sigma^A$. As shown in Fig. 3b, the use of both reconstituted RNAP holoenzymes, RNAP + $\sigma^A$ or RNAP + $\sigma^B$, resulted in clear signals with P*pqo* template, whereas no signals were obtained when RNAP holoenzymes with the ECF sigma factors $\sigma^D$, $\sigma^E$, $\sigma^H$, and $\sigma^M$ were used. We therefore conclude that the P*pqo* promoter (containing −35 and −10 hexamers GTGGCA and CACAAT, respectively) is active with the primary-like sigma factor $\sigma^B$ as well as with primary sigma factor $\sigma^A$.

### Overlapping Specificity of *C. glutamicum* $\sigma^H$ and $\sigma^E$ in Promoter Recognition Proved by In Vitro Transcription

To broaden the testing of overlapping specificity of different *C. glutamicum* sigma factors, the following known $\sigma^H$-dependent promoters were analyzed by in vitro transcription assay: P2*dnaK*, P2*dnaJ2*, and P1*clgR*. As shown in Fig. 4, using templates containing each of these $\sigma^H$-dependent promoters, clear signals were obtained not only with the RNAP + $\sigma^H$ holoenzyme, but also with RNAP + $\sigma^E$. On the other hand, no signal was obtained when RNAP holoenzymes containing primary sigma factor $\sigma^A$, primary-like $\sigma^B$, or other ECF sigma factors, $\sigma^D$ and $\sigma^M$, were used for in vitro transcription assay. These results indicate that the examined $\sigma^H$-dependent promoters were also recognized by $\sigma^E$. The two stress-responding sigma

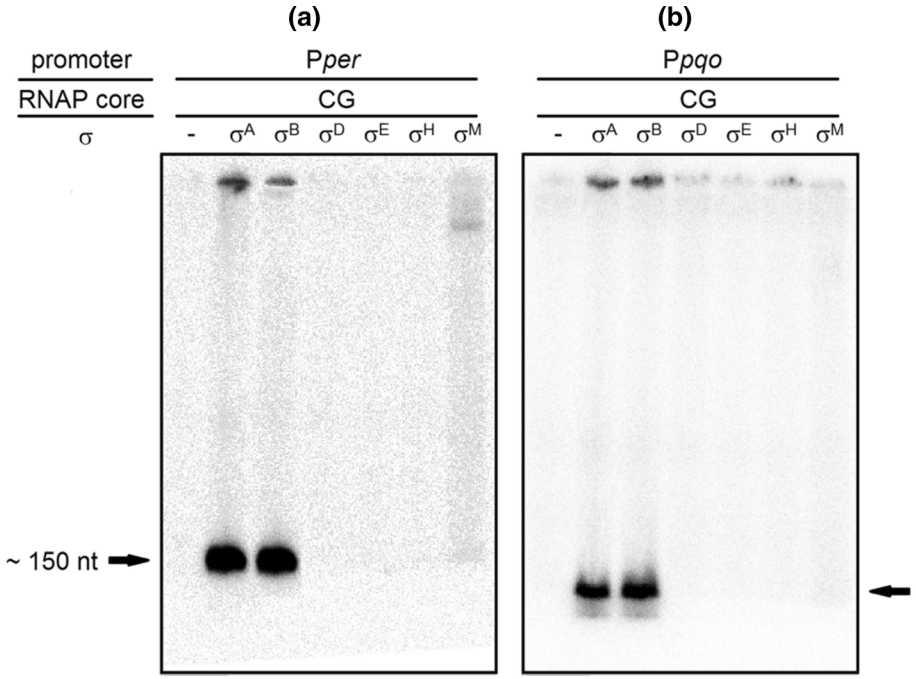


**Fig. 3** In vitro transcription from *C. glutamicum* promoters recognized by $\sigma^A$ and $\sigma^B$. Promoters used as templates and sigma factors in holo-RNAP are indicated at the *top*. The specific transcripts are indicated with *arrows*. (**a**) Promoter P*per*; (**b**) Promoter P*pqo*

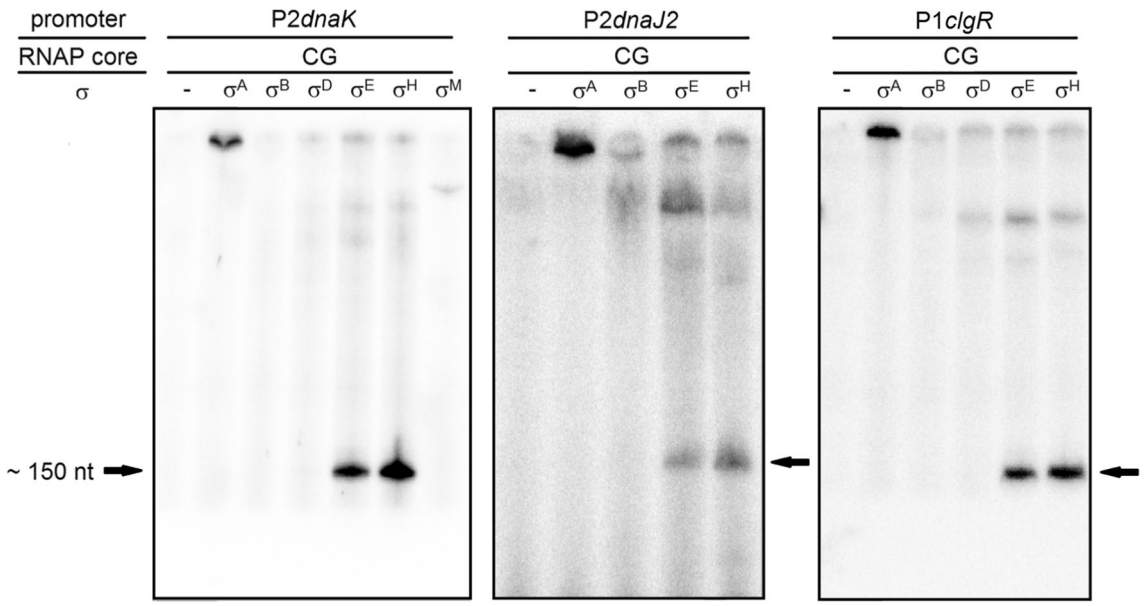


**Fig. 4** In vitro transcription from *C. glutamicum* promoters P2*dnaK*, P2*dnaJ2*, and P1*clgR* recognized by $\sigma^H$ and $\sigma^E$. Promoters used as templates and sigma factors in holo-RNAP are indicated at the *top*. The specific transcripts are indicated with *arrows*. The *upper bands* in some samples probably represent transcripts arising from the vector

factors, $\sigma^H$ and $\sigma^E$, thus overlap in their recognition specificities in these cases. The results suggest that still unknown consensus sequence of *C. glutamicum* promoters recognized by $\sigma^E$ is most probably highly similar to that recognized by $\sigma^H$ (GGAA–19-20 nt–GTT [2]).

## Discussion

In vitro transcription assay was previously found to be the only method suitable for direct defining the *C. glutamicum* promoters recognized by the primary sigma factor $\sigma^A$ since deletion of the *C. glutamicum sigA* gene is lethal [8]. Here we have proved that in vitro transcription assay is a convenient technique for defining the promoters recognized by two different sigma factors (dual promoters) in *C. glutamicum*. Using this method, we found promoters recognized by both primary $\sigma^A$ and primary-like $\sigma^B$ (P*per* and P*pqo*) as wells as promoters recognized by two different stress-responding sigma factors, $\sigma^H$ and $\sigma^E$ (P1*clgR*, P2*dnaK*, and P2*dnaJ2*). Although the use of in vitro transcription assay unequivocally proved recognition of particular promoters by specific sigma factors, dependence of some promoters (P2*dnaK* and P2*dnaJ2*) on specific sigma factors was not apparent when activities of these promoters were measured in *C. glutamicum* mutants with deletions of genes encoding these sigma factors.

The results of the in vitro and in vivo analyses of the promoters P*pqo* (recognized by $\sigma^A$ and $\sigma^B$) and P1*clgR* (recognized by $\sigma^H$ and $\sigma^E$) are generally in agreement. The observed decreased but substantial activities of these promoters in the analyzed deletion mutants (Δ*sigB* and Δ*sigH*, respectively) suggested that in addition to the respective sigma factors ($\sigma^B$ and $\sigma^H$) which control activity of these promoters, other sigma factors can substitute their function. Recognition of these promoters by sigma factors $\sigma^A$ and $\sigma^E$, respectively, was indeed proved by in vitro transcriptional assay. On the other hand, measuring activities of the promoters P2*dnaK* and P2*dnaJ2* (proved to be recognized by $\sigma^H$ and $\sigma^E$ by in vitro assay) in *C. glutamicum* Δ*sigH* and Δ*sigE* did not show clear dependency of these promoters on the respective sigma factors. High activity of the promoters P2*dnaK* and P2*dnaJ2* in the deletion strains showed that an alternative sigma factor in each case was able to substitute the missing sigma factor efficiently. Higher activities of the P2*dnaK* and P2*dnaJ2* promoters in *C. glutamicum* Δ*sigE* suggest that the regulation of activity of sigma factors through their competition for RNAP core or direct competition for binding the promoter sequences is an important regulatory mechanism balancing their functions. SigH is a candidate for global regulator due to its prominent role in the hierarchy of cross-regulations among sigma factors [22]. Its affinity to RNAP core and/or efficiency of RNAP + $\sigma^H$ in transcription from the tested promoters is therefore probably higher than that of $\sigma^E$. Missing competition in the Δ*sigE* strain may result in increased activity of these dual promoters with $\sigma^H$. We have observed this phenomenon also in several other promoters (data not shown).

Recognition of the typical housekeeping promoter P*per* by the alternative sigma factor $\sigma^B$ is in agreement with our observation that P*per* is still highly active in early stationary phase, when the level of $\sigma^A$ decreases sharply and the level of $\sigma^B$ increases [10]. The consensus sequences of $\sigma^A$- and $\sigma^B$-dependent promoters are virtually indistinguishable and $\sigma^B$ is considered a second sigma factor for housekeeping genes [4] or a backup sigma for transition and stationary growth phases [10]. We therefore infer that P*per* is active with both $\sigma^A$ and $\sigma^B$ in vivo. We assume that such overlapping functional specificity of $\sigma^A$ and $\sigma^B$ could be found in many housekeeping promoters.

None of the promoters tested in this study provided signals in in vitro transcription assays when RNAP + $\sigma^D$ or $\sigma^M$ were used. Preliminary in vitro assays in a different project with RNAP + $\sigma^D$ and a few tested DNA templates resulted in clear positive signals (data not shown). These tests confirmed that the purified $\sigma^D$ protein is active. RNAP + $\sigma^M$ was tested with a few promoters which were supposed to be $\sigma^M$-dependent [12], but no positive result was obtained. We found that one of these promoters, P*trxB*, is $\sigma^H$-dependent, which is in agreement with the results of Ehira et al. [5]. The lack of positive signal with RNAP + $\sigma^M$ on P*trxB* template was supported by the results of in vivo analysis using deletion mutants (data not shown). The role of $\sigma^M$ in *C. glutamicum* clearly needs further investigation.

The presented results suggest that the combination of in vivo and in vitro techniques is useful for obtaining reliable data on transcriptional regulation by sigma factors of RNA polymerase.

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**Compliance with ethical standards**

**Conflict of Interests** The authors declare that they have no conflict of interest.

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