Analysis of SOS-Induced Spontaneous Prophage Induction in Corynebacterium glutamicum at the Single-Cell Level

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The genome of the Gram-positive soil bacterium Corynebacterium glutamicum ATCC 13032 contains three integrated prophage elements (CGP1 to -3). Recently, it was shown that the large lysogenic prophage CGP3 (~187 kbp) is excised spontaneously in a small number of cells. In this study, we provide evidence that a spontaneously induced SOS response is partly responsible for the observed spontaneous CGP3 induction. Whereas previous studies focused mainly on the induction of prophages at the population level, we analyzed the spontaneous CGP3 induction at the single-cell level using promoters of phage genes (P_{recA} and P_{int2}) fused to reporter genes encoding fluorescent proteins. Flow-cytometric analysis revealed a spontaneous CGP3 activity in about 0.01 to 0.08% of the cells grown in standard minimal medium, which displayed a significantly reduced viability. A P_{recA}-eyfp promoter fusion revealed that a small fraction of C. glutamicum cells (~0.2%) exhibited a spontaneous induction of the SOS response. Correlation of P_{recA} to the activity of downstream SOS genes (P_{divS} and P_{recN}) confirmed a bona fide induction of this stress response rather than stochastic gene expression. Interestingly, the reporter output of P_{recA} and CGP3 promoter fusions displayed a positive correlation at the single-cell level (p = 0.44 to 0.77). Furthermore, analysis of the P_{recA}-eyfp/P_{int2}-e2-crimson strain during growth revealed the highest percentage of spontaneous P_{recA} and P_{int2} activity in the early exponential phase, when fast replication occurs. Based on these studies, we postulate that spontaneously occurring DNA damage induces the SOS response, which in turn triggers the induction of lysogenic prophages.

Genome sequencing projects have revealed a large amount of prophage DNA in bacterial genomes. Although not all prophage DNA accounts for functional prophages, because it includes degenerated phage remnants, this DNA can have a marked impact on bacterial physiology (1). The biotechnological platform organism Corynebacterium glutamicum is a Gram-positive, bio- tin-auxotroph soil bacterium that is used for the industrial production of more than four million tons of L-glutamate and L-lysine per year (2, 3). As revealed by whole-genome sequencing, C. glutamicum ATCC 13032 possesses three prophages that are integrated into its genome (CGP1 to -3), of which CGP1 and CGP2 are probably degenerated phage remnants (4–6). Previous studies showed that the large prophage CGP3 (187 kb) retains the ability to be excised from the genome and exist as a circular DNA molecule. Interestingly, a small number of wild-type cells showed an expression of genes responsible for the excision of phage DNA, indicating the repressor sites within the prophage is alleviated, leading to repressor cleavage of the repressor LexA, which leads to the derepression of more than 40 SOS genes (SOS response) (14). The life cycle of lambdoid phages is linked to this regulatory pathway. The central repressor cl mimics the autocatalytic center of LexA and thus becomes cleaved upon the induction of the SOS response. Its binding to repressor sites within the prophage is alleviated, leading to an expression of genes responsible for the excision of phage DNA, virion assembly, and release of the infectious phage particles into the extracellular space (13).

In this study, we address the question of whether the spontaneous induction of the lysogenic prophage CGP3 in single C. glutamicum cells is linked to the spontaneous activation of the SOS response. The promoters of genes of the SOS pathway and those encoded by CGP3 were fused to the fluorescent reporter genes eyfp and e2-crimson to analyze the activity of the respective promoters under standard cultivation conditions. Single-cell analysis was performed using flow cytometry and an in-house developed polydimethylsiloxane (PDMS) microfluidic chip setup (15, 16) suit-

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able for observing rare cellular events of interest. We observed a positive correlation between the spontaneous activation of the SOS response and the spontaneous induction of the prophage CGP3, and we postulate a bona fide activation of the SOS response as a prominent trigger leading to prophage excision.

**MATERIALS AND METHODS**

**Bacterial strains, media, and growth conditions.** The bacterial strains used in this study are listed in Table 1. *C. glutamicum* ATCC 13032 was used as the wild-type strain; all strains were cultivated at 30°C. For growth experiments, a glycerin stock culture was streaked onto BHI (brain heart infusion; Difco, BD, Heidelberg, Germany) agar plates. Single colonies were used to inoculate 5 ml liquid BHI medium. After cultivation for 20 h, the preculture was used to inoculate 25 ml CGXII minimal medium (1:50 dilution) for microtiter-scale cultivations, the Biolector microbioreactor system (m2p-labs, Heinsberg, Germany) was utilized for cultivations at an OD600 of 4.

**Construction of promoter fusions.** For construction of the promoter fusions of *P_iac2*, *P_lysin* and *P_{cg2067}* 250 bp upstream of the coding sequence with an additional 10 codons and the 16-bp ribosomal binding site of *pET16* were amplified using the oligonucleotide pairs int2-fwd/int2-rev and cg2067-fwd/cg2067-rev, respectively. The promoter fusions were ligated into the vector pJC1-crimson-term by restriction with BamHI and NdeI.

**Cloning techniques.** For PCR amplification of DNA used for cloning, KOD HotStart polymerase (Merck Millipore, Darmstadt, Germany) was used. DreamTaq (fisher scientific, Schwerte, Germany) was utilized for PCR verification of ligation reactions. Heat shock transformation of *E. coli* was performed as described previously (19). Transformation of *C. glutamicum* was performed by electroporation as described previously (20).

**Isolation and purification of plasmids from *E. coli* cultures was performed using plasmid isolation and purification kits from Qiagen (Qiagen, Hilden, Germany) and thermo scientific (fisher scientific, schwerte, Germany) miniprep kits. Chromosomal DNA of *C. glutamicum* was isolated as described previously (21). DNA sequencing and oligonucleotide synthesis were performed by Eurofins MWG Operon (Ebersberg, Germany). Plasmids and oligonucleotides used in this work are listed in Table 1 and Table 2, respectively. The in-frame deletion mutant of *recA* was collected as described previously (21). DNA sequencing and oligonucleotide synthesis were performed by Eurofins MWG Operon (Ebersberg, Germany). Plasmids and oligonucleotides used in this work are listed in Table 1 and Table 2, respectively. The in-frame deletion mutant of *recA* was constructed as described by Jochmann et al. (22).

**TABLE 1** Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td><em>supE44 ΔlacU169 (φ80lacZD315) hisD17 recA1 endA1 gyrA96 thi-1 relA1</em></td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>C. glutamicum</em></td>
<td></td>
<td></td>
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<tr>
<td>ATCC 13032</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 13032ΔlexA</td>
<td>Biotin-auxotrophic wild type</td>
<td>36</td>
</tr>
<tr>
<td>ATCC 13032ΔlexA: <em>P_{recA}</em>-eyfp</td>
<td>Integration of <em>P_{recA}</em>-eyfp into the intergenic region between cg1121 and cg1122</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
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<tr>
<td>pJC1</td>
<td>Kan’, Amp’, <em>C. glutamicum</em> shuttle vector</td>
<td>38</td>
</tr>
<tr>
<td>pEKEx2- <em>P_{lac2}</em>-cyfp</td>
<td>Kan’, pEKEx2 containing cyfp with pET16 RBS, under the control of <em>P_{lac}</em>, the insert includes the promoter of <em>recA</em> and an additional ribosome binding site (<strong>pET16</strong>) in front of cyfp</td>
<td>This study</td>
</tr>
<tr>
<td>pAN6- <em>e2-crimson</em></td>
<td>Kan’, pAN6 derivative for expression of E2-Crimson under the control of the <em>P_{recA}</em> promoter</td>
<td>6</td>
</tr>
<tr>
<td>pK19mobSacB</td>
<td>Kan’, oriV&lt;sub&gt;E. coli&lt;/sub&gt;, sacB lacZ&lt;sub&gt;A&lt;/sub&gt;, the insert includes the promoter of cis-6, 30 bp of the coding sequence, a stop codon, and an additional ribosome binding site (<strong>pET16</strong>) in front of <em>e2-crimson</em></td>
<td>37</td>
</tr>
<tr>
<td>pK18mobSacB-cg1121/1122</td>
<td>Kan’, oriV&lt;sub&gt;E. coli&lt;/sub&gt;, sacB</td>
<td>6</td>
</tr>
<tr>
<td>pK18mobSacB-cg1121/1122- <em>P_{recA}</em>-cyfp</td>
<td>pJC1 derivative containing the promoter of <em>recA</em> (260 bp) fused to cyfp; the insert includes the promoter of <em>recA</em> and an additional ribosome binding site (<strong>pET16</strong>) in front of cyfp</td>
<td>This study</td>
</tr>
<tr>
<td>pJC1-P&lt;sub&gt;recA&lt;/sub&gt;- <em>e2-crimson</em></td>
<td>pJC1 derivative containing the promoter of <em>recA</em> (441 bp) fused to <em>e2-crimson</em>; the insert includes the promoter of divS, 30 bp of the coding sequence, a stop codon, and an additional ribosome binding site (<strong>pET16</strong>) in front of <em>e2-crimson</em></td>
<td>This study</td>
</tr>
<tr>
<td>pJC1-P&lt;sub&gt;recN&lt;/sub&gt;- <em>e2-crimson</em></td>
<td>pJC1 derivative containing the promoter of <em>recN</em> (207 bp) fused to <em>e2-crimson</em>; the insert includes the promoter of <em>recN</em>, 30 bp of the coding sequence, a stop codon, and an additional ribosome binding site (<strong>pET16</strong>) in front of <em>e2-crimson</em></td>
<td>This study</td>
</tr>
<tr>
<td>pJC1-P&lt;sub&gt;recA&lt;/sub&gt;-e2-crimson</td>
<td>pJC1 derivative containing the promoter of <em>int2</em> (250 bp) fused to <em>e2-crimson</em>; the insert includes the promoter of <em>int2</em>, 30 bp of the coding sequence, a stop codon, and an additional ribosome binding site (<strong>pET16</strong>) in front of <em>e2-crimson</em></td>
<td>This study</td>
</tr>
<tr>
<td>pJC1-P&lt;sub&gt;recN&lt;/sub&gt;-e2-crimson</td>
<td>pJC1 derivative containing the promoter of <em>lysin</em> (250 bp) fused to <em>e2-crimson</em>; the insert includes the promoter of <em>lysin</em>, 30 bp of the coding sequence, a stop codon, and an additional ribosome binding site (<strong>pET16</strong>) in front of <em>e2-crimson</em></td>
<td>This study</td>
</tr>
<tr>
<td>pJC1-P&lt;sub&gt;cg2067&lt;/sub&gt;-e2-crimson</td>
<td>pJC1 derivative containing the promoter of <em>cg2067</em> (250 bp) fused to <em>e2-crimson</em>; the insert includes the promoter of <em>cg2067</em>, 30 bp of the coding sequence, a stop codon, and an additional ribosome binding site (<strong>pET16</strong>) in front of <em>e2-crimson</em></td>
<td>This study</td>
</tr>
</tbody>
</table>

For induction of the SOS response, mitomycin C (Sigma-Aldrich, Seelze, Germany) was added at the appropriate concentrations at an OD<sub>600</sub> of 4.

**Cloning techniques.** For PCR amplification of DNA used for cloning, KOD HotStart polymerase (Merck Millipore, Darmstadt, Germany) was used. DreamTaq (Fisher Scientific, Schwerte, Germany) was utilized for PCR verification of ligation reactions. Heat shock transformation of *E. coli* was performed as described previously (19). Transformation of *C. glutamicum* was performed by electroporation as described previously (20).

Isolation and purification of plasmids from *E. coli* cultures was performed using plasmid isolation and purification kits from Qiagen (Qiagen, Hilden, Germany) and Thermo Scientific (Fisher Scientific, Schwerte, Germany) miniprep kits. Chromosomal DNA of *C. glutamicum* was isolated as described previously (21). DNA sequencing and oligonucleotide synthesis were performed by Eurofins MWG Operon (Ebersberg, Germany). Plasmids and oligonucleotides used in this work are listed in Table 1 and Table 2, respectively. The in-frame deletion mutant of *lexA* in the wild-type strain ATCC 13032 was constructed as described by Jochmann et al. (22).
the ampiclon into the shuttle vector pK18mobsacB_cg1121/22. Promoter fusions of divS and recN were amplified with the oligonucleotides divS_fwd and divS_rev and the oligonucleotides recN_fwd and recN_rev, respectively, using genomic DNA as the template. Additionally, the first 10 codons were amplified along with a stop codon. The coding sequence of the amplicon into the medium-copy-number vector pJC1. Both amplicons were combined by overlap extension PCR. The restriction site NheI was used for ligation into the medium-copy-number vector pJC1.

The P_tac-e2-crimson promoter fusion was constructed according to Hentschel et al. (23) by amplifying 725 bp upstream of the ptsG start codon with oligonucleotides PptsG_fwd and PptsG_rev, introducing BamHI and NdeI restriction sites. The PCR fragment was ligated into pJCl-e2-crimson-term after treatment of both with BamHI and NdeI restriction enzymes.

Fluorescence microscopy. For phase contrast and fluorescence microscopy, samples were analyzed on 1 to 2% agar pads, which were placed on microscope slides and covered by a coverslip. Images were taken on a Zeiss Axiosplan 2 imaging microscope with an AxioCam MRm camera and a Plan-Apochromat 100 magnification, 1.4 numerical-aperture oil differential interference contrast (DIC) objective. Filter sets 46HE and 63HE were used for imaging enhanced yellow fluorescent protein (eYFP) and E2–Crimson fluorescence, respectively. Digital images were acquired and analyzed with the AxioVision 4.6 software (Zeiss, Göttingen, Germany).

Flow cytometry. Flow-cytometric measurements and sorting were performed on a FACSaria II (Becton, Dickinson, San Jose, CA) flow cytometer with 488-nm excitation by a blue solid-state laser and 633-nm excitation by a red solid-state laser. Forward-scatter characteristics (FSC) and side-scatter characteristics (SSC) were detected as small- and large-angle scatters of the 488-nm laser, respectively. eYFP fluorescence was detected using a 502-nm long-pass and a 530/30-nm band pass filter set. E2–Crimson fluorescence was detected using a 660/20-nm band pass filter set. Cell size was determined at a threshold rate of 3,000 to 4,000 events/s and sorted onto BHI agar plates at a threshold rate of 3,000 to 4,000 events/s. Data were analyzed using FlowJo V7.6.5 (Tree Star, Inc., Ashland, OR).

Statistical analysis. Nonparametric tests for the analysis of correlation were performed with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). Spearman’s rank correlation coefficient (the Pearson correlation coefficient of the ranked variables) was used. A perfect (inverse) correlation takes on p values of (−1); totally independent variables take on p values of 0.

**Microfluidic cultivation.** *C. glutamicum* was cultivated in in-house-developed microfluidic cultivation chambers (0.9 μm by 60 μm by 60 μm) arranged in parallel between 10-fold-deeper supply channels. For details on our microfluidic chip setup, see references 15 and 25.

During the experiment, CGXII minimal medium was infused continuously at 300 nl min⁻¹ using a high-precision syringe pump (neMESYS; Cetoni GmbH, Karlsruhe, Germany) with attached disposable syringes (OmniFlex P Tuberculin, 1 ml; Braun Melsungen AG, Melsungen, Germany) to maintain constant environmental conditions. Cell growth and eYFP fluorescence were observed at 10-min intervals by time-lapse imaging with a fully motorized inverted Nikon Eclipse Ti microscope (Nikon GmbH, Düsseldorf, Germany). Chip cultivation was performed at 30°C using a microscope incubator system (PeCon GmbH, Erbach, Germany). The microscope was equipped with a focus assistant (Nikon PFS) to compensate for thermal drift during long-term microscopy, with a Plan Apo λ 100× oil Ph3 DM objective (Nikon GmbH, Düsseldorf, Germany) and a high-speed charge-coupled device (CCD) camera (Andor Clara DR-3041; Andor Technology Plc., Belfast, United Kingdom). An optical filter system (YFPHQ filter system [excitation, 490 to 550 nm; dichroic mirror, 510 nm; absorption filter, 520 to 560 nm]; AHF Analysentechnik AG, Tübingen, Germany) and a mercury light source (Intensilight; Nikon GmbH, Düsseldorf, Germany) were installed for fluorescence microscopy.

**RESULTS**

The prophage CGP3 is spontaneously induced in single cells. In recent studies we observed a spontaneous excision of the prophage CGP3 in a small number of *C. glutamicum* cells cultivated in shake flasks with CGXII minimal medium (5). Transcriptome analysis revealed an upregulation of CGP3 genes (cg1890 to cg2071) upon induction of the SOS response by addition of the DNA-cross-linking antibiotic mitomycin C (A. Heyer and J. Frunzke, personal communication). In an effort to create appropriate tools to monitor prophage activity, we consulted reports on previous microarray experiments to determine which genes are suitable candidates. We constructed plasmid-based promoter fusions of the CGP3 genes cg2071 (integrate, int2), cg1974 (putative lysin), and cg2067 (hypothetical protein) to the coding sequence of the fluorescent protein E2–Crimson. To test their function, wild-type *C. glutamicum* ATCC 13032 cells were transformed with the

**TABLE 2 Oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5′→3′)</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>cg2114_del_1</td>
<td>TGATGCTCGGAGACCTTACATGG</td>
<td>Smal</td>
</tr>
<tr>
<td>cg2114_del_2</td>
<td>TGAAGTCTCCTGACGATCA</td>
<td>NheI</td>
</tr>
<tr>
<td>cg2114_del_3</td>
<td>TGATGCTCGGAGACCTTACATGG</td>
<td>NheI</td>
</tr>
<tr>
<td>cg2114_del_4</td>
<td>GCCTAGATGATCTCCTTAAAGTTAAATACTCTTTTTAATTTGGAAGAGGCGCGCGCG</td>
<td>NheI</td>
</tr>
<tr>
<td>PrecA_pK18_fwd</td>
<td>CGCGGATCCCGGGCGAGAGGGTGAGCGAT</td>
<td>MfeI</td>
</tr>
<tr>
<td>PprecA_YFP_rev</td>
<td>GCTCGACATATGATCTCCTTTTTAATTTGCTTATTGTTTTATTTGGAAGAGGCGCGCGCG</td>
<td>MfeI</td>
</tr>
</tbody>
</table>

*Restriction sites are underlined.*
promoter fusion constructs and treated with 2 μM mitomycin C to induce DNA lesions and subsequently trigger the SOS response. Samples were analyzed by flow cytometry and fluorescence microscopy (shown for P_int2) (Fig. 1A). The treated samples exhibited a highly induced P_int2 activity and morphological changes. The untreated cultures showed no significant P_int2 activity, yet, in agreement with earlier studies, P_int2 was highly induced in a small number of cells. Flow-cytometric analysis of untreated cultures revealed that a fraction of 0.01 to 0.08% of the cells exhibited a 5- to 160-fold higher P_int2 activity than the bulk of the population (Fig. 1B). Single cells with a high fluorescent signal (phage\(^+\)) and cells showing background fluorescence (phage\(^-\)) were sorted onto BHI agar plates, and their survival was assessed after incubation for 24 h. As expected, the survival rate of phage\(^+\) cells was significantly below that of phage\(^-\) cells (survival rates of 23% and 96%, respectively) (Fig. 2). Thus, cells showing an increased P_int2 activity were significantly impaired in their ability to resume growth on plates, which is likely caused by prophage excision and subsequent cell lysis.

**Spontaneous P_reca activity** in single cells. Since the host SOS response is a prominent trigger of lysogenic phases, we constructed a promoter fusion of the recA promoter to effp and integrated it into the genome of *C. glutamicum* ATCC 13032 at the intergenic region of cg1121 and cg1122 to test for a spontaneous SOS induction. As proof of principle, the P_reca-effp strain was cultivated in microtiter scale and the SOS response was induced by addition of mitomycin C in increasing concentrations. A strain with a truncation of lexA, the repressor of SOS genes, served as a reference strain which exhibits a maximally induced SOS response (Fig. 3A, black bar). At low concentrations (15 nM and 100 nM) of mitomycin C and in the ΔlexA strain, P_reca activity showed a bimodal distribution, with the majority of cells showing a slight increase in reporter signal and a smaller fraction of cells shifted toward an even higher signal. This bimodal state was not observed at higher concentrations (500 nM and 1,000 nM mitomycin C) (Fig. 3B). We subjected cells with the integrated P_reca-effp promoter fusion to flow-cytometric analysis to get more detailed insight into single-cell dynamics of the SOS response in *C. glutamicum* populations. Under standard cultivation conditions, we observed a spontaneous activity, analogous to the activities of CGP3 promoters (Fig. 3C). About 0.07 to 0.2% of cells showed a 12- to 18-fold increased reporter signal (SOS\(^+\) cells). These SOS\(^+\) cells had a reduced survival rate (recovery rate of 46% after sorting on BHI agar plates) (Fig. 2). It was tempting to hypothesize that these rare events may act as a bet-hedging strategy to ensure an increased fitness under changing environmental conditions. We sorted SOS\(^+\) cells on agar plates with different DNA damaging conditions yet saw no increased fitness under the tested conditions (data not shown).

**Spontaneous P_reca activity reflects a bona fide SOS response.** Next we tested whether spontaneous P_reca activity and reduced viability were indicative of a bona fide SOS response caused by potentially lethal DNA damage. The reporter strain harboring the integrated promoter fusion P_reca-effp was transformed with plasmids carrying promoter fusions of the two SOS-responsive genes *divS* and *recN*, respectively, fused to the autofluorescent reporter gene e2-crimson. Both genes were previously shown to be upregulated in the deletion mutant ΔlexA (22) or after induction with mitomycin C. Analysis of the dual reporter strain (P_reca-effp integrated into the genome, P_int2-e2-crimson plasmid-borne) by fluorescence microscopy and flow cytometry revealed a correlation of the P_reca signal to P_divS as well as P_reca signals. After gating of SOS\(^+\)
cells, the signals of both reporters were correlated in single cells by using Spearman’s rank correlation coefficient (Fig. 4). Both promoter fusions showed a high correlation ($P_{\text{divS}}$, $\rho = 0.78$, $P < 0.0001$, $n = 165$; $P_{\text{recN}}$, $\rho = 0.85$, $P < 0.0001$, $n = 131$) at the single-cell level. The control promoter fusion $P_{\text{recA}}$-e2-crimson was constructed and introduced into the $P_{\text{recA}}$-eyfp strain to exclude high correlation values due to factors other than sharing the same regulation. SOS+ cells were gated and their $P_{\text{recA}}$ signal correlated to the $P_{\text{ptsG}}$ reporter signal. Both signals displayed a low correlation ($\rho = 0.36$, $P < 0.0001$, $n = 248$). Thus, the strong correlation of $P_{\text{recA}}$ to $P_{\text{divS}}$ and $P_{\text{recN}}$ activities confirmed that a spontaneous $P_{\text{ptsG}}$ activity in single cells leads to a bona fide induction of the downstream SOS cascade.

**Time-lapse analysis of $P_{\text{recA}}$ activation dynamics.** SOS+ cells did not display a uniform fate, with some cells being able to survive on agar plates whereas others were not viable. For a time-resolved analysis of the SOS induction in single cells, we cultivated the $P_{\text{ptsG}}$-eyfp strain in an in-house-developed PDMS microfluidic system enabling spatiotemporal analysis of growing microcolonies by automated time-lapse microscopy. Single cells were seeded into the microfluidic cultivation chambers and cultivated for several generations in standard CGXII minimal medium under non-stressful conditions; images were acquired every 10 min. Again, we observed different fates of SOS+ cells (see Movies S1 to S3 in the supplemental material). Some cells showed a high and continuous reporter signal together with an elongated cell morphology and growth inhibition caused by an activated SOS response (Fig. 5; also, see Movies S1 to S3 in the supplemental material). These bacteria represent cells which undergo severe DNA damage, triggering the SOS response. However, other cells merely showed a pulse of the reporter along with an unaltered growth and cell morphology (Fig. 5; also, see Movies S1 and S2 in the supplemental material). This output either represents activity due to the stochastic binding of repressor molecules or might be triggered by DNA damage which is repaired before a full-blown SOS response is initiated. Furthermore, we observed an additional cell fate which consisted of high induction of the reporter coupled with an elongated cell morphology, growth inhibition, and cell branching (see Movie S3 in the supplemental material). This is analogous to *C. glutamicum* cells which continuously overexpress divS under the control of the promoter $P_{\text{recA}}$ (26), indicating a high concentration of DivS in these cells.

**Correlating prophage activity to an induced SOS response in single cells.** Having established promoter fusions for the analysis of spontaneous CGP3 induction and SOS response, we combined promoter fusions of *recA* and of the prophage genes to correlate

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**FIG 3** $P_{\text{recA}}$ promoter fusion for the analysis of SOS induction in single cells. (A) Dose response plotted as mean eYFP fluorescence at increasing mitomycin C (MmC) concentrations. (B) Offset histogram of *C. glutamicum*::$P_{\text{recA}}$-eyfp cells subjected to increasing concentrations of mitomycin C. *C. glutamicum* ΔlexA was used as a reference strain which exhibits maximal induction of the SOS response. (C) Scatter plot of the strain *C. glutamicum*::$P_{\text{recA}}$-eyfp cultivated under non-stressful conditions. A total of 100,000 cells were analyzed for their size characteristics (forward scatter) and their fluorescent properties. A small fraction of cells exhibits an increased reporter signal (green dots) in comparison to the bulk of the population (black dots). Cells were cultivated in CGXII medium plus 4% glucose in the Biolector system until the stationary phase.

**FIG 4** Correlation of $P_{\text{recA}}$ activity to the activity of promoters of downstream SOS genes. Scatter plots of *C. glutamicum*::$P_{\text{recA}}$-eyfp::pJC1-ΔdivS-e2-crimson (A), *C. glutamicum*::$P_{\text{ptsG}}$-eyfp::pJC1-ΔptsG-e2-crimson (B), and *C. glutamicum*::$P_{\text{recA}}$-eyfp::pJC1-ΔrecN-e2-crimson (C) are shown; *C. glutamicum*::$P_{\text{ptsG}}$-eyfp::pJC1-ΔptsG-e2-crimson served as a control, reflecting the correlation of *recA* expression and an SOS-independent promoter. Cells with high eYFP fluorescence (spontaneous $P_{\text{ptsG}}$ induction and SOS response) were combined and introduced into the $P_{\text{recA}}$-eyfp strain. The signals of both reporters were correlated in single cells by using Spearman’s rank correlation coefficient ($\rho = 0.78$, $P < 0.0001$, $n = 165$; $\rho = 0.85$, $P < 0.0001$, $n = 131$) at the single-cell level. The control promoter fusion $P_{\text{ptsG}}$-e2-crimson was constructed and introduced into the $P_{\text{ptsG}}$-eyfp strain. Both signals displayed a low correlation ($\rho = 0.36$, $P < 0.0001$, $n = 248$). Thus, the strong correlation of $P_{\text{ptsG}}$ to $P_{\text{divS}}$ and $P_{\text{recN}}$ activities confirmed that a spontaneous $P_{\text{ptsG}}$ activity in single cells leads to a bona fide induction of the downstream SOS cascade.

**FIG 5** Correlation of $P_{\text{recA}}$ activity to the activity of promoters of downstream SOS genes. Scatter plots of *C. glutamicum*::$P_{\text{recA}}$-eyfp::pJC1-ΔdivS-e2-crimson (A), *C. glutamicum*::$P_{\text{ptsG}}$-eyfp::pJC1-ΔptsG-e2-crimson (B), and *C. glutamicum*::$P_{\text{recA}}$-eyfp::pJC1-ΔrecN-e2-crimson (C) are shown; *C. glutamicum*::$P_{\text{ptsG}}$-eyfp::pJC1-ΔptsG-e2-crimson served as a control, reflecting the correlation of *recA* expression and an SOS-independent promoter. Cells with high eYFP fluorescence (spontaneous $P_{\text{ptsG}}$ induction and SOS response) were combined and introduced into the $P_{\text{recA}}$-eyfp strain. The signals of both reporters were correlated in single cells by using Spearman’s rank correlation coefficient ($\rho = 0.78$, $P < 0.0001$, $n = 165$; $\rho = 0.85$, $P < 0.0001$, $n = 131$) at the single-cell level. The control promoter fusion $P_{\text{ptsG}}$-e2-crimson was constructed and introduced into the $P_{\text{ptsG}}$-eyfp strain. Both signals displayed a low correlation ($\rho = 0.36$, $P < 0.0001$, $n = 248$). Thus, the strong correlation of $P_{\text{ptsG}}$ to $P_{\text{divS}}$ and $P_{\text{recN}}$ activities confirmed that a spontaneous $P_{\text{ptsG}}$ activity in single cells leads to a bona fide induction of the downstream SOS cascade.

**Correlating prophage activity to an induced SOS response in single cells.** Having established promoter fusions for the analysis of spontaneous CGP3 induction and SOS response, we combined promoter fusions of *recA* and of the prophage genes to correlate...
them at the single-cell level. To this end, the P<sub>recA</sub>-eyfp strain was transformed with the P<sub>int2</sub>, P<sub>divS</sub>, and P<sub>cgIm</sub> fusions. Analysis by flow cytometry again showed the occurrence of spontaneously activated cells for both types of promoter fusions. Fluorescence microscopy revealed that not all cells showed an activation of the prophage promoters when P<sub>recA</sub> was active (Fig. 6). To measure the interdependence of both signals, the Spearman rank correlation coefficient was calculated for both. The highest correlation to SOS<sup>−</sup> cells was observed for P<sub>cgIm</sub> and P<sub>int2</sub> (r<sub>S</sub> = 0.77, P < 0.0001, n = 687; r<sub>S</sub> = 0.57, P < 0.0001, n = 828) (Fig. 6). P<sub>int2</sub> showed the lowest correlation (r<sub>S</sub> = 0.44, P < 0.0001, n = 790) under the tested conditions. Even though the correlation coefficient is not able to reveal a causal link between two processes, it did show a high correlation of the promoters of recA and the prophage genes. Reciprocal analysis of phage<sup>−</sup> cells was performed as well and gave almost identical values (P<sub>cgIm</sub> r<sub>S</sub> = 0.72, P < 0.0001, n = 119; P<sub>int2</sub> r<sub>S</sub> = 0.58, P < 0.0001, n = 565; P<sub>divS</sub> r<sub>S</sub> = 0.45, P < 0.0001, n = 759) (data not shown). This correlation suggests a link between spontaneously induced SOS response and spontaneous activity of CGP3 in single cells. Nevertheless, correlation was lower than that between P<sub>recA</sub> and P<sub>divS</sub>/P<sub>cgIm</sub> indicating that others factors besides the SOS response might influence the activity of the prophage promoters.

Impact of growth phase on spontaneous SOS and prophage activity. The P<sub>recA</sub>-eyfp/pJC1-P<sub>int2</sub>-e2 crimson strain was cultivated in shake flasks, and samples were analyzed until the cells reached stationary growth phase (Fig. 7A) to eliminate the rigidity of measurements at single time points and better assess the characteristics of the reporters during growth. The P<sub>recA</sub> signal showed maximal intensity at the transition to and during the early phase of exponential growth. Activity of P<sub>recA</sub> showed the same behavior. We expected this parallel activation of both promoters, if the SOS response and prophage induction are somehow linked. The number of spontaneously activated cells was measured for all time points as well (Fig. 7B). Whereas the peaks of both reporters’ fluorescent output behaved similarly, the maximal number of spontaneously activated phage<sup>−</sup> and SOS<sup>+</sup> cells showed a temporal disparity. The increase of SOS<sup>+</sup> cells was observed 2 h before the relative amount of phage<sup>−</sup> cells increased.

**DISCUSSION**

The present study on the induction of lysogenic prophages was performed on a population-wide scale. It was shown that the induction of lambdoid phages is typically linked to the host’s SOS response. The underlying bistable switch (13), simplified by the action of the repressor of phage genes cI, is turned toward lytic growth when the host’s SOS response becomes activated. Spontaneous induction of prophages had been observed as far back as the 1950s (11), yet studies since then have not explored this phenomenon in more detail at the single-cell level. Due to the general link between the SOS response and prophage induction, it was tempting to speculate that the cause of spontaneous prophage induction lies in a spontaneously induced SOS response. The data shown in this study suggest that a small fraction of *C. glutamicum* cells grow under standard conditions spontaneously induced expression of prophage genes and that this activation is caused in part by the spontaneous activity of the SOS response in single cells.

During the cultivation of wild-type cells under standard cultivation conditions, we observed single cells that induced the SOS response spontaneously. As the occurrence of spontaneous DNA breakage has previously been reported in studies on *E. coli* (27), we tested this in *C. glutamicum*. In the *E. coli* studies, use of the SOS-inducible promoter of the cell division inhibitor gene *sulA* fused to *gfp* revealed a spontaneous SOS response in about 0.9% of cells. The rate of spontaneous SOS induction that we measured with P<sub>recA</sub>-eyfp lies at about 0.2%. When other LexA-regulated promoters of *C. glutamicum*, such as P<sub>divS</sub>, P<sub>recA</sub>, and P<sub>cgIm</sub>, were used, the fraction of cells exhibiting a spontaneous SOS response lay between 0.1 and 0.5% (data not shown). Considering that further studies in *E. coli* using promoter fusions of *lexA*, *recA*, and *umuDC* showed spontaneous promoter activity in 0.09 to 3.1% of cells (28), our findings are consistent with those reported previously.

We assumed the SOS<sup>+</sup> cells to be impaired in their survival due to potentially lethal DNA damage. The survival rate of SOS<sup>−</sup> cells lay at 46%. Our microfluidic studies showed that an induction of the SOS response leads to the arrest of cell growth in some cells, probably caused by irreparable DNA damage, whereas other cells are able to resume growth after they exhibit P<sub>recA</sub> activity. This activity in turn did not necessarily lead to a full-blown SOS response, as some cells showed the SOS phenotype; others, however, were either unaffected in their growth with a mere pulse of P<sub>recA</sub> activity or were inhibited in their growth but able to recover from the SOS-induced inhibition of cell division. The presence of some cells showing a short pulse of reporter signal, but no growth inhibition, might hint at stochastic fluctuations in the binding of the repressor LexA or at DNA damage which is so minor that it is repaired before a full-blown SOS response is stimulated.

Studies in *E. coli* have revealed that SOS genes show a heterogeneous expression which is independent of RecA and based on
stochastic factors and binding affinities of LexA to SOS boxes (28). To confirm that the spontaneous induction of \( P_{\text{recA}} \) is caused by a bona fide SOS response, we transformed the \( P_{\text{recA}} \) reporter strain with plasmids carrying transcriptional fusions of \( \text{divS} \) and \( \text{recN} \) promoters, respectively. The degree of correlation gained by calculating Spearman’s ranked correlation coefficient is strong (\( \rho_{\text{divS}}, 0.78 \); \( \rho_{\text{recN}}, 0.78 \)). Whereas this is a strong correlation, higher values of \( \rho \) (\( \rho_{\text{CGP3}}, 0.9 \)) might be expected for causal relationships, as they are present within the SOS cascade. Even though \( \text{recA} \) is upregulated after cells encounter DNA damage, this damage might be repaired before the cell division inhibitor \( \text{divS} \) is induced, thus avoiding a premature inhibition of growth. Our microfluidic experiments showed that this might be the case, as a high number of SOS/CGP3 cells are not inhibited in their growth.

The same applies to the correlation values of the prophage reporters which lie below those observed for the correlation to the SOS reporters. An induced SOS response need not seal the fate of genomic excision for CGP3. Rather, an immunity to low levels of DNA damage and an induction upon accumulation or lasting presence of DNA damage would constitute a more reliable warning system telling the prophage when to “leave the sinking ship.” If CGP3 were induced by every event of SOS response, the integration of the prophage would be a rather unstable situation. Thus, a threshold-based model of excision (29), as described for well-studied lambdoid phages, helps to explain the observed deviation from a perfect correlation. Nevertheless, future studies will elucidate the possible influence of so-far unknown regulators that play a role in the induction of CGP3. Microarray studies, for example, revealed that the putative regulator Cg2040 exhibits an inhibitory effect on a set of prophage genes when overexpressed (A. Heyer and J. Frunzke, personal communication).

Finally, we tested our dual promoter fusion strains (\( P_{\text{recA}}\text{-eyfp/pJC1-\text{lysin}-e2-crimson} \)) during a standard flask cultivation experiment to analyze their expression during the course of growth. We observed that \( P_{\text{recA}} \) and \( P_{\text{int2}} \) promoters reached their peaks of activation during exponential growth phase. It is reasonable to assume that this phase of rapid cell growth gives rise to intrinsic DNA damage, which is produced by native DNA polymerases. Indeed, it has been reported that replication fork breakage is a major contributor to double-strand breaks (DSBs), which in turn activate the SOS response (30, 31). The measurement of spontaneous activation of the SOS response and prophage activity showed temporally separated peaks (Fig. 7B). As this disparity occurs in a small number of cells, it is clear that this effect would be masked in the bulk measurement of reporter output, as shown in Fig. 7A.

While the consequence of spontaneously inducing prophages has been reported for Shewanella oneidensis (7) and Streptococcus pneumoniae (8), the effects on \( C. \) glutamicum on a population-wide scale remain unknown. In studies by Bossi and coworkers (32), a spontaneous induction of prophages led to a competitive fitness of the population against other bacteria. Spontaneous

![FIG 6 Correlation of \( P_{\text{recA}} \) reporter activity to the activity of CGP3 genes. Scatter plots of \( C. \) glutamicum \( P_{\text{recA}}\text{-eyfp/pJC1-\text{lysin}-e2-crimson} \) (A), \( C. \) glutamicum \( P_{\text{recA}}\text{-eyfp/pJC1-\text{int2}-e2-crimson} \) (B), and \( C. \) glutamicum \( P_{\text{recA}}\text{-eyfp/pJC1-Pcg2067-e2-crimson} \) (C) are shown. Cells with high eYFP fluorescence (SOS cells) were gated and their E2-Crimson fluorescence plotted against their eYFP fluorescence. Spearman’s rank correlation coefficient (\( \rho \)) was calculated using GraphPad Prism 6. (D) Fluorescence microscopy analysis of cells on agar pads showing the spontaneous induction of SOS and prophage reporters. Cells were cultivated in CGXII medium plus 4% glucose and analyzed at an OD600 of 4.](http://jb.asm.org/)

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The phenomenon of spontaneous prophage excision. We have established a first link between the host’s SOS response and the excision of the genomically integrated CGP3 prophage in single bacterial cells. Further studies will assess the level at which the two phenomena are linked and which other regulatory pathways may feed into the prophage’s decision to excise from the host genome.

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