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The small 6C RNA of *Corynebacterium glutamicum* is involved in the SOS response

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ABSTRACT

The 6C RNA family is a class of small RNAs highly conserved in *Actinobacteria*, including the genera *Mycobacterium, Streptomyces* and *Corynebacterium* whose physiological function has not yet been elucidated. We found that strong transcription of the cgb_03605 gene, which encodes 6C RNA in *C. glutamicum*, was driven by the SigA- and SigB-dependent promoter P_{cgb_03605}. 6C RNA was detected at high level during exponential growth phase (180 to 240 molcules per cell) which even increased at the entry of the stationary phase. 6C RNA level did not decrease within 240 min after transcription had been stopped with rifampicin, which suggests high 6C RNA stability. The expression of cgb_03605 further increased approximately twofold in the presence of DNA-damaging mitomycin C (MMC) and nearly threefold in the absence of LexA. Deletion of the 6C RNA gene cgb_03605 resulted in a higher sensitivity of *C. glutamicum* toward MMC and UV radiation. These results indicate that 6C RNA is involved in the DNA damage response. Both 6C RNA level-dependent pausing of cell growth and branched cell morphology in response to MMC suggest that 6C RNA may also be involved in a control of cell division.

Introduction

Small RNAs (sRNAs) have been detected in all 3 domains of life in unexpectedly large numbers. Such transcripts typically do not encode proteins and are therefore also referred to as non-coding RNAs. sRNAs serve various regulatory functions mediated by diverse mechanisms which also include the action of *cis-* and *trans*-acting sRNAs antisense to target RNAs and sRNAs binding to proteins (see, for example, refs.[1-3] and others). The hitherto identified RNA families are collected in the Rfam online database which enables searching, browsing, and downloading of information as well as annotating RNA sequences using covariance models.[4] More than 2,200 families with over 6 million sequences have already been collected in the Rfam database.[4] Systematic bioinformatics and experimental approaches to predict and detect sRNAs are much faster than their functional characterization.

Among the collated families in the Rfam database, the 6C RNA family is a widespread class for which a physiological function has not yet been found. The sequences of 6C RNAs are widely conserved in *Actinobacteria* including the genera *Mycobacterium, Corynebacterium, Frankia, Nocardia* and *Streptomyces*, respectively.[5] This family, originally discovered by bioinformatics in *S. coelicolor*, has been termed 6C due to at least 6 conserved cytosine residues found in each of 2 loop regions of the conserved ~80 nt stem-loops structure.[5] Transcription of the 6C RNA gene in *S. coelicolor* increases during sporulation. Three different transcripts with different apparent start and stop sites were detected.[6,7] Increased levels of the 2

shorter transcripts were found in a later stage of development associated with spore formation that may reflect a function of 6C RNAs in spore development.[7] Since not all *Actinobacteria* in which the 6C RNA motif was found form spores (e.g. *C. glutamicum*), 6C RNAs were also speculated to be involved in a general dormancy or metabolic slow-down response,[7] for example when cells cease to grow due to environmental conditions. In *M. tuberculosis* the 6C RNA level increased 1.8-fold in response to H_2O_2, which mimics the oxidative stress encountered inside the host macrophage.[8] When overexpressed, a high level of 6C RNA proved to be lethal in *M. tuberculosis* whereas it resulted in very slow growth as well as elongated cells with altered morphology in *M. smegmatis*.[8]

Recently, the presence of 6C RNA was also demonstrated in *C. glutamicum*, a workhorse in industrial biotechnology and model microorganism for medically important related species including *M. tuberculosis, C. diphtheriae* and *C. ulcerans*. Transcriptome profiling by sequencing RNA from *C. glutamicum* cells in the exponential growth phase revealed the presence of a monocistronic 109 nt 6C RNA transcript, which is encoded by gene cgb_03605.[9] The 6C RNA exhibited no changes in abundance under some physical or chemical stresses compared to non-stressed conditions during exponential growth.[10] In our study with *C. glutamicum*, we searched for the physiological function(s) of the 6C RNA by analyzing 6C RNA levels under a variety of conditions and regulatory elements involved in 6C RNA gene expression. We discovered that the function of 6C RNA in *C. glutamicum* is connected to the LexA-dependent SOS response and affects the transient stop of cell division.

Results

6C RNA is present throughout growth phases, highly abundant and very stable

Recently, a comprehensive transcriptome analysis of *C. glutamicum* showed that the genomic region matching the 6C RNA motif (locus designated cgb_03605) is expressed as a monocistronic transcript of 109 nt which in the exponential growth phase exhibited no significant changes in abundance under selected stress conditions compared to non-stressed conditions.[9,10] Here we analyzed 6C RNA transcript abundance throughout growth in a batch cultivation including the stationary phase. We found by Northern blot analysis that 6C RNA is always present in *C. glutamicum* grown in the CGXII medium with glucose and exhibits transiently approximately twofold increased levels when cells enter the stationary phase (Fig. 1). To assess the amount of 6C RNA in *C. glutamicum* in terms of a number of molecules per cell, we conducted quantitative Northern blot analysis with a T7 RNAP-generated 6C RNA *in vitro* transcript as calibration standard (0.8 ng to 3.2 ng). Based on 2 biological replicates we found a range of 180 to 240 6C RNA molecules per cell in the mid-exponential growth phase ($OD_{600} \sim 5.5$). This reflects that in *C. glutamicum* 6C RNA is highly abundant compared to the mean mRNA abundance[11] and exhibits transcript levels similar to, for example, the dual-function sRNA SR1 from *Bacillus subtilis*.[12]

The transient increase in 6C RNA abundance when cells enter the stationary phase and cease to divide may be caused by transcriptional regulation, by sigma factor dependencies and by transcript accumulation due to transcript stability. To assess the 6C RNA transcript stability fr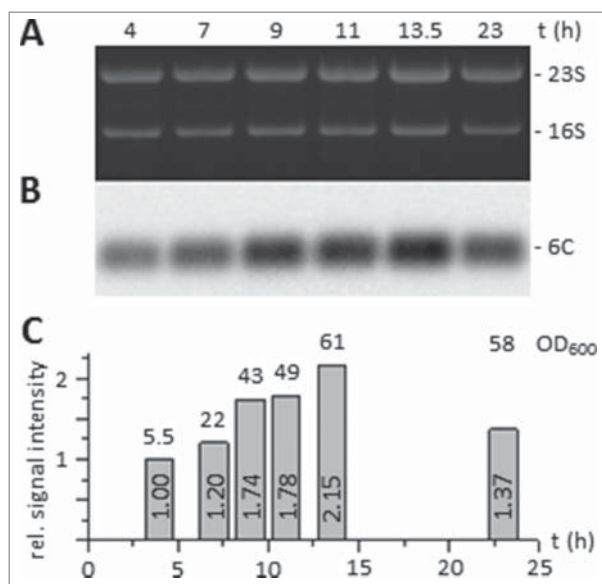om *C. glutamicum* during growth, we analyzed the half-life by blocking the RNA synthesis with rifampicin, which was added after 6 hours of growth in the mid-exponential phase at an OD_{600} of 5 to 6. As a result, growth stopped within 2 hours at an OD_{600} of 10 to 12, whereas control cultures reached a final OD_{600} of about 50. In the Northern blot analysis using total RNA isolated from cells before the addition of rifampicin and up to 4 hours after addition, we did not observe a significant decrease in the signal intensity of the 6C RNA bands (Fig. 2). That indicates that the 6C RNA transcript exhibits a half-life far longer than 120 to 240 min under the conditions tested. As a control, we used the *gltA* mRNA encoding citrate synthase, an enzyme of the central metabolism. As expected, the level of *gltA* mRNA strongly decreased within only 5 min after the addition of rifampicin, and became almost undetectable after 15 min (Fig. 2). These results demonstrate that 6C RNA is very stable in *C. glutamicum*.

Promoter activity of cgb_03605 is SigA- and SigB-dependent

We then analyzed the sigma factor dependence of the 6C RNA promoter (P_{cgb_03605}) using a transcriptional fusion of P_{cgb_03605} with the *gfp*uv reporter gene in the promoter test vector pEPR1 and overexpression of various genes encoding sigma factors (*sigA, sigB, sigE, sigH* and *sigM*) cloned in the expression vector pEC-XT99A. The sequences CATAAT at the -10 and TTGTCT at the -35 region relative to the transcriptional start site (Fig. 3) detected by RNA sequencing[9] suggest that cgb_03605 is transcribed from a housekeeping promoter, i.e. a promoter recognized by RNA polymerase (RNAP) with SigA.[13] The promoter consensus sequence for SigB-dependent promoters, which are mainly active during the transition from the exponential to the stationary growth phase, is indistinguishable from that of SigA-dependent promoters.[13-15] In the reporter assays only the overexpression of *sigA* resulted in an increased activity of P_{cgb_03605} in the exponential phase (Fig. 4A). The results were similar for both defined CGXII medium with glucose and rich medium ($2 \times TY$). To test further a possible role of SigB in transcription of the cgb_03605 gene in the stationary phase, we analyzed P_{cgb_03605} activity in a *sigB* deletion strain ($\Delta sigB$) and WT carrying pEPR1-P_{cgb_03605}. According to the fluorescence provided by the *gfp*uv reporter, P_{cgb_03605} activity was high during the exponential phase and increased in the stationary phase in the WT (Fig. 4B). The activity of the promoter was similar during the exponential phase in $\Delta sigB$, however, much lower in comparison with the WT in the stationary phase (24 h and 27 h, Fig. 4B). This result suggested that SigB is involved in transcription of cgb_03605 in later stages of the batch culture. Thus, P_{cgb_03605} seems to be mainly SigA-dependent in the exponential phase and partially SigB-dependent during transition to the stationary phase according to the results of the *in vivo* analysis. To confirm these results, we further tested the activity of P_{cgb_03605} using the *in vitro* transcription system with the purified RNAP core and a sigma factor from *C. glutamicum*.[16] A PCR fragment carrying P_{cgb_03605} was used as a template and tested with SigA, SigB, SigE, SigH or SigM applied to *in vitro* reactions. Only SigA and SigB resulted in strong signals, whereas SigE, SigH and SigM provided no signals at all (Fig. 4C). These results suggest that in addition to RNAP+SigA also RNAP+SigB recognizes the promoter of

Figure 1. Presence of 6C RNA in *C. glutamicum* WT during batch cultivation in CGXII glucose minimal medium. (A) 23S and 16S rRNA bands to verify equal sample load. A representative formaldehyde agarose gel (1.5 %) loaded with 1.2 μg of total RNA from cells harvested at different times is shown. (B) Representative Northern blot analysis showing bands of 6C RNA. (C) 6C RNA levels (values in bars) quantified from Northern blot analysis (B) using AIDA. The value at the early exponential phase (4 h) was set to 1. Numbers above bars represent OD_{600} of the culture at the time of cell harvest. Data represent mean values from 2 biological replicates.
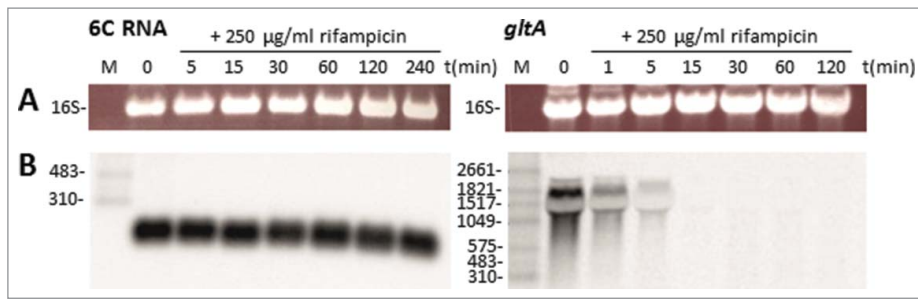
Figure 2. Northern blot analysis for determining the half-life of 6C RNA (left) and for comparison *gltA* mRNA (right). *C. glutamicum* was cultivated in CGXII medium with glucose. Time refers to minutes after the addition of rifampicin (0 min). One.5 μg (left) and 10 μg (right) of total RNA were loaded onto the gels to detect 6C RNA and *gltA* mRNA, respectively. (A) 16S rRNA bands on representative Nylon membranes after blotting of separated RNA from agarose gels as a control of the RNA transfer. (B) Representative Northern blots showing the detected 6C RNA and *gltA* transcripts, respectively. The *gltA* gene is transcribed from 2 promoters, resulting in 2 *gltA* transcripts of about 1520 nt and 1756 nt, respectively.[39] Transcript bands were visualized with specific DIG-labeled antisense ssDNA probes. M: DIG-labeled RNA nt size marker.
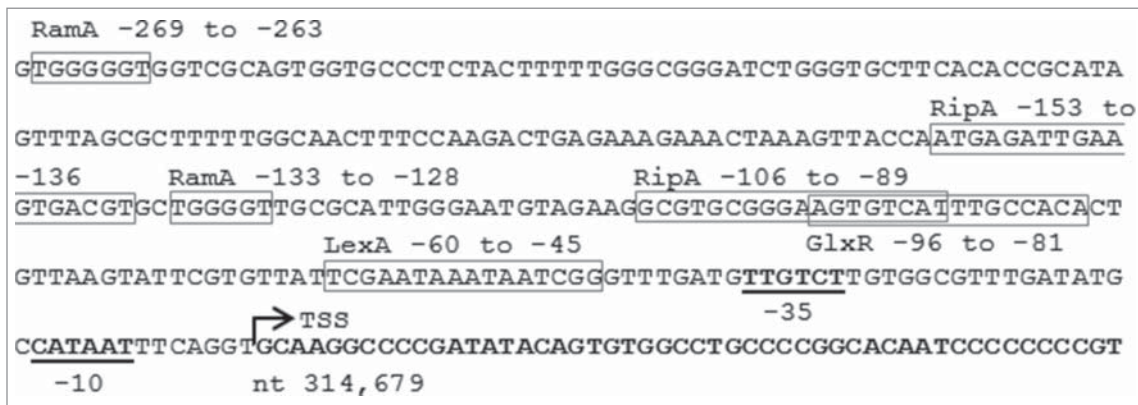

Figure 3. The promoter region and 5′-end of cgb_03605 from *C. glutamicum* WT which is transcribed into 6C RNA (bold). Transcriptional start (TSS, nt 314,679 of the NCBI *C. glutamicum* sequence, accession BX927147) is indicated with an arrow.[10] The proposed −35 and −10 regions are in bold and underlined. The binding site of the transcriptional regulator GlxR is boxed.[17] Putative binding sites for RamA (consensus motive $HG_{(4-6)}Y$ or $AC_{(4-5)}D$[52]), RipA (consensus motive $RRGCGN_4RYGAC$[53]) and LexA (consensus motive TcGAAmAnnTGTtCGA[20]) are also boxed.
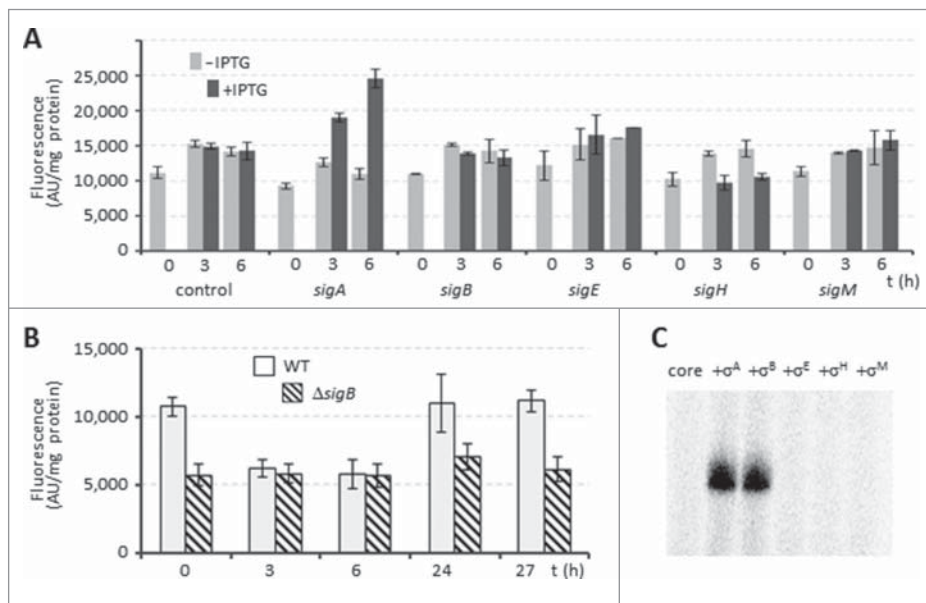

Figure 4. Activity of P_{cgb_03605} *in vivo* (A, B) and *in vitro* (C). Cells were grown in CGXII medium with glucose. (A) Effect of sigma factor overexpression on the P_{cgb_03605} promoter activity. Specific fluorescence levels provided by the *gfp*uv reporter under control of P_{cgb_03605} in *C. glutamicum*/pEC-XT99A/pEPR1-P_{cgb_03605} (control), as well as *C. glutamicum* strains/pEC-XT99A-*sigA/B/E/H/M*/pEPR1-P_{cgb_03605} to overexpress a sigma factor gene (*sigA/B/E/H/M*). (B) Effect of the absence of SigB on the P_{cgb_03605} promoter activity. Specific fluorescence levels provided by the *gfp*uv reporter under control of P_{cgb_03605} in *C. glutamicum* WT and in Δ*sigB* carrying the plasmid pEPR1-P_{cgb_03605}. (C) Detected transcripts after *in vitro* transcription from P_{cgb_03605} with reconstituted *C. glutamicum* RNAP core and holo-RNAPs containing a sigma factor as indicated (autoradiogram of a representative SDS-PAGE gel).

cgb_03605 and may drive transcription under specific conditions *in vivo*.

Transcriptional regulators which bind to the 6C RNA promoter region

The global transcriptional regulator GlxR was reported to bind to the intergenic region between the protein-coding gene cg0360 and cgb_03605.[17] Our inspection of the P_{cgb_03605} promoter sequence furthermore revealed the presence of putative binding sites for the transcriptional regulators RamA, RipA, and LexA (Fig. 3). To determine experimentally which regulators bind to P_{cgb_03605} and thus possibly control cgb_03605 transcription, we conducted DNA affinity chromatography experiments with crude protein extracts from *C. glutamicum* and DNA fragments containing P_{cgb_03605} or P_{cg0896}, the latter serving as a control of specificity. Affinity-enriched protein samples were subjected to SDS-PAGE analysis. After Coomassie-staining several protein bands became visible and were identified by peptide mass fingerprinting using MALDI-TOF-MS analysis (Fig. 5). The transcriptional regulators GlxR, OxyR, RamB and GntR1 could be detected in only one replicate with P_{cgb_03605}. The transcriptional regulators LexA, CitB and AtlR, and the putative DNA-binding excisionase protein Cg0492 were specifically and reproducibly enriched and detected with the P_{cgb_03605} DNA fragment in 2 independent DNA affinity chromatography experiments. RamA was enriched with both fragments, P_{cgb_03605} and P_{cg0896}. We verified the binding of RamA and LexA to P_{cgb_03605} by EMSAs.

The *C. glutamicum* RamA protein was overexpressed as a hexahistidyl-tagged fusion protein in *E. coli* BL21(DE3) using the plasmid pET28-RamAx6His and purified.[18] The direct interaction of RamA-His$_6$ with fragment FP_{cgb_03605} could be confirmed by the retardation observed at an 8-fold molar excess of RamA-His$_6$, yet only one retarded band was observed under the conditions tested (Fig. 6A, B). The functionality of purified RamA-His$_6$ was confirmed with a promoter fragment of the *aceA-aceB* intergenic region (P_{aceAB}) as a positive control[18] and the promoter region of *pgi* (P_{pgi}) as a negative control.[19] While P_{pgi} was not shifted, 2 distinct retardations of the P_{aceAB} fragment were observed with an increasing molar excess of RamA-His$_6$ (Fig. 6B) due to the 2 RamA binding sites present in the P_{aceAB} fragment. To further support that RamA is involved in the regulation of the 6C RNA gene, we compared P_{cgb_03605} activities in a *ramA* deletion strain ($\Delta ramA$) and the WT using the *gfp*uv reporter for P_{cgb_03605}. The activity of P_{cgb_03605} was similar in both strains during the exponential phase, but in the stationary phase (23 h, 26 h) it was up to 1.9-fold higher in the $\Delta ramA$ strain compared to the WT (Fig. 6C). The activity of the P_{dapA} promoter used as a control did not differ significantly between the 2 strains throughout growth.

The *C. glutamicum* LexA protein was overexpressed as a hexahistidyl-tagged fusion protein in *E. coli* BL21(DE3) using the plasmid pET15b-cg2114 and purified.[20] To demonstrate the relevance of the predicted LexA operator sequence in the 6C RNA promoter region DNA band shift assays were carried out with the tagged LexA protein and the PCR fragments F1 and F2 or the double-stranded oligonucleotides OP1, OP2 and OP3 (Fig. 6A). Fragment F1 without the predicted LexA operator was
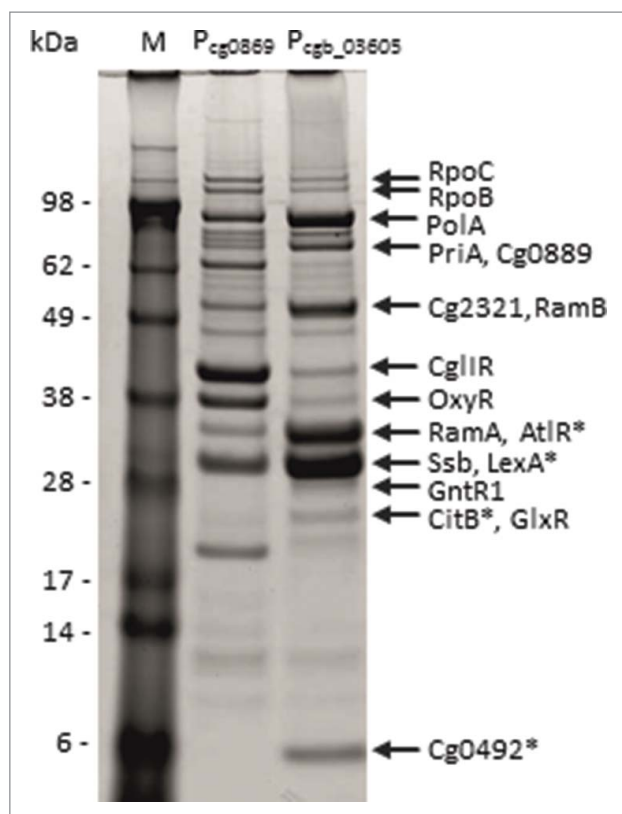

Figure 5. Enrichment and identification of proteins, which bind to the cgb_03605 promoter region, by DNA affinity chromatography and mass spectrometry. The protein extract was obtained from *C. glutamicum* WT cells cultivated in CGXII glucose minimal medium until the mid-exponential phase (OD$_{600}$ of 5-6). Aliquots of the crude protein extract were incubated with magnetic beads loaded with DNA fragments carrying the cgb_03605 promoter (P_{cgb_03605}) or as a control the cg0869 promoter (P_{cg0869}). Proteins were eluted in high-salt buffer and separated by SDS-PAGE (10 %). After Coomassie-staining, protein bands were excised, digested in gel with trypsin, and identified by peptide mass fingerprinting using MALDI-TOF-MS. Bands labeled with an asterisk indicate proteins that were reproducibly detected in 2 independent experiments. M: Pre-stained protein size marker SeeBlue Plus2. RpoC (Cg0577, 147.3 kDa), RNA polymerase β' subunit; RpoB (Cg0576, 128.8 kDa), RNA polymerase β subunit; PolA (Cg1525, 96.8 kDa), DNA polymerase I; PriA (Cg1805, 74.6 kDa), primosome assembly protein PriA or N' replication factor Y; Cg0889 (75.1 kDa), putative DNA helicase RecQ; Cg2321 (51.1 kDa), DNA polymerase III ε subunit; RamB (Cg0444, 53.9 kDa), transcriptional regulator involved in acetate metabolism; CglIR (Cg1997, 39.8 kDa), type II restriction endonuclease; OxyR (Cg2109, 35.0 kDa), hydrogen peroxide-sensing transcriptional regulator; RamA (Cg2831, 30.8 kDa), transcriptional regulator of acetate metabolism; AtlR (Cg0146, 30.9 kDa), transcriptional regulator of arabitol metabolism; Ssb (Cg3307, 23.3 kDa), single-strand DNA-binding protein; LexA (Cg2114, 27.3 kDa), transcriptional repressor of SOS-response; GntR1 (Cg2783, 27.2 kDa), transcriptional regulator of gluconate catabolism and pentose phosphate pathway; CitB (Cg0090, 23.4 kDa), 2 component response regulator of citrate homeostasis; GlxR (Cg0350, 25.0 kDa), cAMP-dependent global transcriptional regulator; Cg0492 (7.1 kDa), extremely conserved putative DNA-binding excisionase protein.

not shifted, whereas fragment F2 containing the predicted LexA operator shifted at a 15-fold molar excess of LexA (Fig. 6D). The 32-mer ds-oligonucleotide OP1 containing the predicted LexA operator plus 8 bp upstream and downstream was completely shifted at a 10-fold molar excess of LexA. In contrast, ds-oligonucleotides OP2 and OP3, immediately located upstream or downstream of the predicted LexA binding site with only 1 or 2 bp overlap were not retarded even at a 20-fold molar excess of LexA. These *in vitro* results support the relevance of the sequence TCGAATAAATAATCGG as a LexA operator within the
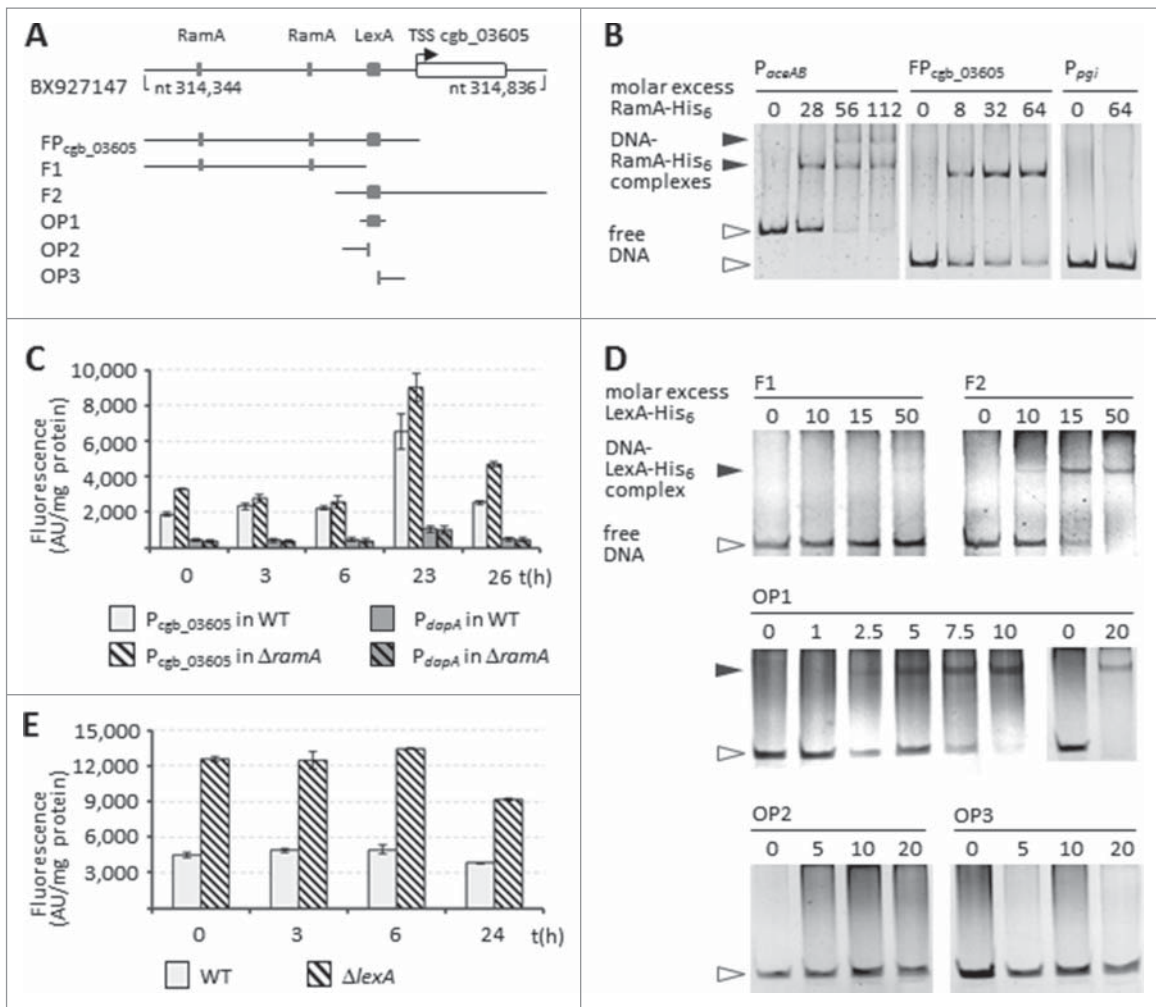
Figure 6. Electrophoretic mobility shift assays (EMSA) with recombinant RamA and LexA proteins and various DNA fragments of the P_{cgb_03605} region. (A) Schematic representation of *C. glutamicum* WT genomic DNA (BX927147) from nt 314,344 to 314,836. Regions of the predicted RamA and LexA operator sequences are indicated as gray boxes (for the sequences, see Fig. 3). TSS: Transcriptional start site at nt 314,679 of the cgb_03605 gene (framed) which is transcribed into 6C RNA.[10] Regions covered by the DNA fragments FP_{cgb_03605} (337 bp), F1 (271 bp), F2 (258 bp) as well as OP1 (32 bp), OP2 (30 bp), and OP3 (31 bp) indicate the absence or presence of predicted operator sequences within the tested DNA fragments. (B) EMSAs with purified RamA-His$_6$ protein. The DNA fragments P_{aceAB} (586 bp, positive control), FP_{cgb_03605} or P_{pgi} (337 bp, negative control) were incubated without protein or with an increasing molar excess of purified RamA-His$_6$. The samples were subjected to electrophoresis on 10 % native polyacrylamide gels and stained with SYBR Green I. (C) Effect of the absence of RamA on the P_{cgb_03605} promoter activity. Specific fluorescence levels provided by the *gfp*uv reporter under control of P_{cgb_03605} in *C. glutamicum* WT and in $\Delta ramA$ carrying the plasmid pEPR1-P_{cgb_03605}. (D) EMSAs with purified LexA-His$_6$ protein. The DNA fragments F1 and F2 (PCR products) as well as the double-stranded oligonucleotides OP1, OP2, and OP3 were incubated with an increasing molar excess of purified LexA-His$_6$. The samples were subjected to electrophoresis on 2 % agarose gels and stained with SYBR Green I. (E) Effect of the absence of LexA on the P_{cgb_03605} promoter activity. Specific fluorescence levels provided by the *gfp*uv reporter under control of P_{cgb_03605} in *C. glutamicum* WT and in $\Delta lexA$ carrying the plasmid pEPR1-P_{cgb_03605}.

P_{cgb_03605} region upstream and close to the −35 region (Fig. 3). To further support that LexA is involved in the regulation of the 6C RNA gene, we also compared P_{cgb_03605} activities in a *lexA* deletion strain ($\Delta lexA$) and the WT using the *gfp*uv reporter. The activity of P_{cgb_03605} in the $\Delta lexA$ strain was up to 2.8-fold higher than in the WT during growth and in the stationary phase (Fig. 6E). These results indicate that transcription from P_{cgb_03605} is repressed by LexA.

Taken together, the results suggest that in *C. glutamicum*, 6C RNA expression may be regulated or fine-tuned by several transcriptional regulators including RamA and LexA with the latter 2 repressing the P_{cgb_03605} activity. RamA is a transcriptional regulator of acetate metabolism and also acts on a global scale as repressor or activator of genes enconding enzymes of the central metabolism.[19] LexA is the key transcriptional regulator that represses SOS response genes which are induced under certain DNA-damaging conditions.[20]

6C RNA is involved in the SOS response and affects cell morphology

To test whether 6C RNA is generally required for growth or for certain stress responses, we deleted cgb_03605 and its promoter region in *C. glutamicum* WT, which resulted in the Δcgb_03605 strain. If LexA indeed regulates P_{cgb_03605} *in vivo*, 6C RNA is expected to be involved in the SOS response. We therefore screened for a phenotype of Δcgb_03605 under various conditions, including DNA-damage stress caused by mitomycin C (MMC) and UV radiation. Besides this, growth in complex media (LB, BHI) and in defined CGXII medium with alternative substrates (acetate, glucose, sucrose, fructose, ribose, gluconate, L-lactate, pyruvate, propionate, or citrate) was tested. In modified CGXII medium with glucose as substrate we also tested the effect of acidic condition (pH 5.5), shortage of metal ions (1/10 Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, and Ni^{2+}

compared to standard CGXII medium), limitation of iron (1/36) or biotin (1/100), and stress by an excess of metal ions (3-fold Fe^{2+} and Cu^{2+}), respectively. Of all the conditions tested, only the presence of MMC and exposure to UV radiation affected the growth of Δcgb_03605. Thus, 6C RNA is not generally required for growth of *C. glutamicum*, yet appears to be indeed involved in the SOS response. In the presence of MMC (0.75 μM), 6C RNA appears to be advantageous to more rapidly overcome a transient growth stop at an OD_{600} of 9 to 10 to reach the final OD_{600} (Fig. 7A). When 6C RNA is absent, growth paused much longer at an OD_{600} of 9 to 10. When exposed to UV radiation, the Δcgb_03605 mutant forms less viable cells than the wild type, as revealed by a dilution series on agar plates (Fig. 7B). Both stress experiments suggest that 6C RNA is advantageous when dealing with SOS stress. For complementation studies we constructed the plasmid pJC1-cgb_03605 carrying the 6C RNA gene under control of its native promoter region. Expression from this plasmid results in a 5-fold increase in 6C RNA level in Δcgb_03605/pJC1-cgb_03605 compared to the WT (Fig. 7C). On the one hand, plasmid-based expression of 6C RNA basically restored growth in the presence of MMC, since *C. glutamicum* Δcgb_03605/pJC1-cgb_03605 and the wild-type reference reached similar OD_{600} values after 23 h of cultivation, while the control Δcgb_03605/pJC1 did not and still remained in the pausing phase (Fig. 7D). On the other hand, under SOS stress the increased expression of 6C RNA elicits another growth phenotype showing an earlier pause in growth at a much lower OD_{600} of 2-3 (Fig. 7D). The same growth results were obtained with the wild-type strain carrying pJC1-cgb_03605 or pJC1, thus the different pause in growth is specific to 6C RNA expression levels and not related to unknown secondary mutations in the cgb_03605 deletion strain. When exposed to UV radiation, the expression of 6C RNA from pJC1-cgb_03605 specifically restored the number of viable cells of the Δcgb_03605 background to a level similar to the wild-type control (Fig. 7E).

To assess the influence of LexA, RamA and SigB on 6C RNA expression under SOS-inducing mitomycin C stress (0.75 μM) we again used the *gfp*uv reporter to measure P_{cgb_03605} activity. In the presence of MMC the P_{cgb_03605} activity in the WT, in $\Delta ramA$, and in $\Delta sigB$ was similarly increased up to 2.2-fold in the exponential phase compared to the absence of MMC, while increase of P_{cgb_03605} activity in $\Delta lexA$ was much lower (Table 1). This suggests that the increase of P_{cgb_03605} activity in the presence of MMC in early stages of the batch culture is mainly LexA-dependent. Under SOS-inducing condition with MMC, the P_{cgb_03605} activity in $\Delta lexA$ was always higher than in the WT (Table 1) suggesting that transcription from P_{cgb_03605} in the WT is not fully derepressed by LexA in the presence of MMC or that also other regulators interfere. In later stages of the batch culture with MMC, the P_{cgb_03605} activity was higher in $\Delta ramA$ and lower in $\Delta sigB$ than in the WT
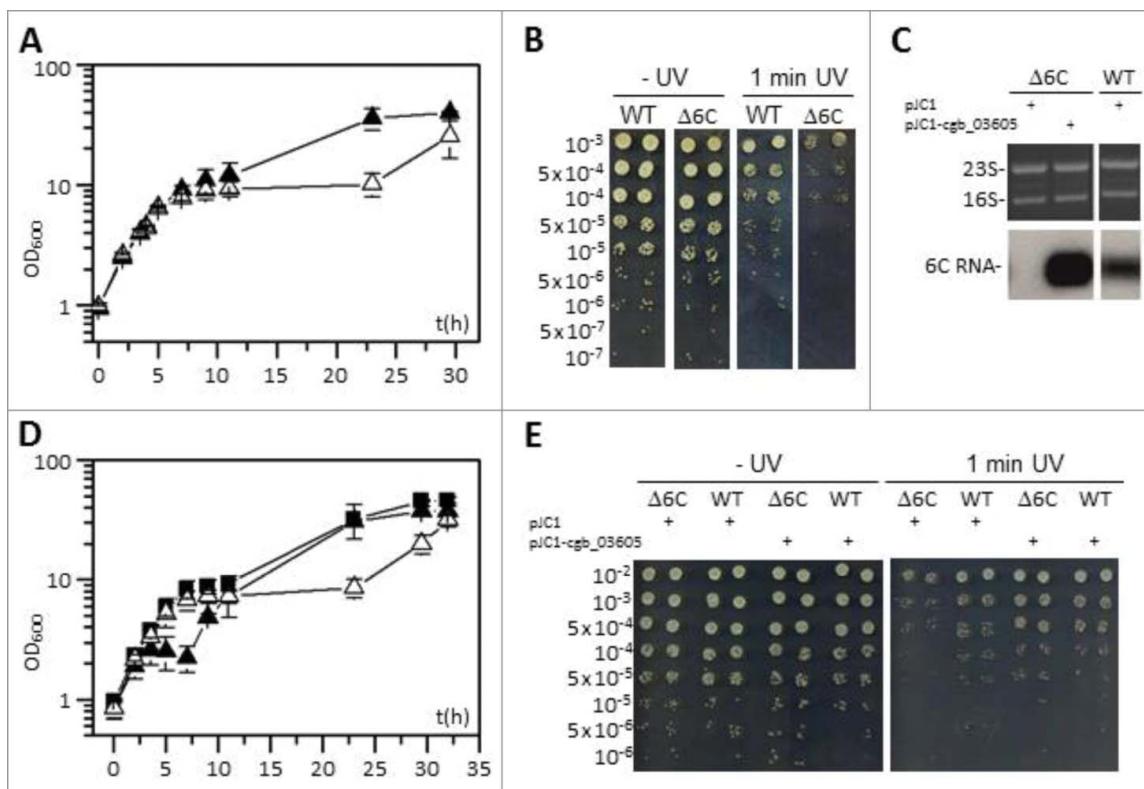

Figure 7. Growth of *C. glutamicum* strains under non-stress and stress conditions and P_{cgb_03605} promoter activities. (A) Growth of WT (▲) and Δcgb_03605 (△) in CGXII glucose minimal medium with glucose in the presence of 0.75 μM mitomycin C. (B) Growth of dilution series of WT and Δcgb_03605 (Δ6C) on agar plates (CGXII glucose minimal medium) after 24 h of incubation without or with exposure to UV radiation (1 min) prior to incubation. (C) Northern blot analysis of 6C RNA level in *C. glutamicum* Δcgb_03605/pJC1-cgb_03605, *C. glutamicum* Δcgb_03605/pJC1 as control, and *C. glutamicum*/pJC1 as wild-type reference. One.2 μg of total RNA was loaded for each sample. Upper image displays part of the agarose gel showing the rRNA bands. Lower image displays part of the membrane showing detected 6C RNA bands. (D) Growth of complementation strain *C. glutamicum* Δcgb_03605/pJC1-cgb_03605 (▲), *C. glutamicum* Δcgb_03605/pJC1 (△), and *C. glutamicum*/pJC1 as a reference (■) in the presence of 0.75 μM mitomycin C. (E) Growth of dilution series of WT and Δcgb_03605 (Δ6C) strains carrying plasmid pJC1-cgb_03605 or empty vector pJC1 as a control on agar plates (CGXII glucose minimal medium) after 48 h of incubation with or without exposure to UV radiation (1 min) prior to incubation.

Table 1. Relative activities of the 6C RNA promoter P_{cgb_03605}. The relative activities were calculated from specific fluorescence levels provided by the *gfp*uv reporter under control of P_{cgb_03605} in *C. glutamicum* WT, $\Delta lexA$, $\Delta ramA$ and $\Delta sigB$ carrying the plasmid pEPR1-P_{cgb_03605}. Cells were grown in CGXII medium with glucose in the absence of MMC or with 0.75 μM of MMC. Values represent averages with standard deviation from triplicate measurements. - not determined.

t (h)	WT+MMC/WT	$\Delta lexA$+MMC/$\Delta lexA$	$\Delta lexA$+MMC/WT+MMC	$\Delta ramA$+MMC/$\Delta ramA$	$\Delta ramA$+MMC/WT+MMC	$\Delta sigB$+MMC/$\Delta sigB$	$\Delta sigB$+MMC/WT+MMC
3	1.52 ± 0.03	1.06 ± 0.04	1.78 ± 0.07	1.59 ± 0.01	1.15 ± 0.01	1.16 ± 0.03	0.78 ± 0.02
6	2.20 ± 0.19	1.32 ± 0.07	1.63 ± 0.08	2.02 ± 0.16	0.91 ± 0.07	1.69 ± 0.18	1.14 ± 0.12
23	—	—	—	1.53 ± 0.06	1.77 ± 0.08	—	—
24	1.81 ± 0.03	1.80 ± 0.03	2.39 ± 0.02	—	—	2.12 ± 0.47	0.62 ± 0.14
26	—	—	—	2.14 ± 0.31	1.59 ± 0.24	—	—
27	—	—	—	—	—	1.80 ± 0.16	0.44 ± 0.04

background (Table 1). That was also observed in the absence of MMC (Fig. 6C, E) and suggests a general contribution of RamA to repression of P_{cgb_03605} as well as SigB-dependent transcription in later stages of the batch culture.

The 6C RNA level-dependent pause in growth in the presence of MMC suggest that 6C RNA may be involved in or interfere with the halting of cell division during the SOS response. Such an influence may result in altered cell morphology, and we therefore examined the cells under a microscope with staining (Fig. 8). We found that already in the absence of MMC the overexpression of cgb_03605 in the wildtype resulted in elongated cells and the presence of free DNA in the extracellular space, which indicates damage to the cell envelope or cell lysis. The morphological differences caused by the overexpression of cgb_03605 were even more prominent in the presence of MMC. Additionally, branched cells with irregularly distributed septa were observed under DNA damage condition when cgb_03605 is overexpressed.

Discussion

In *C. glutamicum* we found high levels of the 6C RNA throughout growth and strong promoter activity of P_{cgb_03605}, which looks to be a strong housekeeping promoter with a good −10 and −35 region.[13] That fits to its high SigA-dependent activity shown using the *gfp*uv reporter system. The transient increase in 6C RNA level when cells enter the stationary phase could be attributed to the high apparent stability of >240 min. Stem-loop regions of secondary structures, which are a characteristic of 6C RNAs, can stabilize RNAs by hindering 3′-to-5′ exoribonucleolytic degradation,[21,22] yet the very high stability may also be caused by binding to target protein, thereby protecting 6C RNA from degradation. The RNA chaperone Hfq binds many sRNAs which can stabilize some of these, but small RNA half-lives can also be skewed by rifampicin treatment if the small RNA acts by base pairing and is co-degraded with its target.[23] However, in *C. glutamicum* a Hfq protein candidate is not
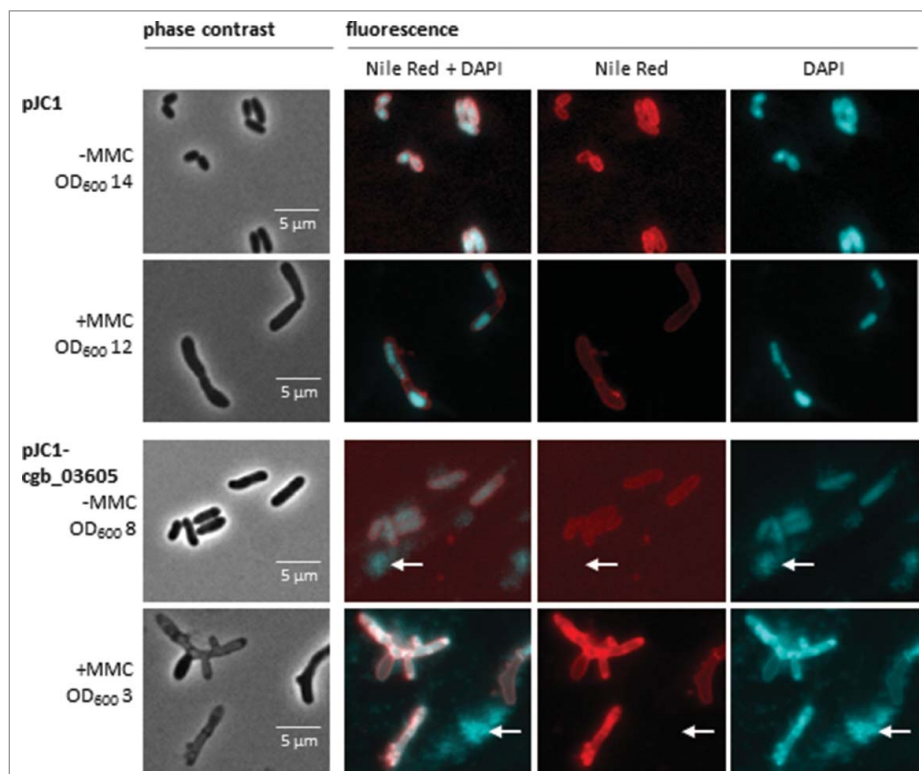

Figure 8. Microscopic analysis of *C. glutamicum* cells. *C. glutamicum*/pJC1-cgb_03605 overproducing 6C RNA and *C. glutamicum*/pJC1 (control) were cultivated in defined CGXII medium with glucose without mitomycin C (−MMC) or with 0.75 μM (+MMC). Samples were taken after 6 h of cultivation. OD_{600} values of the cultures at the sampling time are given. Cells were stained with Nile Red and DNA with 4′,6-diamidino-2-phenylindole (DAPI). White arrows indicate positions of free DNA.

known yet. The SigB-dependent expression of cgb_03605 can also contribute to the transient increase of 6C RNA level in *C. glutamicum* when cells enter the stationary phase. Since the activity of SigB depends significantly on the physiological control, *sigB* overexpression during exponential growth did not result in higher P$_{cgb\_03605}$ activity. The results with the Δ*ramA* strain indicate that RamA may act as a repressor of P$_{cgb\_03605}$ in the stationary phase. This is surprising in view of the positions of the predicted RamA binding sites upstream of the −35 region from which activation by RamA could be expected. It may indicate that other RamA binding sites exists in the promoter region resulting in the repression or the RamA effect is indirect via another transcriptional regulator in the absence of RamA. The SigB dependence furthermore ensures expression of cgb_03605 also under stress condition such as DNA damage caused by mitomycin C (MMC), as suggested by the P$_{cgb\_03605}$ activity measurement using the Δ*sigB* strain. Additionally, in *C. glutamicum* the 6C RNA gene is derepressed by LexA during the SOS response which requires an increase in 6C RNA level for optimal adaptation and survival to DNA damage according to our results. Interestingly, DNA-damaging MMC is a naturally occurring secondary metabolite which was originally isolated from *Streptomyces* species in which 6C RNA was also originally found.[5,24] As a soil bacterium, the producer cells themselves and other soil bacteria such as *C. glutamicum* have to cope with and protect themselves from MMC. In *M. tuberculosis* MMC stress did not altered the expression of the 6C RNA gene.[25] However, MMC and UV radiation can damage DNA, which then can trigger the SOS response. Generally, SOS genes are repressed by LexA to varying degrees that depend on the position and exact sequence of the SOS box and the strength of the promoter.[26] In *C. glutamicum*, the LexA-binding site which we identified is near to the −35 region about 50 bp upstream of the transcriptional 6C RNA start. Therefore LexA is expected to only partially repress the expression of cgb_03605, which is in accordance with our experimental data.

Two other bacterial sRNAs have been reported so far to be involved in LexA-dependent SOS response: *agrB* and transcript variant *istR*-1 from *E. coli*. These antisense sRNAs regulate the expression of toxin-antitoxin (TA) systems that can inhibit growth of *E. coli* by base-pairing to their target mRNA that inhibits translation (*dinQ*) or stimulates mRNA degradation (*tisAB*).[27-29] However, the 6C RNA from *C. glutamicum*, which is highly abundant throughout growth, is not such an antisense RNA and TA systems are not obvious by the annotations of *C. glutamicum* genomes nor have been reported yet. The 6C RNA's main role in *Actinobacteria* must be different. In *C. glutamicum*, the deletion as well as the overexpression of 6C RNA did not affect growth under standard conditions, yet overexpression resulted in elongated cells which additionally are branched in the presence of DNA-damaging MMC inducing the SOS response. In *M. smegmatis*, the overexpression of 6C RNA also resulted in elongated cells, while in *M. tuberculosis* the overexpression of 6C RNA proved to be lethal.[8] For *C. glutamicum*, the elongated cell morphology resembles that of a *lexA* deletion mutant in which we found an almost 3-fold increase in 6C RNA promoter activity.[20] Together the morphological alterations suggest that 6C RNA may be involved in some aspect of cell division in *Actinobacteria*. More specifically, in *C. glutamicum* the 6C RNA levels

appear to be critical for the transient stop of cell division after induction of the SOS response. The SOS response leads to an inhibition of Z-ring formation to give the bacterium time to complete the repairs of damaged DNA to avoid offspring with damaged genomes when cells resume their growth.[30,31] When 6C RNA is overexpressed, an earlier pause in growth and many branched *C. glutamicum* cells were observed after induction of the SOS response with MMC. That morphology very much resembles the branched phenotype observed when the cell division suppressor DivS inhibiting Z-ring formation is overexpressed in *C. glutamicum*.[20,32] The morphological defects of DivS overexpression are similar to mutants with reduced FtsZ levels,[33] yet could not be complemented by overexpression of FtsZ alone.[30] Recently, the RNase III mediated degradation of *mraZ* mRNA was shown to be also required for cell division in *C. glutamicum* and the absence of RNAse III resulted in cell elongation as 6C RNA overexpression did.[34] The transcriptional regulator MraZ represses *ftsEX* expression in *C. glutamicum*. FtsE putatively plays a role in promoting Z-ring assembly and FtsX is described as a regulator of peptidoglycan hydrolases.[30] Further studies are required to uncover the role of 6C RNA in cell division and whether mRNAs or proteins or both are targeted.

Materials and methods

Bacterial strains, plasmids and cultivation conditions

The bacterial strains and plasmids used are listed in Table 2. *C. glutamicum* was cultivated in 50 ml of defined CGXII medium [35] with 4 % (w/v) glucose as the sole carbon and energy source or complex medium (2 × TY, BHI)[36] in a 500 ml baffled shake flasks on a rotary shaker (120 rpm, 30°C). The main cultures were inoculated to an optical density OD$_{600}$ of 0.5 to 1 from precultures after washing the cells with 0.9 % NaCl. *Escherichia coli* DH5α was cultivated in LB medium (37°C). Where appropriate, kanamycin (25 μg/ml for *C. glutamicum* and 50 μg/ml for *E. coli*) and/or tetracycline (10 μg/ml) was added to the cultures. Mitomycin C was added to CGXII medium to a final concentration of 0.75 μM. For RNA decay assays, rifampicin was dissolved in methanol and added to CGXII medium to a final concentration of 250 μg/ml. Isopropyl-β-D-1-thiogalactopyranoside (IPTG, 1 mM) was added to cultures to induce the expression of *sig* genes cloned in pEC-XT99A. Growth was monitored by measuring the OD$_{600}$ with a UV-1800 spectrophotometer (Shimadzu). For UV radiation stress, aliquots of cell culture exponentially grown to an OD$_{600}$ of 5 in CGXII medium (4 % glucose) were taken and diluted to an OD$_{600}$ of 1. This reference cell suspension was used to generate a dilution series down to 10^{-7}. Volumes of 4 μl from each dilution step were dropped twice onto agar plates to generate replicate spots. Plates were incubated for drop-drying (5 min), exposed to UV (1 min) or not (− UV) and incubated for cell growth (30°C, 24 h).

Recombinant DNA work

The enzymes for DNA manipulations were obtained from Fermentas. Oligonucleotides were synthesized by Eurofins MWG

Table 2. Strains and plasmids used in this study.

Strain or plasmid	Host / relevant characteristics / use	Reference
C. glutamicum		
WT	ATCC 13032, wild type	ATCC
Δcgb_03605	WT derivative, deletion of 6C RNA gene and its promoter region	This work
ΔsigB	WT derivative, deletion of sigB	[45]
ΔlexA	WT derivative, deletion of lexA	[20]
ΔramA	WT derivative, deletion of ramA	[18]
E. coli		
DH5α	cloning	[46]
BL21(DE3)	heterologous expression	[47]
Plasmid		
pUCBM21	*E. coli* pUC19 derivative, Amp^R	[48]
pUCBM21-cgb_03605	pUCBM21 derivative, 6C RNA gene with T7 promoter for in vitro transcription	This work
pK19mobsacB	Kan^R; *E. coli* vector for construction of insertions and deletions in *C. glutamicum* (pK18 oriV_{Ec}, sacB, lacZα)	[49]
pK19mobsacB-Δ6C	Kan^R, pK19mobsacB derivative containing a 1027 bp overlap-extension PCR product for deletion of 6C RNA gene cgb_03605 and its promoter region	This work
pJC1	*E. coli* and *C. glutamicum* / Kan^R	[50]
pJC1-cgb_03605	pJC1 derivative with 6C RNA gene and its native promoter region	This work
pEPR1	*E. coli* and *C. glutamicum* / gfpuv as reporter, Kan^R / promoter test vector	[43]
pEPR1-P_{cgb_03605}	pEPR1 with P_{cgb_03605}, 189 nt fragment from −162 to +27 relative to TSS / promoter analysis	This work
pEC-XT99A	*E. coli* and *C. glutamicum* / trc promoter, Tet^R / expression	[51]
pEC-XT99A-sigA	pEC-XT99A with sigA / expression	This work
pEC-XT99A-sigB	pEC-XT99A with sigB / expression	This work
pEC-XT99A-sigE	pEC-XT99A with sigE / expression	This work
pEC-XT99A-sigH	pEC-XT99A with sigH / expression	This work
pEC-XT99A-sigM	pEC-XT99A with sigM / expression	This work
pET28-RamAx6His	*E. coli* / ramA, Kan^R / production of His_6-tagged RamA from *C. glutamicum* in *E. coli* BL21(DE3)	[18]
pET15b-cg2114	*E. coli* / cg2114 (lexA), Amp^R / production of His_6-tagged LexA from *C. glutamicum* in *E. coli* BL21(DE3)	[20]

Operon or Biolegio (Table 3). PCR, DNA restriction, ligation and transformation of *E. coli* were carried out according to standard protocols.[36] PCR products were generated using KOD Hot Start (Novagen) or GoTaq DNA polymerase (Promega). Plasmids were isolated from *E. coli* DH5α using a QIAprep Spin Miniprep Kit (Qiagen) or GeneJET^{TM} Plasmid Miniprep Kit (Fermentas). *C. glutamicum* was transformed by electroporation.

For construction of plasmid pUCBM21-cgb_03605 the DNA sequence of the 6C RNA gene (cgb_03605) was amplified from genomic DNA of *C. glutamicum* WT using the primer pair ivTr_6C_fw / ivTr_6C_rv, introducing the T7 promoter sequence and a *Psi*I site for the 3′-blunt end omitting the last 4 T bases. The *Xba*I-digested pUCBM21 plasmid and PCR fragment were ligated and used for transformation of *E. coli* DH5α.

For deletion of the 6C RNA gene in *C. glutamicum* WT the up- and downstream regions were amplified using the primer pairs 6C_P1fw / 6C_P2rv and 6C_P3fw / 6C_P4rv, respectively, followed by crossover PCR of the 2 PCR products with 6C_P1fw / 6C_P4rv. The resulting fragment was cloned to obtain the plasmid pK19*mobsacB*-Δ6C used to generate the strain *C. glutamicum* Δcgb_03605 (deletion from −58 to +106 bp relative to the transcriptional start) via a 2-step homologous recombination method.[37] For the expression of cgb_03605 in *trans* from its native promoter, the plasmid pJC1-cgb_03605 was constructed by cloning a *Bam*HI/*Sal*I fragment obtained by PCR with the primer pair nP_6Cfw / nP_6Crv into the plasmid pJC1.

The promoter region of cgb_03605 (P_{cgb_03605}) was cloned as a *Pst*I/*Bam*HI PCR fragment (189 nt, primer pair nP_6Cfw2 / nP_6Crv2) in the promoter test vector pEPR1 to determine the promoter activity using the *gfp*uv reporter. To overexpress the sigma factor genes, these genes were cloned in the IPTG-inducible expression vector pEC-XT99A analogously to those described recently.[38] Fragments carrying the genes *sigA*, *sigB*,

sigE, sigH and *sigM* were amplified by PCR using the respective the SigA/B/E/H/M primer pairs. All plasmid inserts or resulting modified genomic locations in constructed *C. glutamicum* strains were confirmed by DNA sequencing (Eurofins MWG Operon).

In vitro *transcription and preparation of DIG-labeled ssDNA probe*

In vitro transcription with purified *C. glutamicum* RNA polymerase (RNAP) was essentially carried out as described.[16] *C. glutamicum* holo-RNAP, reconstituted from a purified RNAP core and the respective sigma factors (SigA, SigB, SigE, SigH or SigM), was tested for transcription *in vitro*, which was initiated from the P_{cgb_03605} promoter. The respective DNA template was obtained as a PCR fragment (169 nt) with the primer pair P6C_IVTfv and P6C_IVTrv. α^{32}P-UTP labeled transcripts were separated in a 7 % polyacrylamide gel. The autoradiogram of the gel was scanned using a Molecular Imager FX (Bio-Rad).

For *in vitro* transcription with T7 RNAP (RiboMax, Promega) plasmid pUCBM21-cgb_03605 was linearized by *Psi*I digestion and used as a template for the generation of 6C RNA followed by DNAse treatment (15 min, 37°C). The 6C RNA *in vitro* transcript was purified using phenol/chloroform extraction. 6C RNA concentrations were determined using the Qubit fluorometer and the Qubit RNA BR assay kit (Life Technologies).

Single-stranded digoxigenin-labeled DNA (ssDIG-DNA) complementary to the 6C RNA transcript was generated by PCR. First, a DNA template containing the 6C RNA sequence was generated from chromosomal DNA using the primer pair NB_fw / NB_rv. The resulting PCR product (−28 to +139 bp relative to transcriptional start) was used in a second PCR with

Table 3. Oligonucleotides used in this study. Restriction sites are underlined.

Name	5′–3′ DNA sequence	Cloning site
ivTr_6C_fw	TCTAGA**TAATACGACTCACTATAGG**CAAGGCCCCGATATACAGTG, T7 promoter sequence used is given in bold	*Xba*I
ivTr_6C_rv	AGATTATAAGGGACGGCCCGCGCCT	*Psi*I
6C_P1fw	TTTAAGTCGACAGTGACCACCGAATGCATGG	*Sal*I
6C_P2rv	**CCCATCCACTAAACTTAAACAG**AATAACACGAATACTTAACAGTGTGG, overlap with 6C_P3fw is given in bold	–
6C_P3fw	**TGTTTAAGTTTAGTGGATGGG**TTTCTAAACTAGTGCCCTTAGCAG, overlap with 6C_P2rv is given in bold	–
6C_P4rv	AATTTGAATTCGTTGTTTGTCATCTCTGCCTAAAGC	*Eco*RI
nP_6Cfw	TTTAAGGATCCTGGCAACTTTCCAAGACTGAG	*Bam*HI
nP_6Crv	AAAATTGTCGACCACAGAAAATCTGCTAAGGGCA	*Sal*I
nP_6Cfw2	AACTGCAGTTACCAATGAGATTGAAG	*Pst*I
nP_6Crv2	TAGGATCCAGGCCACACTGTATATCG	*Bam*HI
SigAF	GCGAATTCATCCTCAGCATCACTC	*Eco*RI
SigAR	TGTCTAGACGAACCAAAGCAACAG	*Xba*I
SigBF	AGGAATTCGTTGAACCTCTTGAAC	*Eco*RI
SigBR	GCTCTAGAACTCGCCGGTAAAATA	*Xba*I
SigHF	AAGAATTCTGCAGATAGTCAACACGCATTTTCGAAAGGGGC	*Eco*RI
SigHR	AATCTAGACGAGTCGCTGCGGTTGAGA	*Xba*I
SigMF	AAGAGCTCTGCAGGCCCATACTAAGCCGCATAA	*Sac*I
SigMR	AATCTAGATCAAAGTAGTAAACGTAAAAGTGC	*Xba*I
SigEF	AAGAATTCGTCGACAGGATGAGGATCGTTTCGCATGAAAAAGAAGTCCCGAGATGACGCACCCG	*Eco*RI
SigER	AATCTAGAAAGTTCCCTGTCCGCACCAC	*Xba*I
P6C_IVTfw	CTGAGAATTCAACTAAAGTTACCAATGAG	*Eco*RI
P6C_IVTrv	TCGGAAGCTTGCACCTGAAATTATG	*Hind*III
NB_fw	TGGCGTTTGATATGCCATAATTTCAGGTGC	–
NB_rv	ACAGAAAATCTGCTAAGGGCACTAGTTTAG	–
P6C_fw	CGTAGGCGTAGGTGGAACTC	–
P6C_rv	**GAGGAGTCGTCGATGTGGAGACC**CACACTGTATATCGGGGCCTT, overlapping Biotin_rv sequence below is given in bold	–
Biotin_rv	BITEG-GAGGAGTCGTCGATGTGGAGACC	–
P6C_rv2	GCACCTGAAATTATGGCATATC	–
PaceAB_fw	TCAAGTCGACTTCCTTAAGTGCTGATTCG	–
PaceAB_rv	CGGGATCCCGCTCCTTTTAAAGCATGGG	–
Ppgi_fw	CCTACCTTCTCGATCCCTTCTC	–
Ppgi_rv	CCTGGGTGGTCGAAATGTC	–
LexA_F1_rv	CACGAATACTTAACAGTGTGGC	–
LexA_F2_fw	GGAAGTGTCATTTGCCACACTG	–
LexA_F2_rv	CACTTTCGAATGCAGAGGCACAG	–
LexA_OP1fw	CGTGTTATT**TCGAATAAATAATCGG**GTTTGATGpredicted LexA operator sequence(+) is given in bold	–
LexA_OP1rv	CATCAAAC**CCGATTATTTATTCGA**ATAACACGpredicted LexA operator sequence(−) is given in bold	–
LexA_OP2fw	TTGCCACACTGTTAAGTATTCGTGTTAT**TC**overlap with predicted LexA operator sequence(+) is given in bold	–
LexA_OP2rv	**GA**ATAACACGAATACTTAACAGTGTGGCAAoverlap with predicted LexA operator sequence(−) is given in bold	–
LexA_OP3fw	**G**GTTTGATGTTGTCTTGTGGCGTTTGATATGoverlap with predicted LexA operator sequence(+) is given in bold	–
LexA_OP3rv	CATATCAAACGCCACAAGACAACATCAAAC**C**overlap with predicted LexA operator sequence(−) is given in bold	–

200 μM dATP, 200 μM dCTP, 200 μM dGTP, 160 μM dTTP, and 30 μM DIG-11-dUTP (Roche) with the primer NB_rv (0.6 μM) to generate the respective ssDIG-DNA. For the detection of *gltA* mRNA as a control, the respective ssDIG-DNA was generated as described.[39]

Isolation of RNA, Northern blot analysis and absolute quantification

For the isolation of total RNA, cells were harvested by centrifugation and disrupted using Zirconia/Silica beads followed by phenol/chloroform/isoamylalcohol extraction as described.[40] For the separation of RNA by electrophoresis, RNA loads were denatured (10 min, 65°C) in loading buffer (30 % formamide, 20 % glycerol, 2.7 % formaldehyde, 4 mM EDTA pH 8.0, 10 μg/ml ethidium bromide, 0.6 % bromophenol blue) and subsequently separated on a 1.2 % formaldehyde agarose gel. Gels were used for blotting the RNA onto a nylon membrane (Roche) by downward capillary transfer in 5 × SSC (0.75 M NaCl, 75 mM tri-sodium citrate, pH 7) followed by UV cross-linking. Prehybridization (60 min, 45°C) was carried out with 10 ml hybridization buffer supplemented with 0.1 mg/ml salmon sperm DNA. Hybridization was performed overnight at 51°C in hybridization buffer containing a DIG-labeled RNA-specific ssDNA probe generated by PCR. For posthybridization (at RT), membranes were washed twice (2 × 5 min in 2 × SSC, 0.5 % SDS; 2 × 15 min in 1 × SSC, 0.5 % SDS, 50°C) and then incubated in blocking solution (30 min). For detection, membranes were incubated for 30 min in 20 ml antibody solution (1 μl anti-DIG-AP conjugate from Roche in 10 ml blocking solution), washed twice (15 min) in 100 ml wash buffer, and incubated for 5 min in 20 ml detection buffer and then 5 min in CDP-Star working solution (Roche). Signals were detected using a CCD camera (LAS-3000 Image analyzer, Fujifilm) and quantified using the software AIDA. All Northern blot analyses and subsequent calculations were performed in triplicate from independent cell cultures. For estimating the 6C RNA molecules per cell, the signal intensities obtained with isolated RNA from a known number of *C. glutamicum* WT cells (2.8 x10^8 cells / ml / 1 OD$_{600}$) were compared to the signal intensities of varying amount of *in vitro*-generated 6C RNA.

DNA affinity purification and protein identification

For the DNA affinity purification of proteins which bind specifically to the 6C RNA promoter region, the region was amplified

from chromosomal DNA of *C. glutamicum* WT using primer pair P6C_fw / P6C_rv. The resulting DNA fragment (P$_{cgb\_03605}$, −336 to +23 relative to transcriptional start) was purified and used as a template in a second PCR using the primer pair P6C_fw / Biotin_rv to introduce a biotin tag via a TEG linker (Eurofins MWG Operon). The resulting 381 bp DNA fragment was purified and coupled to streptavidin-tagged magnetic Dynabeads (Invitrogen) as described.[41] Cell-free crude protein extracts of *C. glutamicum* WT were prepared from cells grown to an OD$_{600}$ of 5 to 6 in CGXII medium with 4 % glucose. Cells were mechanically disrupted 6 times at 172 MPa using a French pressure cell (SLM Aminco) after adding protease inhibitor cocktail (Complete Mini, Roche). Intact cells and cell debris were removed by centrifugation (40 min, 5,000 × *g*, 4°C). DNA affinity purifications with crude protein extracts and DNA-coupled beads in the respective buffers were carried out as described.[41] The eluted proteins were separated with the Bis-Tris SDS-NuPAGE® gel system (Invitrogen) and visualized by Coomassie staining. Protein bands were subjected to MALDI-TOF peptide mass fingerprinting for protein identification as described.[42]

Electrophoretic mobility shift assays

For electrophoretic mobility shift assays (EMSAs) with PCR products or with oligonucleotides the proteins RamA-His$_6$ and LexA-His$_6$ were produced and purified as described.[18,20] The DNA fragment FP$_{cgb\_03605}$ (337 bp) covering the promoter region of the 6C RNA gene was generated by PCR with the primer pair P6C_fw / P6C_rv2, the positive control P$_{aceAB}$ (586 bp) with PaceAB_fw / PaceAB_rv, and the negative control P$_{pgi}$ (337 bp) with Ppgi_fw / Ppgi_rv. The DNA fragment F1 was generated by PCR using the primer pair P6C_fw / LexA_F1_rv, and DNA fragment F2 using LexA_F2_fw / LexA_F2_rv. To obtain the dsDNA fragments OP1, OP2, and OP3 the complementary oligonucleotide pairs LexA_OP1fw / LexA_OP1rv, LexA_OP2fw / LexA_OP2rv, and LexA_OP3fw / LexA_OP3rv, respectively, were annealed.

For EMSAs with RamA-His$_6$, 85 ng of DNA fragments were incubated with an increasing molar excess of protein in binding buffer (20 mM HEPES, 30 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$, 1 mM EDTA, 1 mM D,L-1,4-dithiothreitol, 0.2 % (w/v) Tween 20, pH 7.6) with 0.05 μg poly[d(I-C)] (Roche) in a total volume of 20 μl for 20 min at RT. Subsequently, samples were loaded onto a native 10 % polyacrylamide gel and separated. EMSAs with LexA-His$_6$ were performed as described.[20] 60 ng of DNA fragment F1 or F2 were incubated for 20 min at RT with an increasing molar excess of LexA-His$_6$. For EMSAs with OP1, OP2, or OP3, respectively, 0.25 pmol of dsDNA were incubated for 30 min at 30°C with an increasing molar excess of purified LexA-His$_6$. Subsequently, samples were loaded onto agarose gel and separated. All gels were stained with SYBR Green I and visualized with a UV-transilluminator at 254 nm (Quantum gel documentation system, Peqlab).

GFP fluorescence intensity measurements

The P$_{cgb\_03605}$ promoter activity was determined using a transcriptional fusion of the cgb_03605 promoter region with the *gfp*uv reporter in the promoter-probe vector pEPR1.[43] The cells were washed with PBS buffer with 2-mercaptoethanol (3 mM) and phenylmethylsulfonyl fluoride (PMSF, 0.1 mM) and disrupted using a FastPrep homogenizer (MP Biomedicals). The fluorescence of the cell extract was measured with a Saphire2 spectrophotometer (Tecan) (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).

Cell microscopy and staining

For cell microscopy, 4 μl of cell cultures were pipetted onto an agarose pad on a microscopic glass slide and observed using a Zeiss Axio Imager.M2 microscope (Zeiss). For a combined staining of *C. glutamicum* cells with DAPI (Sigma-Aldrich) and Nile Red (Life Technologies), samples of cell cultures were first diluted in an appropriate volume of standard PBS buffer and then mixed with the appropriate volume of staining solution as described.[44] Mixtures were incubated for 10 min in the dark before imaging. All images were acquired using the software AxioVision (Zeiss).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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