**INTRODUCTION**

The SOS response is the major bacterial stress mechanism to cope with DNA damage provoked by mutagenic agents. The SOS regulon of *Escherichia coli* comprises 31 experimentally proven damage-inducible (*din*) genes (Fernández de Henestrosa et al., 2000). Microarray analyses identified upregulation of 163 genes when *E. coli* cells were exposed to UV light (Berney et al., 2006). The SOS regulon of the Gram-positive model organism *Bacillus subtilis* consists of 63 (hitherto known) genes organized in 23 operons (Friedberg et al., 1995; Goranov et al., 2006). As for *E. coli*, two major players regulate the SOS response in *B. subtilis*. The LexA homologue DinR blocks transcription of the *din* genes under non-inducing conditions (Groban et al., 2005; Miller et al., 1996). Subsequent to DNA damage, RecA (activated by ssDNA) facilitates the autoproteolytic cleavage of the dimeric repressor protein DinR (Little, 1984; Love & Yasbin, 1984; Lovett et al., 1994), eventually leading to the expression of the *din* genes (Love & Yasbin, 1984). As part of the general SOS response, Gram-negative as well as Gram-positive bacteria stall cell division. In *E. coli*, SulA inhibits formation of the Z-ring at the divisome by directly blocking FtsZ polymerization, whereas in *B. subtilis*, YneA presumably suppresses recruitment of late division proteins to the divisome by an as-yet-unidentified mechanism (Bi & Lutkenhaus, 1993; Huisman et al., 1984; Kawai et al., 2003; Kawai & Ogasawara, 2006; Mukherjee et al., 1998). For termination of the SOS response – to regain the cell division capacity – such division blockage has to be abrogated. In *E. coli*, SulA is degraded by the Lon protease to resume cell division (Mizusawa et al., 1983). In *B. subtilis*, the YneA protein appears to be inactivated after signal peptide cleavage when the cleaved-off protein is released into the medium and degraded (Mo & Burkholder, 2010).

Since *Bacillus megaterium* differs from the model organism *B. subtilis* in a number of important aspects with respect to coping with DNA damage, such as the very high level of UV resistance and the existence of two *recA* genes as well as two *ftsZ* copies (English & Vary, 1986; Eppinger et al., 2011; Nahrstedt et al., 2005a), we were eager to learn more about the *recA*-dependent inhibition of cell division (ICD) as part of the SOS response in *B. megaterium* and about the players involved.

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**yneA mRNA instability is involved in temporary inhibition of cell division during the SOS response of *Bacillus megaterium***

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The SOS response, a mechanism enabling bacteria to cope with DNA damage, is strictly regulated by the two major players, RecA and LexA (*Bacillus* homologue DinR). Genetic stress provokes formation of ssDNA-RecA nucleoprotein filaments, the coprotease activity of which mediates the autocatalytic cleavage of the transcriptional repressor DinR and ensures the expression of a set of *din* (damage-inducible) genes, which encode proteins that enhance repair capacity, accelerate mutagenesis rate and cause inhibition of cell division (ICD). In *Bacillus subtilis*, the transcriptional activation of the *yneAB–ynzC* operon is part of the SOS response, with YneA being responsible for the ICD. Pointing to its cellular function in *Bacillus megaterium*, overexpression of homologous YneA led to filamentous growth, while ICD was temporary during the SOS response. Genetic knockouts of the individual open reading frames of the *yneAB–ynzC* operon increased the mutagenic sensitivity, proving – for the first time in a *Bacillus* species – that each of the three genes is in fact instrumental in coping with genetic stress. Northern- and quantitative real-time PCR analyses revealed – in contrast to other *din* genes (exemplified for *dinR, uvrBA*) – transient mRNA-presence of the *yneAB–ynzC* operon irrespective of persisting SOS-inducing conditions. Promoter test assays and Northern analyses suggest that the decline of the ICD is at least partly due to *yneAB–ynzC* mRNA instability.

**Abbreviations:** ICD, inhibition of cell division; MMC, mitomycin C.

A supplementary table and two supplementary figures are available with the online version of this paper.
 METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. Cultivation was carried out at 37°C in Luria–Bertani (LB) broth or minimal medium containing 100 ml 10 x salt solution per litre (60 g Na₂PO₄ 1⁻, 30 g KH₂PO₄ 1⁻, 10 g NH₄Cl 1⁻, 5 g NaCl 1⁻, pH 7.4), 0.2% glucose, 1 mM MgSO₄ 2⁻, 0.02% Casamino acids, 0.1 mM CaCl₂, 0.01% yeast extract and 0.2 μg MnSO₄ ml⁻¹, pH 7.4. Leucine auxotrophic cells were cultivated in minimal medium containing 0.5% (w/v) glucose, 11 mM MnSO₄, 100 ml KP buffer (440 mM KH₂PO₄, 803 mM K₂HPO₄, pH 7.2) and 100 ml salt solution [151 mM (NH₄)₂SO₄, 8 mM MgSO₄ and 34 mM trisodium citrate] per litre. For selection of recombinant E. coli cells, media were supplemented with ampicillin (100 μg ml⁻¹). Recombinant B. megaterium cells were selected with tetracycline (1.25 or 12.5 μg ml⁻¹), erythromycin (0.3 or 5 μg ml⁻¹) or chloramphenicol (4.5 μg ml⁻¹); low concentrations in combination with the non-permissive temperature of 42°C served for obtaining single-copy states as a result of chromosomal integration; high concentrations of the respective antibiotics at the permissive temperature of 30°C selected cells with freely replicating (multi-copy) plasmids. The SOS response was induced by treating cells with 0.2–0.4 μg mitomycin C ml⁻¹ (MMC; Calbiochem).

DNA techniques and transformation methods. Molecular cloning procedures in E. coli were carried out as described by Sambrook & Russell (2001). Plasmids used in this study are listed in Table 1. Plasmid DNA was purified using JETSTAR columns (Genomed). Genomic DNA from Bacillus strains was isolated as previously described (Nahrstedt & Meinhardt, 2004). For in vitro amplification of DNA, PCRs contained 200 μM dNTPs, 100 ng template DNA, 1 pmol of each primer and 1 U Taq (New England Biolabs) or Phusion (Finnzymes Thermo Fisher Scientific) DNA polymerase according to the manufacturer’s recommendations. Primer design was based on the B. megaterium genome database Megabac 9 (www.megabac.tu-bs.de). Primers are listed in Table S1 (available in Microbiology Online). Purification of restriction fragments or PCR products after gel electrophoresis was carried out using the QIAquick Gel Extraction kit (Qiagen). Plasmids were introduced into B. megaterium cells by PEG-mediated protoplast transformation (Brown & Carlton, 1980; Meinhardt et al., 1989; Vorob’eva et al., 1980). Nucleotide sequences were determined by the dye-deoxy chain-termination method (Sanger et al., 1977) using fluorescent-labelled dideoxynucleotides and the BigDye Terminator v3.1 sequencing kit (Applied Biosystems) in combination with an ABI Prism capillary sequencer, model 3700.

RNA techniques. For isolation of RNA, B. megaterium cells were grown in minimal medium to the OD₅₆₅ of 1.8. When required, cultures were supplemented with 0.2 μg MMC ml⁻¹. Cell samples were taken at 30 min intervals, and RNA was isolated using hot phenol as previously described (Nahrstedt & Meinhardt, 2004). For Northern blots, 20 μg of total RNA from B. megaterium was separated in a 1.5% (w/v) formaldehyde agarose gel with MOPS running buffer and subsequently transferred to nylon membranes by vacuum blotting. Sizes were estimated by comparison to the RNA Molecular Weight Marker 1 (Boehringer). Signal detection was performed using CDP-Star (Roche Diagnostics). RNA probes were generated by

Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source/reference</th>
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<td>Escherichia coli DH5α</td>
<td>endA hsdR17 (rK mK+) supE44 thi-1 recA1 gyrA96 relA1 (lacIYSZ-) argF U169 deoR F’ (Φ80dIacZ Δ (lacZ) M15)</td>
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<td>Bacillus megaterium DSM 319</td>
<td>Wild-type (plasmid-free)</td>
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<td>Schmidt et al. (2005)</td>
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<td>Plasmids</td>
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<td>pMM1522</td>
<td>Shuttle vector for cloning in E. coli (Ap&lt;sup&gt;R&lt;/sup&gt;) and gene expression in B. megaterium (Tc&lt;sup&gt;R&lt;/sup&gt;), derivative of pMM1520 with additional BsrGI site</td>
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<td>E. coli cloning vector; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>E. coli/Bacillus-shuttle vector for gene replacement, Amp&lt;sup&gt;R&lt;/sup&gt; Em&lt;sup&gt;R&lt;/sup&gt; ori&lt;sup&gt;α&lt;/sup&gt;</td>
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<td>Bacillus knock out vector, Em&lt;sup&gt;R&lt;/sup&gt; ori&lt;sup&gt;α&lt;/sup&gt;</td>
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<td>pHV33</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt; (E. coli) Tc&lt;sup&gt;R&lt;/sup&gt; (Bacillus) Cm&lt;sup&gt;R&lt;/sup&gt; (E. coli and Bacillus)</td>
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<td>Integrative promoter test vector; ΔclyC::ΔbgaM, Tc&lt;sup&gt;R&lt;/sup&gt;, Ap&lt;sup&gt;R&lt;/sup&gt; ori&lt;sup&gt;α&lt;/sup&gt; E. coli ori&lt;sup&gt;α&lt;/sup&gt;</td>
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<td>pSPT19</td>
<td>Vector for in vitro transcription of yneA</td>
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directed ligation of an internal PCR fragment using primers dinR3/dinR4, for the dinR probe, or the entire sequence of yneA into pSPTyneA, respectively. The resulting products, pSPTdinR and pSPTyneA, were linearized with BsiWI and subsequently used for in vitro transcription using the DIG RNA labelling kit (Roche Diagnostics). For Northern experiments concerning mRNA stability, RNA was isolated from cells growing exponentially in minimal medium 20 min post SOS induction by MMC. Cells were collected prior to (t0) and after (t2–20) addition of 100 µg rifampicin ml⁻¹ by pelleting the samples in equal volume of killing buffer (0.13 % NaN₃, 2 mM Tris/HCI, 1 mM MgCl₂). RNA was isolated by disrupting the cells with a dismembrator (see below) and applying TRIzol reagent (Life Technologies) following the manufacturers’ recommendations. Total RNA (3 µg) was separated on a 1.2 % formaldehyde agarose gel and blotted by capillary transfer to a positively charged nylon membrane (Roche Diagnostics). RNA integrity was checked by staining the membrane with methylene blue (0.2 % methylene blue, 0.3 M trisodium citrate). RNA sizes were estimated with the aid of the RiboRuler High Range RNA Ladder (Thermo scientific). RNA probes were synthesized by in vitro transcription with T7 polymerase (Roche Diagnostics) and PCR-generated T7 polymerase promoter sequence introduced into the template by the reverse primer (see Table S1; dinR: dinR_NB1/dinR_NB2; yneAB–yneC. yneA_p/yneA_NB2). Hybridization and immunological detection were performed by applying CDP-Star following the manufacturer’s recommendations (Roche) with a hybridization and stringency wash temperature of approximately 72 °C. For real-time experiments, RNA was isolated by applying the High pure RNA isolation kit (Roche Diagnostics) with the following modifications: 2.5 ml culture pellets resuspended in 400 µl Tris/HCl (pH 8) were transferred into 1.5 ml cryo-tubes (Nalgene) filled with 300 mg acid-washed glass beads (150–212 µm; Sigma-Aldrich) and disrupted for 3 min at 2700 r.p.m in a Mikro-Dismembrator S (Sartorius). cDNA treatment of RNA samples was performed with the RNase-free DNase I Set (Qiagen). Purification of cDNA I-treated RNA samples was carried out by isopropyl alcohol precipitation. RNA samples were quantified with the Nanodrop ND-1000 UV-VIS spectrophotometer (Peqlab Biotechnologie). The absence of DNA was confirmed by PCR. cDNA samples (2 µg) were applied for cdNA synthesis with the RevertAid H M Inus First Strand cDNA Synthesis kit using random hexamer primers (Fermentas). Primers for real-time experiments were designed using Primer Express v3 (Applied Biosystems) and purchased from Invitrogen. RT-PCR were performed in a 48-well plate on a StepOne Real-Time PCR System (Invitrogen) with a total volume of 20 µl, containing 2 × Fast SYBR Green Master Mix (Applied Biosystems by Life Technologies), 0.2 µl each primer at 100 nM, 20 ng cDNA and nuclease-free demineralized water. The one-step amplification protocol started with an initial denaturation at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. Fluorescence data were collected during the 60 °C annealing and extension step. Finally, a melt curve was performed with a temperature range from 60 to 95 °C. The results were visualized and analysed with StepOne software v2.0.2 (Applied Biosystems by Life Technologies). Non-template controls (NTC) served as negative controls and the B. megaterium rpoB gene served as an endogenous control for normalization. Relative gene expression levels were determined by applying the CΔ method. All samples were normalized against the uninoculated culture at time point 0, which served as the calibrator. Duplicate real-time analyses were performed in three independent experiments.

Overexpression of yneA. For construction of an yneA expression vector, a 428 bp fragment, carrying the promoterless yneA ORF, was amplified by PCR with primers E-yneA_rev and E-yneA_for. The fragment was cut with SnaB1 and SpeI and ligated into the likewise linearized vector pMM1522. In the resulting xylene-inducible pMMyneA, YneA is fused to five N-terminal amino acids of XylA. Induction was done with 0.5 % (w/v) xylene.

Gene disruption and deletion experiments. The temperature-sensitive shuttle vector pSKE194 (Nahrstedt et al., 2005b) was used for targeted gene disruption and deletion of yneA and yneC. For disruption of yneA, vector pSKEyneA::cat was constructed: a 905 bp fragment, consisting of the yneA gene sequence with parts of the flanking sequences of dinR and yneB, was amplified with primer pair ynea1/ynea2 and cloned into the Smal linearized vector pUCBM20. A Smal site was inserted into the resulting vector in the centre of yneA by mutagenesis PCR using the primer yneA_mut1/yneca2. Subsequently, a chloramphenicol resistance cassette, amplified from the plasmid pHV33 with primers cat1/cat2 was inserted into the generated Smal site. The resulting vector was cut with PstI to excise the yneA disruption cassette, which was ligated into the PstI site of the shuttle vector pSKE194. For the clean deletion of yneA, a vector was constructed by amplifying two recombination flanks from chromosomal DNA of B. megaterium: flank A was amplified with primers Bmeg_ynea1/Bmeg_ynea2 and flank B was created with primers Bmeg_ynea3/Bmeg_ynec4. Due to an inserted half Smal site by the primer Bmeg_ynec2, both flanks could be subsequently ligated into a Smal site of pUCBM20. The resulting yneA deletion cassette was subcloned into the single PstI site of shuttle vector pSKE194 resulting in pAyneA. Cloning of the deletion vector pAyneC was performed as for pAyneA, except applying primer pairs Bmeg_ynec1/Bmeg_ynec2 and Bmeg_ynec3/Bmeg_ynec4 for flank A and B. The vector for deletion of yneB was constructed by ligating the yneB-region, amplified by primer pair Bmeg_ynec1/Bmeg_ynec2 from chromosomal DNA of B. megaterium, into the EcoRV-linearized pEO07 vector (Borgmeier et al., 2012). From the resulting plasmid, a PCR product was amplified by primer pair Bmeg_ynec2/Bmeg_ynec3, which was ligated to gain pAyneB.

B. megaterium clones carrying the freely replicating vector were selected on agar plates containing 5 µg erythromycin ml⁻¹ at the permissive temperature of 30 °C, whereas clones with a chromosomally integrated vector were selected with 0.3 µg erythromycin ml⁻¹ at the non-permissive temperature of 42 °C. For curing, single colonies with the integrated vector were subcultivated on LB plates without antibiotics at 42 °C. Plasmid-free clones were screened by PCR with primer pair dinR6/ynec1 for the yneA disruption, dinR6/Bmeg_ynec2 for the yneA deletion, Bmeg_ynec1/Bmeg_ynec4 for the yneB deletion or Bmeg_ynec3/ynec4 for the yneC deletion, respectively. The yneA disruption mutant was additionally screened on LB plates containing chloramphenicol (4.5 µg ml⁻¹).

Complementation of the yneA::cat disruption mutant was performed by introducing the yneA expression vector pMMyneA into the mutant followed by comparative inspection in a MMC survival assay with the wild-type strain and the disruption mutant carrying the empty plasmid pMM1522. 0.1 % xylene served to ensure yneA expression.

Construction of a promoter test system and β-galactosidase assay. The intergenic region between dinR and yneA was amplified from chromosomal DNA of B. megaterium with primer pair interBm1/interBm2. The 120 bp amplicon was inserted into the Smal linearized promoter test vector ppts to result in plasmid pptsyneA, in which the PdinR gene is fused to the β-galactosidase-encoding bgalA gene of B. megaterium that can be monitored in the background of the β-galactosidase-negative strain B. megaterium MS021 (Schmidt et al., 2005). Clones carrying the multi-copy plasmid were selected on LB plates containing 12.5 µg tetracycline ml⁻¹. Plasmid pptsyneA was also integrated into the chromosomal leuC locus (Meinhardt et al., 1994) to generate B. megaterium MS061. Replacement of the chromosomal leuC locus by the PyneA test cartridge was monitored by PCR with primers leuC_anker2/leuC_anker4 and phenotypically by leucine auxotrophy. Strains were cultivated in 50 ml minimum medium with or without antibiotics at 30°C at 180 r.p.m. to an OD566 of 1. Subsequently, this was divided into two subcultures, one...
of which was treated with 0.2 μg mitomycin C ml⁻¹ to induce the SOS response. During 3 h of further cultivation, 500 μl samples were taken every 30 min in duplicates. The promoter activity was quantified by measuring β-galactosidase activity using ONPG as the substrate, essentially as described by Schmidt et al. (2005). For the calculation of Miller units, the cell density was determined (Miller, 1972). Promoter test assays were performed in duplicates in three independent experiments.

UV and MMC survival measurements. LB cultures of B. megaterium cells were adjusted to the same optical density prior to plating of appropriate dilutions on LB plates containing 0.3 μg MMC ml⁻¹ or LB plates free of any supplements for UV irradiation. Plated cells were irradiated with UV and kept overnight in the dark at 37 °C. Survival was calculated by relating the number of colonies on UV irradiated/MMC-containing plates to those on non-irradiated/MMC-free LB plates. Quantitative sensitivity assays were performed as triplicates. For rapid qualitative UV tests, cultures were adjusted to identical optical densities and diluted 2:1 in 15 mM NaCl. Dilutions (10 μl) on LB plates were exposed to different UV intensities. For qualitative MMC testing, 7 μl of undiluted and diluted (10⁻¹–10⁻⁴) cultures were spotted onto LB plates containing increasing MMC amounts (0.2–0.4 μg ml⁻¹).

Microscopy and cell length distribution. For fluorescence microscopy and cell length distributions, samples were taken at timely intervals as indicated in the respective figures. Microscopy was performed with an Olympus BX51 fluorescence microscope and a CC12 Soft Imaging Camera (Olympus Soft Imaging Solutions). Image analysis was performed with analySIS FIVE software (Olympus Soft Imaging Solutions) and cell sizes were measured with the included length determination tool.

RESULTS

The dinR–yneAB–ynzC region in B. megaterium resembles that of B. subtilis (Kawai et al., 2003) and that is why we focused on these loci to genetically investigate the SOS-induced ICD. The tricistronic operon (yneAB–ynzC) is adjacent and reversely orientated to the dinR gene (for a

![Fig. 1. Schematic representation of the yneAB–ynzC operon in the wild-type B. megaterium DSM 319 and knockout mutants. Open reading frames are depicted as arrows; hairpins indicate factor-independent transcriptional terminators. (a) The yneAB–ynzC operon in B. megaterium DSM 319 (wt). Transcripts are sketched as solid arrows underneath the operon. Sequence details of the intergenic promoter region of yneAB–ynzC and dinR: probable promoter elements (−10, −35 region) and ribosome-binding sites (S/D) are underlined. DinR boxes are depicted inversely. (b) yneA insertion (MS067) and deletion (MS081) mutants. (c) yneB deletion (MS075) and (d) ynzC deletion (MS074) mutants.](http://mic.sgmjournals.org)
schematic representation see Fig. 1a). Based on the genetic organization and in conclusion with the identified mRNA sizes (not shown here, but see Figs 5 and 7), transcription of the dinR gene and the yneAB–ynzC operon starts from a shared intergenic promoter region in which three putative binding sites for the DinR repressor (DinR boxes, Fig. 1a) were identified. DinR Box I/II and DinR Box III have two and three mismatches respectively, but clearly agree with the consensus sequence 5′-CGAACRNRYGTTYC-3′ in B. subtilis (Winterling et al., 1998). The predicted B. megaterium DinR protein is 80% identical to the corresponding protein of B. subtilis, whereas the inversely orientated yneA of B. megaterium encodes a protein that merely exhibits 30% identity to the corresponding polypeptide of B. subtilis.

Although the overall identity is seemingly low, the presence of the signal peptide as well as the LysM domain suggests similar cellular functions as for the B. subtilis YneA protein (Bateman & Bycroft, 2000; Bendtsen et al., 2004; Kawai et al., 2003; Mo & Burkholder, 2010).

To functionally analyse the yneAB–ynzC operon, two different experimental approaches were followed: (i) construction of knockout mutants and (ii) inducible overexpression. In Fig. 1, the genetic structures of knockout mutants MS067 (yneA::cat), MS081 (ΔyneA), MS075 (ΔyneB) and MS074 (ΔynzC) are depicted. When the yneA mutants MS067 and MS081 were analysed with respect to MMC and UV-light resistance, their increased sensitivity to the DNA damaging agents became obvious. Furthermore, results obtained with the yneB and ynzC mutants tested with MMC proved – for the first time in a member of the genus Bacillus – SOS functionality of both genes, each contributing to cope with genetic stress (Fig. 2). Since no polar effects on the transcription of downstream genes were detected in real-time PCR experiments as well as in MMC sensitivity assays when compared to the deletion mutant MS081 and a mutant comprising the whole operon (data not shown), we used disruption mutant MS067 (yneA::cat) in our experiments. When the MS067 mutant was complemented with the pMMyneA vector and checked for MMC sensitivity, the yneA mutation turned out to be solely responsible for the MMC sensitive phenotype (Fig. S1).

While growth was not affected, the inducible overexpression of YneA – as for B. subtilis (Kawai et al. 2003) – resulted in the ICD eventually leading to giant cells up to 100 μm in size (Fig. 3). However, large cells were hardly observable in wild-type cultures exposed to SOS-inducing conditions. Hence, the precise determination of the cell length distribution in MMC-treated cultures was systematically performed including the yneA knockout mutant.

**Fig. 2.** Sensitivity of yneAB–ynzC mutants to mutagenic agents. (a) B. megaterium DSM 319 (wt) and yneA, yneB and ynzC knockout mutants on media with increasing MMC concentrations. (b) Survival of B. megaterium DSM 319 (wt) and the yneA disruption mutant MS067 (yneA::cat) treated with 0.3 μg MMC ml⁻¹. (c) B. megaterium DSM 319 (wt) and the yneA disruption mutant MS067 (yneA::cat) after UV irradiation (100 J m⁻²).
MS067 (yneA::cat) as the negative control. Size distributions of treated and non-treated cells did not differ at the beginning of the experiment (Fig. 4). After 120 min, the sizes of the (MMC-treated or untreated) yneA::cat mutant cells still matched the sizes of the untreated wild-type cells, while MMC-treated wild-type cells were almost double in length. Surprisingly, however, after 270 min size differences for the cells exposed to MMC and the control could no longer be seen. Apparently, inhibition of cell division (ICD) did not persist though cells were still exposed to MMC.

To check whether SOS transcripts fade away when ICD ceases, we performed Northern analyses of MMC-exposed cells. Since dinR itself is damage-inducible, its expression was analysed in parallel to yneA. As depicted in Fig. 5, transcription of both genes (yneA and dinR) was enhanced 30 and 60 min post MMC addition. Constant high levels of the dinR transcript up to 120 min were indicative of a persisting SOS response. However, transcripts of the yneA gene could only be seen up to 60 min post MMC addition.

To precisely address transcript levels, quantitative real-time PCR analyses were performed including the yneA operon, the adjacent dinR (see Fig. 1a) and – as an experimentally proven SOS-locus in B. megaterium (Nahrstedt & Meinhardt, 2004) – the uvrBA operon. The results shown in Fig. 6 in general agree with the Northern analyses: as anticipated, transcript levels of all genes increased significantly after induction. The transcript level of the yneA operon became highest after 30 to 60 min and then rapidly dropped whereas transcripts of the other damage-inducible genes (dinR, uvrB, uvrA) were still abundant, reaching a maximum after 90 min when the transcripts of the former had almost completely declined (for the control see Fig. S2).

As SOS gene regulation in bacteria is rather often due to promoter regulation (Au et al., 2005; Winterling et al., 1998), we performed reporter gene analyses of the yneA promoter by making use of the bgaM gene, which encodes...
the *B. megaterium* β-galactosidase (Schmidt et al., 2005; Strey et al., 1999). Accordingly, for both the single- and multi-copy state (chromosomally integrated and vector based, respectively), a decline in transcription initiation is not likely to cause the reduction of the yneA-transcripts during the SOS response as – over the time-course checked – the reporter assays proved progressively increasing promoter activity in MMC-treated cultures (Fig. 7a). To further check whether transcript instability could be involved in the shut-off of the SOS-induced ICD we performed Northern blot analyses with RNA from rifampicin treated cells. In contrast to the dinR (0.62 kb) transcript, which was visible for at least 10 min after the addition of rifampicin (Fig. 7c), the yneAB–ynzC transcript (1.35 kb) was almost undetectable already after 1 min (Fig. 7d).

**DISCUSSION**

Since overexpression of homologous YneA in *B. megaterium* led to a filamentous cell phenotype, the protein’s potential to act as the inhibitor of cell division as for *B. subtilis* (Kawai et al., 2003) became evident. However, unlike the latter, induced overexpression of YneA (Mo & Burkholder, 2010) in *B. megaterium* did not abolish single colony formation on solid medium (not shown). Cell length distributions in the MMC-treated wild-type and respective knock out mutants demonstrated that YneA is

![Fig. 4. Time-course of cell length distribution after SOS induction. *B. megaterium* DSM 319 (wt) cells (left side) and yneA disruption mutant MS067 (*yneA::cat*) (right side). Cells were cultivated until mid-exponential growth phase and subdivided into a control without MMC (grey bars) and with 0.2 μg MMC ml⁻¹ (black bars).](image-url)
responsible for the ICD during the SOS response as there was no detectable size increase of the yneA knockout mutant. Since B. megaterium possesses two recA genes, of which recA2 is apparently essential (Nahrstedt et al., 2005a), it is at present not feasible to check the possible interdependency with RecA, which acts, when activated by DNA damage, as a coprotease for DinR (LexA) degradation.

The isolation of E. coli lexA mutants constitutively executing the SOS response was only achievable when the expression of the inhibitor of cell division, SulA, was in parallel turned off (Friedberg et al., 1995; Mount, 1977), whereas in B. subtilis the respective dinR knockout mutants were readily obtainable (Hajjema et al., 1996; Kawai et al., 2003; McKenzie et al., 2000). However, in our hands, the deletion of dinR was not feasible in B. megaterium (not shown), once more suggesting differences from B. subtilis.

The microscopically proven transient ICD as part of the B. megaterium SOS response agrees with Northern and quantitative real-time PCR data, which – with MMC being present along the way – evidenced only a temporary occurrence of the yneA transcript. Indeed, as the SOS response held up, dinR and uvrBA displayed high expression levels for a rather long period of time, i.e. for 120 min post addition of MMC. Since the results obtained from the promoter test analyses suggest the yneA transcript disappearance not being due to a down-shift in transcription initiation, Northern analyses on transcript instability pointed to rapid yneA–ynzC mRNA turnover that is likely to be involved in the abrogation of ICD.

Besides transient expression, different induction levels of the three genes of the tricistronic operon are noteworthy: yneB transcript signals became most intense, while those for ynzC were only rather poorly enhanced. From B. subtilis transcriptome analyses, such different mRNA levels of a number of genes from several din operons, including the yneA–ynzC operon, were found to correlate negatively

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**Fig. 5.** Transient expression pattern of the yneA transcript detected by Northern blot analysis. Cells were cultivated in minimal medium until the mid-exponential growth phase and subdivided into a control culture (−) and a culture supplemented with 0.2 μg MMC ml⁻¹ (+). Samples were taken every 30 min up to 120 min after MMC treatment. Transcripts were detected with probes specific for yneA and dinR.

**Fig. 6.** Transcription of SOS-induced genes relatively quantified by real-time PCR. (a) Schematic representation of the dinR–yneAB–ynzC locus and the uvrBA locus. ORFs are depicted as arrows, the direction of which corresponds to their transcriptional orientation. Amplicons for relative quantification are given as solid squares. (b) Expression patterns of the repressor encoding dinR, the yneAB–ynzC operon and the uvrBA operon, respectively, of B. megaterium cells 0 to 120 min after addition of MMC (0.2 μg ml⁻¹). Error bars denote RQmin and RQmax.
with the genes’ relative distance to the promoter (Au et al., 2005). However, the high levels of yneB transcripts of MMC-treated B. megaterium cells suggest more complex regulation.

As the predicted yneB gene product displays the catalytic domain of the serine recombinase family, YneB could well be involved in recombinational DNA-repair during the SOS response (Au et al., 2005). Indeed, the functional analyses of the yneB and the ynzC genes by genetic knockouts performed in this study evidenced their participation in the SOS-dependent survival of DNA damage.

In E. coli, the permanent expression of the SOS response-dependent inhibitor of cell division (SulA) is lethal (Schoemaker et al., 1984). During the SOS response, permanent cell elongation due to SulA expression is overridden by the action of the Lon protease (Gottesman et al., 1981; Mizusawa et al., 1983). Consistently, Lon-negative E. coli cells grow as long filaments, and Lon-negative mutants, when constantly exposed to conditions inducing the SOS response, display a lethal phenotype that can, however, be bypassed by a loss of function mutation within the sulA gene (Friedberg et al., 1995; McKenzie et al., 2000; Mount, 1977). Although there has been no lethal phenotype described to date in B. subtilis, the fact that yneA-overexpression led to the lack of the capability to form colonies on agar plates is remarkable (Mo & Burkholder, 2010). Since YneA was reported to be inactivated due to signal peptide cleavage along with its proteolytic degradation, it was hypothesized that YneA degradation is responsible for the time limited ICD. However, a mutation in the C-terminal part stabilizing the full-sized YneA protein nevertheless resulted in the

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**Fig. 7.** Analyses of yneAB–ynzC expression and mRNA stability. (a) Promoter test assay of the yneAB–ynzC promoter (PyneA) making use of the bgaM reporter gene (B. megaterium β-galactosidase). Values are given as Miller units (MU). Squares correspond to single-copy expression (■; PyneA-bgaM fusion chromosomally integrated into the leuC locus); triangles correspond to multi-copy expression (△; PyneA-bgaM fusion in vector pptsyneA). Cultures were grown until mid-exponential phase and subsequently divided into the control (open symbols) and a culture supplemented with 0.2 μg MMC ml⁻¹ (closed symbols). (b–d) Northern analysis of yneAB–ynzC and dinR transcript (in)stabilities. Total RNA was isolated from wild-type cells in the presence or absence of 0.2 μg MMC ml⁻¹ prior to (~10, 0 min) and after (1, 2, 5, 10 min) the addition of rifampicin. The integrity of total RNA was checked by methylene blue staining (b). Transcript stability was checked with RNA probes specific for the dinR (~0.62 kb) transcript (c) and the yneAB–ynzC (~1.35 kb) transcript (d). Positions of the full-length transcripts and rRNA bands are indicated (solid arrowheads).
transient elongation phenotype (Mo & Burkholder, 2010) and that is why we assume another mechanism is possibly involved in controlling ICD duration. Since yneAB–ynzC transcripts occur only temporarily during the B. megaterium SOS response and are clearly less stable than the dinR transcripts, while transcription initiation from the respective promoter still persists, a regulation mechanism that may include mRNA instability is conceivable.

Recently, the essential RNase Y, part of the so-called RNA-degradosome, was discovered in B. subtilis (Lehnik-Habrink et al., 2011; Shahbabian et al., 2009). RNase Y as well as RNase J1 were identified as key enzymes for mRNA turnover. Depletion of RNase Y led to the accumulation of ~550 mRNAs and to the disappearance of ~350 mRNAs. Among the most stabilized transcripts were those responsible for the adaptation to environmental stress such as biofilm formation or to alternative nutrient consumption such as Amadori catabolism (Deppe et al., 2011a, b; Lehnik-Habrink et al., 2011). In E. coli, the functional homologue of RNase Y, RNase E, was recently shown to be involved in the regulation of the SOS response in such a way that the downregulation of RNase E precluded the initiation of the SOS response (Manasherob et al., 2012). Overexpression of a polypeptide interacting with the C-terminal part of RNase E, the RraA protein (a probable regulator of RNase E) rescued the cells from the negative impact on the SOS response caused by RNase E depletion. Interestingly, overexpression of RraA led to a filamentous phenotype, just as for RNase E-depleted cells, suggesting a link between RNase E modulated mRNA instability and ICD. Though the impact of the major RNases of B. subtilis and other bacilli has not yet been analysed under SOS-inducing conditions, tiling arrays performed with B. subtilis RNase J1, RNase III and RNase Y depletion mutants (Durand et al., 2012) are in line with the assumption that the yneAB–ynzC instability depends on these major RNases when the cells are not exposed to DNA damage. However, their role in transcript turnover, particularly for SOS-induced ICD through destabilization of the yneAB–ynzC mRNA, remains to be elucidated.

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