Population Heterogeneity in *Corynebacterium glutamicum* ATCC 13032 Caused by Prophage CGP3[∇]

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The genome of *Corynebacterium glutamicum* type strain ATCC 13032 (accession number BX927147) contains three prophages, CGP1, CGP2, and CGP3. We recently observed that many genes within the CGP3 prophage region have increased mRNA levels in a *dtxR* deletion mutant that lacks the master regulator of iron homeostasis (J. Wennerhold and M. Bott, J. Bacteriol. 188:2907–2918, 2006). Here, we provide evidence that this effect is due to the increased induction of the prophage CGP3 in the *dtxR* mutant, possibly triggered by DNA damage caused by elevated intracellular iron concentrations. Upon induction, the CGP3 prophage region is excised from the genome and forms a circular double-stranded DNA molecule. Using quantitative real-time PCR, an average copy number of about 0.1 per chromosome was determined for circular CGP3 DNA in wild-type *C. glutamicum*. This copy number increased about 15-fold in the *dtxR* mutant. In order to visualize the CGP3 DNA within single cells, a derivative of the wild type was constructed that contained an array of *tet* operators integrated within the CGP3 region and a plasmid-encoded YFP-TetR fusion protein. As expected, one to two fluorescent foci that represented the chromosomally integrated CGP3 prophage were detected in the majority of cells. However, in a small fraction (2 to 4%) of the population, 4 to 10 CGP3 DNA molecules could be observed in a single cell. Interestingly, the presence of many CGP3 copies in a cell often was accompanied by an efflux of chromosomal DNA, indicating the lysis of the corresponding cell. However, evidence for the formation of functional infective CGP3 phage particles could not be obtained.

In the course of bacterial genome sequencing, it was recognized that the genomes of many species contain integrated prophages that usually can be recognized due to differences from their host genome in GC content, oligonucleotide frequencies, or codon usage and the presence of genes with bacteriophage homologs of known function (e.g., integrases) (4). *Corynebacterium glutamicum* is a predominantly aerobic, biotin-auxotrophic gram-positive soil bacterium that was isolated in Japan because of its ability to excrete L-glutamate under biotin-limiting growth conditions (17). It is used today for the industrial production of more than two million tons of amino acids per year, mainly L-glutamate and L-lysine. Additionally, this species has become a model organism of the *Corynebacterineae*, a suborder of the *Actinomycetales*, which also comprises the genus *Mycobacterium*. An overview of the current knowledge on *C. glutamicum* can be found in a recent monograph (5). The genome of the *C. glutamicum* type strain ATCC 13032 has been sequenced two times independently and deposited in GenBank with the accession numbers BA000036 (12) and BX927147 (14). According to the criteria mentioned above and in contrast to the statement that *C. glutamicum* lacks prophages (3), three prophages were identified in the latter genome (designated CGP1, CGP2, and CGP3), and they are highly diverse in size and the grade of degeneration (13). The smaller prophages, CGP1 and CGP2 (13.5 and 3.9 kbp, respec-

tively), most probably are highly degenerated and no longer able to form functional bacteriophages. In contrast, the CGP3 element is one of the largest known prophages (~187 kbp), constituting ~6% of the entire *C. glutamicum* ATCC 13032 genome. The insertion site of the CGP3 prophage, a tRNA-Val gene, is easily detectable by a 26-bp direct repeat flanking the CGP3 element, a cluster of tRNA genes at its left border, and a phage-type integrase at its right border (*int2*). In the other *C. glutamicum* genome sequence (GenBank accession number BA000036), a fourth prophage, named CGP4, of 23.5 kb is inserted into the CGP3 prophage region, and it represents a major difference between these two sequences.

The isolation of bacteriophages was reported for several *C. glutamicum* strains, which were previously designated *Brevibacterium flavum*, but to our knowledge not for the strain ATCC 13032 (22, 24, 30). Most of the so-far characterized coryne-phages are temperate phages that have been isolated after UV induction (15, 22, 24).

In a recent study, we identified the regulon of the transcriptional regulator DtxR of *C. glutamicum* (33). This protein was first identified in *Corynebacterium diphtheriae*, in which it represses the transcription of the phage gene *tox* (which encodes diphtheria toxin) as well as a variety of genes involved in iron acquisition (18, 29). Our studies revealed that DtxR is the master regulator in a complex regulatory network that controls iron homeostasis in *C. glutamicum* (1, 33). Remarkably, a transcriptome comparison of wild-type (WT) *C. glutamicum* and a *dtxR* deletion mutant showed that the mRNA level of many genes located within the CGP3 prophage region (cg1890 to cg2071) was significantly increased in the *dtxR* deletion mutant (33). We therefore wondered whether this result was due to an

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>C. glutamicum</i>		
ATCC 13032	Biotin-auxotrophic WT	17
13032 Δ dtxR	In-frame deletion of the <i>dtxR</i> gene	33
13032::pLAU44-CGP3-Spec	13032 derivative containing plasmid pLAU44-CGP3-Spec integrated into the cg1905-cg1906 intergenic region	This work
13032::pLAU44-control-Spec	13032 derivative containing plasmid pLAU44-control-Spec integrated into the cg1857-cg1859 intergenic region	This work
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gvrA96</i> <i>thi-1</i> <i>relA1</i>	Invitrogen
Plasmids		
pEKEx2	Kan ^r ; <i>C. glutamicum</i> / <i>E. coli</i> shuttle vector for regulated gene expression (P _{tac} , <i>lacI</i> ^r , pBL1 <i>oriV</i> _{Cg} , pUC18 <i>oriV</i> _{Ec})	6
pEKEx3	Spec ^r ; pEKEx2 derivative containing a spectinomycin resistance gene instead of the kanamycin resistance gene	Laboratory stock
pEKEx2-yfp-tetR	Kan ^r ; pEKEx2 derivative containing <i>yfp-tetR</i> , encoding a YFP-TetR fusion protein under the control of the <i>tac</i> promoter	This work
pLAU44	Amp ^r Gm ^r ; plasmid containing two sets of <i>tetO</i> arrays	19
pLAU44-CGP3-Spec	Spec ^r ; pLAU44 derivative containing a 500-bp insert of the cg1905-cg1906 intergenic region for recombination into the genome	This work
pLAU44-control-Spec	Spec ^r ; pLAU44 derivative containing a 500-bp insert of the cg1857-cg1859 intergenic region for recombination into the genome	This work

induction of the CGP3 prophage in the mutant that results in the amplification of the phage genome.

In this work, we provide evidence that the CGP3 prophage still is able to excise from the *C. glutamicum* genome and to exist as a circular double-stranded phage DNA molecule. Fluorescence microscopy was used as a novel approach to visualize the number of CGP3 DNA molecules within single cells and to complement the data obtained by molecular genetic studies.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. All strains and plasmids used in this work are listed in Table 1. The *C. glutamicum* type strain ATCC 13032 (GenBank accession number BX927147) (14) was used as the WT. Strain Δ dtxR is a derivative containing an in-frame deletion of *dtxR* (cg2103) (33). For growth experiments, 5 ml of brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI) was inoculated with colonies from a fresh BHI agar plate and incubated for 6 h at 30°C and 170 rpm. After being washed with 5 ml 0.9% (wt/vol) NaCl, the cells of this first preculture were used to inoculate a 500-ml shake flask containing 60 ml CGXII minimal medium (16) with 4% (wt/vol) glucose as the carbon and energy source, 30 mg/liter 3,4-dihydroxybenzoate as the iron chelator, and either 1 μ M FeSO₄ (iron starvation) or 100 μ M FeSO₄ (iron excess). The trace element solution with iron salts omitted and the FeSO₄ solution were always added after autoclaving. This second preculture was incubated overnight and then used to inoculate the main culture to an optical density at 600 nm (OD₆₀₀) of ~1. The main culture contained the same iron concentration as the second preculture. For all cloning purposes, *Escherichia coli* DH5 α (Invitrogen) was used as the host. *E. coli* strains were cultivated aerobically in Luria-Bertani medium at 37°C. When appropriate, the medium contained kanamycin (25 μ g/ml for *C. glutamicum* and 50 μ g/ml for *E. coli*) or spectinomycin (250 μ g/ml for *C. glutamicum* and 100 μ g/ml for *E. coli*).

General DNA techniques. The enzymes for recombinant DNA work were obtained from Roche Diagnostics (Mannheim, Germany) or New England Biolabs (Frankfurt, Germany). The oligonucleotides used in this study are listed in Table 2 and were obtained from Operon (Cologne, Germany). Routine methods like PCR, restriction, or ligation were carried out according to standard protocols (27). Plasmids from *E. coli* were isolated with the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany). *E. coli* was transformed by the RbCl method (10), and *C. glutamicum* was transformed by electroporation (31). DNA sequencing was performed with a Genetic Analyzer 3100-Avant (Applied Biosystems, Darm-

stadt, Germany). Sequencing reactions were carried out with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems).

Isolation of chromosomal and phage DNA. Chromosomal DNA from *C. glutamicum* was prepared as described previously (7). For the isolation of circular

TABLE 2. Oligonucleotides used in this study

Oligonucleotide and purpose	Sequence ^a (5'–3')
Overexpression of <i>yfp-tetR</i>	
tetR-YFP-for	TATATAGGATCCAAGGAGATATAGATAT GGTGTCTAGATTAGATAAAAAG (BamHI)
tetR-YFP-rev	TATATAGAATTCTTATCTAGACTTGTACA GCTCGTCC (EcoRI)
CGP3-tetO-for	TATATACTCGAGATCTGGTGTTCACAGA TAGCAAGG (XhoI)
CGP3-tetO-rev	TATATACTGCAGTTTCCCCTGAAATATTA GATTGC (PstI)
control-tetO-for	TATATA CTGAG GTTCTAAGCCCCTCTT CTTTCAC (XhoI)
control-tetO-rev	TATATA CTGAG TTCTGGATCCTTCAT TTCAGAG (PstI)
Spec-tetO-for	TATATACTGCAGTATCTAGATAAAAAAT TTAGAGCC (PstI)
Spec-tetO-rev	TATATAGCTAGACCAATTAGAATGAAT ATTCCC (NheI)
Investigation of prophage induction	
prophage-1-for	GCAGAGTTCGCACGAGTGTGAGCG
prophage-2-rev	CACGTACTTTGCACGGATTCTGTCGG
prophage-3-for	GGTAGCTGGGGGAGTTGCGAGGGCG
prophage-4-rev	CCGCAAGGTCCTATGGCTCAGTGG
prophage-5-for	CATCCCTTGAAGGCTGAAGAAGC
prophage-6-rev	CACGTACTTTGCACGGATTCTGTCGG
qRT-PCR	
Phage-LC-for	CCCACGTTACCCCCACAAACG
Phage-LC-rev	CTAAAATGAAGCCATCGCGACC
ddh-LC-for	ACGTGCTGTTCTGTGCATGG
ddh-LC-rev	GCTCGGCTAAGACTGCCGCT
hdh-LC-for	AGCCGAAACGTTGACCTGTAC
hdh-LC-rev	AAGTTGGTGGTGCCGTTAACG

^a In some cases, oligonucleotides were designed to introduce recognition sites for restriction endonucleases (recognition sites are underlined, and restriction endonucleases are indicated in parentheses).

CGP3 phage DNA, 0.5 g (wet weight) cells of the $\Delta dtxR$ mutant was disrupted as described for the isolation of chromosomal DNA (7), and the DNA was isolated with the reagents of the QIAprep Spin Miniprep kit that are used for plasmid isolation. After the denaturation of protein and chromosomal DNA, the phage DNA was precipitated with 0.9 volumes of cold isopropanol and centrifuged (20 min, $15,000 \times g$, 4°C). Subsequently, the DNA was washed with $500 \mu\text{l}$ 70% (vol/vol) ethanol. After centrifugation (15 min, $15,000 \times g$, 4°C), the DNA was air dried (approximately 30 min at room temperature) and resuspended in 100 to $200 \mu\text{l}$ elution buffer (10 mM Tris-HCl, pH 8.5).

Plasmid construction. For the tagging of the CGP3 prophage region with *tetO* arrays, a 500-bp fragment from the DNA region between cg1905 and cg1906 was amplified using oligonucleotides CGP3-tetO-for and CGP3-tetO-rev, which introduced an XhoI and a PstI restriction site, respectively. Additionally, a DNA fragment containing the spectinomycin resistance cassette from pEKEx3 was amplified with oligonucleotides Spec-tetO-for and Spec-tetO-rev, which introduced restriction sites for PstI and NheI, respectively. Both PCR products were cloned into the vector pLAU44, resulting in plasmid pLAU44-CGP3-Spec. The pLAU44 vector was a kind gift of David J. Sherratt (University of Oxford, United Kingdom) and contains 240 copies of the 19-bp *tetO* operator of transposon Tn10 (19). After the transformation of WT *C. glutamicum* with pLAU44-CGP3-Spec, transformants were selected on BHI plates with $250 \mu\text{g/ml}$ spectinomycin. As the plasmid is unable to replicate in *C. glutamicum*, transformants should have integrated pLAU44-CGP3-Spec via homologous recombination within the cg1905-cg1906 intergenic region of the chromosome. Transformants were assayed for the correct insertion of the *tetO* arrays by PCR using the oligonucleotides control-tetO-for and Spec-tetO-rev (Table 2).

For the synthesis of a yellow fluorescent protein-TetR (YFP-TetR) fusion protein in *C. glutamicum* under the control of the *tac* promoter, a *yfp-tetR* fusion was amplified by PCR using the vector pLAU53 (19) as the template and the oligonucleotides tetR-YFP-for and tetR-YFP-rev, which introduce BamHI and EcoRI restriction sites, respectively. The resulting PCR product was cloned into the vector pEKEx2 (6). The recombinant plasmid pEKEx2-yfp-tetR was transferred via electroporation into strain *C. glutamicum* WT::pLAU44-CGP3-Spec. Transformants were selected for kanamycin and spectinomycin resistance.

Copy number determination using qRT-PCR. The copy number of the circular phage DNA molecule was determined by quantitative real-time PCR (qRT-PCR) as described previously (20). Each sample contained $1 \mu\text{g}$ template DNA, $10 \mu\text{l}$ of $2\times$ QuantiTect SYBR green PCR master mix (Qiagen, Hilden, Germany), and specific forward and reverse oligonucleotides (final concentration, $0.5 \mu\text{M}$) (Table 2) and was supplemented with H_2O to a final volume of $20 \mu\text{l}$. The CGP3-specific oligonucleotides were designed to amplify a 150-bp fragment located near the 5' junction (downstream of cg1890) within the CGP3 DNA, and the oligonucleotides specific for a non-prophage part of the genome were designed to amplify a 150-bp fragment of the *ddh* gene (cg2900). Amplification was carried out in $20\text{-}\mu\text{l}$ capillaries using a LightCycler type 1.0 instrument (Roche Diagnostics). Serial 10-fold dilutions ($1 \text{ ng}/\mu\text{l}$ to $100 \text{ fg}/\mu\text{l}$) of a phage DNA preparation ($10 \text{ ng}/\mu\text{l}$), prepared as described above, and a genomic DNA preparation ($10 \text{ ng}/\mu\text{l}$) were used to establish a standard curve. A negative control was set up by replacing the template DNA with H_2O in order to determine the lower detection limit. RT-PCR was performed with the following cycling conditions: preincubation at 95°C for 15 min, followed by 50 cycles of 94°C for 15 s, 62°C for 20 s, 72°C for 15 s, 80°C for 15 s, and 72°C for 5 s. Upon the completion of 50 cycles, a melting curve analysis was performed (from 60 to 95°C in 15 min) to confirm the specific amplification of PCR products. Subsequently, the copy number per genome was calculated for each sample by using the following equation:

$$\frac{\text{size of chromosomal DNA (bp)} \times \text{amount of plasmid DNA (pg)}}{\text{size of phage DNA (bp)} \times \text{amount of genomic DNA (pg)}}$$

In order to check that the derived copy number values were reliable, a positive control was performed using a template of known copy number. For this purpose, the copy number of the *hom* gene (cg1337) in strain *C. glutamicum* MH20-22B-DR17, which contains four *hom* copies in the genome (26), was determined. An average copy number of 4.2 ± 0.3 was determined for *hom* by qRT-PCR.

Fluorescence microscopy. Cells for microscopy were grown in CGXII minimal medium with 4% glucose under iron excess conditions ($100 \mu\text{M}$ FeSO_4) to an OD_{600} of 2, and then the synthesis of the YFP-TetR protein was induced by the addition of 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Cells were repeatedly analyzed by fluorescence microscopy until fluorescent foci appeared (approximately 30 min after IPTG addition). For phase-contrast and fluorescence microscopy, 1 to $3 \mu\text{l}$ of a culture sample was placed on a microscope slide that was coated with a thin 1% agarose layer and covered by a coverslip. For

FIG. 1. Increased mRNA levels of genes located within the CGP3 prophage region in a *C. glutamicum* *dtxR* deletion mutant compared to those of the WT ATCC 13032. The graph shows the mRNA ratio ($\Delta dtxR/\text{WT}$) of genes located within (cg1890 to cg2071) or adjacent to (cg1848 to cg1889 and cg2072 to cg2109) the CGP3 prophage region in the *C. glutamicum* genome, as determined by DNA microarray analysis in a previous study (33). The two strains were cultivated in CGXII minimal medium with 4% glucose under iron excess ($100 \mu\text{M}$ FeSO_4). The CGP3 prophage region is shaded in gray.

DNA staining, a $10\text{-}\mu\text{l}$ culture sample was mixed with $2 \mu\text{l}$ 4',6'-diamidino-2-phenylindole (DAPI; Sigma, Munich, Germany) solution (containing $1 \mu\text{g/ml}$ 50% glycerol). Images were taken on a Zeiss AxioImager M1 that was equipped with a Zeiss AxioCam HRm camera. YFP fluorescence was monitored using filter set 46 HE, and DAPI fluorescence was examined with filter set 49. An EC Plan-Neofluar $\times 100$ -magnification, 1.3-numeric-aperture oil immersion Ph3 objective was used. Digital images were acquired and analyzed with AxioVision 4.6 software (Zeiss, Göttingen, Germany). Final image preparation was done using Adobe Photoshop 6.0 (Adobe Systems Incorporated).

RESULTS

Demonstration of autonomous circular CGP3 phage DNA in *C. glutamicum*. In a recent study, we compared the transcriptomes of WT *C. glutamicum* and a mutant (strain $\Delta dtxR$) that lacked the transcriptional regulator DtxR, which was found to be the master regulator of iron homeostasis in this species (33). In these studies, most genes located within the CGP3 prophage region (cg1890 to cg2071) of the ATCC 13032 genome showed a more than twofold-increased mRNA level in the $\Delta dtxR$ mutant, whereas the mRNA levels of the genes located next to the CGP3 region (cg1849 to cg1979 and cg2072 to cg2109) were about the same in the WT and mutant (Fig. 1) (33). The vast majority of the 175 open reading frames located within the CGP3 region encode hypothetical proteins, and only a few code for proteins with known or putative functions, e.g., those for a transcriptional regulator involved in gluconate catabolism and glucose uptake (*gntR2*) (8), a restriction modification system (*cglIM*, *cglIR*, and *cglIIR*) (28), transposases, and proteins with known phage homologs, such a phage-type integrase (for an overview, see Table 3). As only two of the genes within the CGP3 region were identified as direct target genes of DtxR (cg1930 and cg1931), we asked whether the increased mRNA level of the CGP3 genes in the *dtxR* deletion mutant result from an induction and subsequent replication of the prophage DNA.

To test for prophage induction, the presence of circular CGP3 DNA molecules and the presence or absence of the CGP3 region in the genome was tested by PCR using several combinations of oligonucleotides (Table 2) and identical

TABLE 3. Genes within the CGP3 prophage region coding for proteins with known or putative functions or related to known phage proteins

Locus	Gene	Annotation
cg1893		Putative acetyltransferase
cg1903		Putative ABC transporter, ATP-binding protein
cg1904		Putative efflux transporter
cg1907		Putative phosphopantothienoylcysteine decarboxylase
cg1929	<i>res</i>	Site-specific recombinase, resolvase family
cg1930		Putative secretory serine protease
cg1932		Putative protein tyrosine phosphatase
cg1935	<i>gntR2</i>	Transcriptional regulator, GntR family (8)
cg1950		Transposase
cg1951		Transposase (partial)
cg1956	<i>recJ</i>	Single-stranded DNA-specific exonuclease
cg1959	<i>priP</i>	Putative prophage DNA primase
cg1961		Putative phage-associated protein
cg1963		Phage-related DNA helicase
cg1965		Putative phage-associated protein
cg1974		Putative cell wall endopeptidase with LysM peptidoglycan binding domain
cg1980		Putative ATPase
cg1982		Putative Clp-ATPase with similarity to <i>Bacillus subtilis</i> ClpE
cg1985		Putative DNA helicase
cg1990		Putative hydrolase with NUDIX domain
cg1991		Putative phage protein related to gp52 of <i>Streptomyces</i> phage phiC31
cg1996	<i>cgIIIM</i>	Type II restriction-modification system, methylation subunit
cg1997	<i>cgIIR</i>	Type II restriction-modification system, restriction subunit
cg1998	<i>cgIIR</i>	Type II restriction-modification system, restriction subunit
cg2004		Putative phage protein, similar to a protein of <i>Lactobacillus</i> phage gle
cg2009		Putative Clp-ATPase with similarity to <i>E. coli</i> ClpA
cg2024		Putative phage-related nuclease
cg2040		Putative transcriptional regulator
cg2043		Putative serine protease
cg2064		DNA topoisomerase I
cg2065		Putative helicase
cg2070	<i>'int2</i>	Putative phage integrase, C-terminal fragment
cg2071	<i>int2'</i>	Putative phage integrase, N-terminal fragment

amounts of genomic DNA isolated from the WT and the $\Delta dtxR$ mutant (Fig. 2). Amplification using oligonucleotides prophage-1-for and prophage-2-rev resulted in a PCR product of ~2.5 kb in the $\Delta dtxR$ deletion mutant and also produced a lower yield in WT *C. glutamicum*. This product can be obtained only if the ends of the CGP3 prophage are closed to a circular phage DNA molecule. A DNA sequence analysis of this PCR fragment confirmed the existence of circular phage DNA molecules in *C. glutamicum*. The ends of the circular phage DNA were connected via a 26-bp direct repeat flanking the CGP3 element (GCTGGTTCTGAACCCAGCTAGGACCAC). PCR with oligonucleotides prophage-3-for and prophage-6-rev led to a product of about 200 bp in the $\Delta dtxR$ mutant and with a much lower yield in the WT. This PCR product can be obtained only if the CGP3 region has been excised from the *C. glutamicum* genome. A DNA sequence analysis of this fragment confirmed the excision of the CGP3 prophage region, which left just one of the 26-bp direct repeats back in the chromosome. Further PCRs that were performed with oligonucleotide pairs prophage-3-for/prophage-4-rev and prophage-5-for/prophage-6-rev led to products of about 500 and 1,200 bp, respectively, which can be formed only from genomic DNA containing the CGP3 prophage. The observation that the yields of these two fragments were comparable between the WT and $\Delta dtxR$ mutant indicated that the prophage still was

integrated in the majority of genomes present in the total DNA preparation. In summary, these results clearly showed that, in a small fraction of WT cells and presumably in a higher fraction of $\Delta dtxR$ mutant cells, the CGP3 prophage is excised from the genome and exists as a double-stranded circular phage DNA molecule (Fig. 3).

Determination of the average copy number of circular CGP3 DNA molecules relative to chromosomal DNA molecules. qRT-PCR has been described as a valuable tool for the determination of plasmid copy numbers in bacteria (20). Therefore, we used this method to determine the copy number of circular CGP3 phage DNA molecules in WT *C. glutamicum* and the $\Delta dtxR$ mutant and to quantify the differences observed between both strains. To ensure that only the circular phage DNA molecule served as the templates for copy number determination, the oligonucleotides used for qRT-PCR (Table 2) were designed to anneal in the CGP3 element at the very left and right borders, respectively, and they point in opposite directions. As a consequence, no product is obtained from the integrated prophage. The oligonucleotide pair used for the quantification of genomic DNA targeted the gene *ddh* (cg2900), which encodes *meso*-diaminopimelate dehydrogenase. Different dilutions of total DNA of WT *C. glutamicum* or the $\Delta dtxR$ mutant were used as the template for amplification. DNA was extracted from cells cultivated in CGXII minimal medium with 4% glucose under iron excess (100 μ M FeSO₄) or under iron limitation (1 μ M FeSO₄) and harvested at an OD₆₀₀ of 5 to 6 by following the conditions used for the

FIG. 2. Evidence for the presence of autonomous circular CGP3 phage DNA molecules in *C. glutamicum* ATCC 13032 and the $\Delta dtxR$ mutant. PCRs with the indicated combinations of oligonucleotides prophage-1-for and prophage-6-rev (Table 2) and total DNA isolated from the WT or the $\Delta dtxR$ mutant were used to test whether CGP3 prophage DNA exists as a circular DNA molecule (primer combination prophage-1-for/prophage-2-rev) and is excised from the *C. glutamicum* genome (primer combinations prophage-3-for/prophage-4-rev, prophage-3-for/prophage-6-rev, and prophage-5-for/prophage-6-rev). The upper part of the figure shows the relative positions of the oligonucleotides on the chromosomal DNA. The PCR products obtained were separated on 1% agarose gels and stained with ethidium bromide (lower part of the figure).

FIG. 3. Preliminary model of CGP3 integration and induction in *C. glutamicum*. The lower part shows CGP3 in its prophage state as it is integrated into the *C. glutamicum* ATCC 13032 genome, as found by genome sequence analysis (14). The insertion site is detectable as a 26-bp direct repeat flanking the CGP3 element (black boxes designated *attL* and *attR*; their sequences are given below the boxes). After the induction of the CGP3 prophage, recombination takes place at the direct repeats, resulting in the excision of an autonomous circular CGP3 phage DNA molecule. In the published genome sequence, the *int* gene encoding a phage-type integrase contains a frameshift, resulting in a protein of 277 amino acid residues that represents the N-terminal part and a protein of 48 amino acid residues that represents the C-terminal part.

previous DNA microarray experiments (33). With this method, the average copy number of circular CGP3 DNA per genome was determined to be 0.11 ± 0.03 and 0.07 ± 0.01 for WT *C. glutamicum* grown under iron excess and iron limitation, respectively. In the $\Delta dtxR$ mutant, the copy number of the circular phage DNA was increased about 15-fold under iron excess (1.74 ± 0.32) and 8-fold under iron limitation (0.59 ± 0.15). These results confirmed the conclusions from the previous PCR experiments that, in WT *C. glutamicum*, a very small fraction of the cells contains circular CGP3 DNA. Either this fraction or the copy number of circular CGP3 molecules per cell increases up to 15-fold in the $\Delta dtxR$ mutant, which also explains the significant increase of the mRNA level of CGP3 genes observed in the transcriptome comparison of WT *C. glutamicum* and the $\Delta dtxR$ mutant (Fig. 1). As a control, we also determined the copy number of the *hom* gene (cg1337), which encodes homoserine dehydrogenase, in the *C. glutamicum* strain MH20-22B-DR17 (26). This strain has been genetically modified to contain four copies of the *hom* gene in the genome. qRT-PCR gave a copy number of 4.20 ± 0.6 , confirming the reliability of the method.

Estimation of the number of CGP3 DNA molecules per cell using fluorescence microscopy. The qRT-PCR data indicated that a maximum of 10% of the cells in a population of WT *C. glutamicum* contain a single copy of circular CGP3 phage DNA. However, this percentage can be significantly lower if

the number of circular CGP3 phage DNA molecules per cell is above 1. In order to visualize the CGP3 DNA within cells and to estimate the CGP3 DNA copy number per cell, an array of approximately 240 *tetO* operator regions of transposon Tn10 (*tetO* array) was inserted into the intergenic region between cg1905 and cg1906 within the CGP3 prophage by using plasmid pLAU44-CGP3-Spec (see Materials and Methods). The resulting strain, 13032::pLAU44-CGP3-Spec, was transformed with plasmid pEKEx2-yfp-tetR, which contains a gene encoding a fusion protein of the YFP and the repressor TetR of Tn10 under the control of a *tac* promoter. This fusion protein should bind specifically to the integrated *tetO* arrays (19) and, thus, should allow the estimation of the number of CGP3 DNA molecules within single cells by counting the fluorescent foci. Cells of strain 13032::pLAU44-CGP3-Spec/pEKEx2-yfp-tetR were grown in CGXII minimal medium with 4% glucose. At an OD_{600} of about 2, the expression of the *yfp-tetR* fusion gene was induced by the addition of 0.1 mM IPTG, and 20 to 40 min later the cells were analyzed by fluorescence microscopy. As expected, one or two fluorescent foci were detected in the majority of cells (>80%), which most likely represented the chromosomally integrated CGP3 DNA copies (Fig. 4A). In these cells, the chromosomal DNA stained with DAPI was easily detectable as a distinct region within the cell (Fig. 4B, row 1). In about 6% of the cells three fluorescent foci were observed, and in about 5% of the cells 4 to 10 foci were observed (Fig. 4B, rows 2 to 4, and C). Interestingly, the presence of such a high number of foci often was accompanied by an efflux of chromosomal DNA, indicating the lysis of one or more cells in the corresponding area. According to two-dimensional (2D) analysis, only fluorescent foci in the focal plane of the camera can be detected. To get insights on the total number and the localization of YFP-TetR foci, 3D analyses were performed. In several cases, these studies revealed more than 10 fluorescent foci in a single cell, which were randomly distributed throughout the cytoplasm (Fig. 5).

In order to exclude the possibility that the presence of 4 to 10 foci per cell is due to the presence of multiple genomes per cell or to an artifact, such as the formation of YFP-TetR inclusion bodies, a control strain was constructed in which the *tetO* arrays were inserted outside the CGP3 prophage region in the *C. glutamicum* chromosomal DNA. For this purpose, plasmid pLAU44-control-Spec was constructed, which contains a 500-bp fragment that covers the intergenic region between cg1859 and cg1857. The control strain 13032::pLAU44-control-Spec/pEKEx2-yfp-tetR was cultivated in the way described above and was analyzed by fluorescence microscopy. The majority of the cells contained one or two fluorescent foci (approximately 300 cells were counted in total) (Fig. 4C), and the maximal number of foci visible in the control strain was three in about 2% of the cells. The fourth one, which is expected to exist, presumably was located in a different layer of the cell. Thus, the high number of fluorescent foci visible in some of the cells of strain 13032::pLAU44-CGP3-Spec/pEKEx2-yfp-tetR most likely is due to the presence of multiple copies of CGP3 DNA.

The results described above confirmed the assumption that a small fraction of *C. glutamicum* WT cells harbors multiple copies of circular CGP3 phage DNA molecules. Although some of these cells appeared to have become leaky, as indi-

FIG. 4. Fluorescence microscopy of *C. glutamicum* cells containing a *tetO* array integrated into the CGP3 (pro)phage DNA (between cg1905 and cg1906) and a plasmid (pEKEx2-yfp-tetR) encoding a YFP-TetR fusion protein under the control of the *tac* promoter. Cells were grown in CGXII minimal medium with 4% glucose to an OD₆₀₀ of ~2, and then the synthesis of YFP-TetR was induced with 0.1 mM IPTG until fluorescent foci appeared (20 to 40 min). Additionally, the nucleoid was stained with DAPI (blue). (A) Representative cells for the various types of cells observed. (B) In most cases, zero to three CGP3 foci could be detected per cell (row 1), and in these cells the nucleoid is visible as a distinct region. In a few cases, 4 to 10 CGP3 foci were observed in a single cell (rows 2 to 4). The presence of such a high number of foci often was accompanied by an efflux of DNA, indicating the lysis of the cells. (C) Fluorescent foci per cell were counted in strain 13032::pLAU44-CGP3-Spec/pEKEx2-yfp-tetR (gray bars) and in the control strain 13032::pLAU44-control-Spec/pEKEx2-yfp-tetR, which contained the *tetO* array inserted outside of the CGP3 region between cg1859 and cg1857 (black bars). Two classes of cells were defined: those containing zero to three foci per cell and those containing more than three foci per cell. The percentage of each class in both strains is given in the graph. Approximately 1,000 cells were evaluated for the CGP3-tagged strain, and about 300 cells were evaluated for the control strain.

cated by the efflux of chromosomal DNA, neither a complete lysis of liquid cultures nor plaque formation on agar plates has been observed during many years of experimental work with this strain, which argues against the formation of infective CGP3 phage particles.

DISCUSSION

In this work, we provide strong evidence for the existence of circular CGP3 DNA molecules in *C. glutamicum*. The initial hint for this finding was the observation that the mRNA level of genes located within the CGP3 prophage region was significantly increased in a *dtxR* deletion mutant that lacked the master regulator of iron homeostasis (Fig. 1). Subsequently, it could be shown that this is due to an induction of the CGP3 prophage in *C. glutamicum*. Upon induction, the CGP3 DNA

becomes excised from the genome and exists as a double-stranded DNA phage with cohesive ends, like the majority of other known corynephages (2, 22, 24, 30). Until now, the CGP3 prophage DNA (~187 kbp) was supposed to exist exclusively as a genomic island integrated into the *C. glutamicum* genome.

The site of the integration of the CGP3 prophage into the *C. glutamicum* genome, a tRNA-Val gene located at the 5' junction, can be easily identified due to the direct 26-bp repeats flanking the CGP3 prophage (5'-GCTGGTTCGAACCCAGC TAGGACCAC-3'). The specific site of integration into a bacterial genome is determined primarily by the integrase enzyme, which catalyzes site-specific recombination between the phage recognition site (*attP*) and a short sequence of bacterial DNA (*attB*) (Fig. 3) (9). The gene encoding the integrase of the CGP3 prophage is located at the 3' junction but seems to be disrupted by a frameshift mutation, with *int2'* (cg2071) encod-

FIG. 5. 3D analysis of *C. glutamicum* cells containing a *tetO* array integrated into the CGP3 DNA region. For a 3D analysis, two cells with multiple YFP-TetR foci were selected, and a Z-stack image series was taken. (A) The mid-cell focal plane is shown in phase contrast (top left), YFP channel (top right), DAPI (bottom left), and as a merged image (bottom right). The scale bar is 1 μm . (B) A deconvolved Z-stack series of the YFP channel is shown with increments of 0.15 μm . (C) A 3D reconstruction of this Z-stack series. The white ellipses illustrate the outlines of the two cells. Several YFP-TetR foci are distributed randomly throughout the 3D space of the cell.

ing the N-terminal part and *int2* (cg2070) encoding the C-terminal part of the integrase (13). The finding that the CGP3 prophage still is able to excise from the *C. glutamicum* genome indicates, however, that a functional integrase still can be formed. We amplified the portion of the *int* gene that con-

tained the frameshift from genomic DNA of our ATCC 13032 WT strain and the ΔdtxR mutant and sequenced the resulting two PCR products (data not shown). There was no difference from the published genome sequence (13), but this does not exclude the possibility that the frameshift is reversed in the small fraction of cells with an induced CGP3 prophage. It also might be possible that the N-terminal portion of the integrase is sufficient for its activity or that the N- and the C-terminal parts reconstitute an active integrase. The above-mentioned 26-bp direct repeat was shown to be the connection site of the circular phage DNA molecule, and one repeat remained in the bacterial genome after the excision of the CGP3 prophage. Thus, it is very likely that crossing over occurs at these 26-bp sites, which form a so-called core-type binding site that is recognized by the phage integrase (9).

The fluorescence microscopy of cells with a YFP-tagged CGP3 region was used to shed light on the heterogeneity of a typical WT *C. glutamicum* culture. Whereas in most cells zero, one, or two fluorescent foci were detected, a small fraction of the bacteria contained eight or even more foci within a single cell (Fig. 4 and 5). The absence of fluorescent foci most likely is caused by an insufficient induction of the *yfp-tetR* gene in the corresponding cells. Two to four foci can be explained by the ongoing replication of the chromosomal DNA in exponentially growing cells, as confirmed by tagging a non-prophage region of the genome. The existence of more than four fluorescent foci within one cell, however, must be due to an induction of the CGP3 prophage and the subsequent replication of its genome.

Interestingly, the cells containing many phage DNA molecules apparently were prone to lysis, as indicated by an obvious efflux of DAPI-stained chromosomal DNA from these cells (Fig. 4B, rows 2 to 4). Double-stranded DNA phages often accomplish lysis by the expression of a muralytic enzyme, called endolysin, that is encoded by the phage genome, which degrades peptidoglycan (21, 34). The action of endolysins usually depends on holins, small membrane proteins that oligomerize in the cytoplasmic membrane and enable the access of the endolysins to the peptidoglycan (32). Whereas no genes encoding putative holins were identified within the CGP3 DNA, it does contain four genes that code for putative proteases or hydrolases (Table 3). An increased expression of these genes might be responsible for cell lysis and the efflux of chromosomal DNA.

The observation that cells with many CGP3 DNA copies can lyse leads to the question of whether intact and fully functional CGP3 phage particles are formed that are released by the lysis of the cell. Homologs of typical phage morphogenic genes, e.g., genes encoding coat proteins, tail fibers, or shaft-building proteins, could not be identified so far, but this does not exclude the possibility that such proteins are encoded by the many hypothetical genes present in the CGP3 DNA. The extreme diversity of phage genes in bacterial genome sequences is a known feature of double-stranded DNA phages (4, 11). An argument against the formation of infective CGP3 phages is the fact that, in decades of studies with *C. glutamicum* ATCC 13032 and derivatives, neither a complete lysis of cells in liquid cultures nor the formation of plaques on lawns has been observed. However, the possibility that such phages are formed after the spontaneous induction of CGP3 in a small percentage

of the population but cannot infect and lyse other cells of the culture still carrying the CGP3 prophage cannot be completely excluded.

Our PCR experiments showed that the CGP3 DNA region is excised from the chromosomal DNA. If this happens at a time point shortly before cell division, cells without CGP3 DNA might be formed. Such cells should have a strong advantage over CGP3-carrying cells, as they have lost the burden of 187 kb of DNA. The fact that *C. glutamicum* has retained the CGP3 prophage indicates the presence of either essential genes or addition modules within the CGP3 DNA. The gene *cg1907* encodes a putative phosphopantothienoylcysteine decarboxylase, which is involved in coenzyme A biosynthesis, and might be regarded as an essential gene. However, there is another gene present in the *C. glutamicum* genome, *cg1807*, which also encodes this enzymatic function, and therefore *cg1907* is unlikely to be essential. On the other hand, type II restriction-modification systems have been shown to serve as addition modules (23). In that context, the restriction enzyme functions as a toxin while the DNA methylase plays the role of an antitoxin. The antitoxin protects the targets of the toxin, which are the recognition sequences in the genome. When the genes encoding the restriction-modification enzymes get lost, the dilution of the methylase leads to unprotected target sites in the chromosome that subsequently are cleaved by the restriction enzyme, which leads to cell death. Thus, the *cgIIM-cgIIR* genes present in the CGP3 DNA could serve as an addition module that is responsible for the maintenance of the CGP3 genomic island.

DNA microarray analysis, as well as the determination by qRT-PCR of the copy number of the circular phage DNA molecule in relation to that of the chromosomal DNA, indicated that the fraction of cells with an induced CGP3 prophage is about 15-fold higher in a *dtxR* deletion mutant than in the WT. Alternatively, the number of phage genomes per cell might be increased 15-fold, which, however, seems unlikely. Several attempts to integrate the *tetO* array into the CGP3 DNA region of the $\Delta dtxR$ mutant were not successful, and therefore it was not possible to determine the percentage of cells with more than four CGP3 genomes and the copy number of CGP3 genomes per cell. In the $\Delta dtxR$ mutant, the expression of a large set of genes encoding high-affinity iron uptake systems is strongly increased even under conditions of sufficient iron supply (1, 33). Although the DNA microarray data did not provide obvious evidence for the induction of an oxidative stress response in this mutant, two genes presumably involved in DNA repair (*cg1318* and *cg1319*) were threefold induced in the $\Delta dtxR$ strain, which might be a hint at DNA damage caused by elevated intracellular iron concentrations (33). DNA damage can be caused by various stresses, including exposure to reactive oxygen species, UV light, or genotoxic agents, such as some classes of antibiotics. A search in our in-house microarray database (25) showed slightly increased mRNA levels of the CGP3 genes after the exposure of WT *C. glutamicum* to UV light (data not shown). Thus, DNA damage might contribute to CGP3 induction, but clearly, further studies are required to unequivocally identify the trigger(s) of the induction process.

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