Evidence for two recA genes mediating DNA repair in *Bacillus megaterium*

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Isolation and subsequent knockout of a recA-homologous gene in *Bacillus megaterium* DSM 319 resulted in a mutant displaying increased sensitivity to mitomycin C. However, this mutant did not exhibit UV hypersensitivity, a finding which eventually led to identification of a second functional recA gene. Evidence for recA duplicates was also obtained for two other *B. megaterium* strains. In agreement with potential DinR boxes located within their promoter regions, expression of both genes (recA1 and recA2) was found to be damage-inducible. Transcription from the recA2 promoter was significantly higher than that of recA1. Since a recA2 knockout could not be achieved, functional complementation studies were performed in *Escherichia coli*. Heterologous expression in a RecA null mutant resulted in increased survival after UV irradiation and mitomycin C treatment, proving both recA gene products to be functional in DNA repair. Thus, there is evidence for an SOS-like pathway in *B. megaterium* that differs from that of *Bacillus subtilis*.

**INTRODUCTION**

The multifunctional RecA protein is involved in a number of cellular processes related to DNA metabolism and repair (Roca & Cox, 1990). As the major player in homologous recombination, RecA binds to single-stranded (ss) DNA forming a nucleoprotein filament, which scans the DNA double helix for homologous sequences and catalyzes strand exchange. Such recombinational processes are crucial for repair of two-strand damage and play a particularly important role in replication restart following collapse of replication forks (Cox, 1999). Besides this direct function in recombinational repair (RR), RecA also has a key regulatory role, as it activates the so-called SOS response, an inducible DNA-repair system which has been intensively studied in the Gram-negative bacterium *Escherichia coli* (Radman, 1975). The *E. coli* SOS system contains a set of at least 40 different damage-inducible (din) genes scattered all over the chromosome (Courcelle et al., 2001). Under normal conditions, transcription of din genes is blocked by the SOS repressor protein LexA binding to specific sequences (SOS boxes) located within their promoter regions. Following exposure to DNA-damaging agents, single-stranded DNA regions are generated due to replication blockage or enzymic processing of broken DNA ends (Sassanfar & Roberts, 1990). Bound to such ssDNA, RecA acquires an active conformation, and thus promotes autocatalytic cleavage of LexA (Little, 1991). As cleaved LexA no longer binds to SOS boxes, transcription of din genes, to which lexA and recA themselves belong, is permitted, eventually resulting in various cellular activities directly or indirectly involved in DNA repair, such as increased recombination, nucleotide excision repair (NER), translesion DNA synthesis (error-prone DNA replication), and inhibition of cell division. After repair has taken place, RecA is no longer activated, and the increasing level of uncleaved LexA leads to repression of the regulon.

Although the basic regulatory principles of the SOS network composed of LexA and RecA are considered to be a universal phenomenon in prokaryotes, recent studies of analogous systems in species from other bacterial taxa have revealed major variations from the *E. coli* model. While the SOS box of *E. coli* and most other members of the γ-subclass of Proteobacteria consists of the 16 bp palindrome CTGTN8ACAG (Walker, 1984), the consensus sequence of the LexA-binding site in *Xanthomonadaceae* clearly differs (Campoy et al., 2002). Deviating SOS boxes were also identified in the α- and δ-subdivisions of Proteobacteria (Tapias et al., 2002; Jara et al., 2003; Campoy et al., 2003). Furthermore, such differences appear not to be restricted to various repressor-binding sequences, but also include the number of din genes constituting the SOS regulon. While analyses of available genomic sequences revealed recA being present throughout the bacteria, lexA homologous genes, however, were found to be absent in some species (Liveris et al., 2004). Furthermore, for other bacteria two copies of lexA were reported to exist (Jara et al., 2003).

In the well-studied Gram-positive bacterium *Bacillus subtilis*, the transcriptional repressor of din genes is the LexA.
homologue DinR, which binds to a palindromic (termed the DinR box) with the consensus sequence CGAACR-NRYGTTTCG (Winterling et al., 1998). Furthermore, by analogy with E. coli, RecA functions as the activator of SOS induction (Yasbin et al., 1991). So far, six loci [recA, dinR, uvrBA (formerly dinA), dinB, tagC (formerly dinC) and yneAB–ynzC] have been shown to be under DinR control (Marrero & Yasbin, 1988; Raymond-Denise & Guillen, 1991; Cheo et al., 1991; Kawai et al., 2003). Compared to the multitude of din genes found in E. coli, the number of as yet identified and proven din loci in B. subtilis is low, suggesting that many din genes remain to be discovered. Expression of the B. subtilis recA gene is not only derepressed under SOS conditions as in E. coli, but is also activated during development of natural competence (Haijema et al., 1996; Hamoen et al., 2001). Such SOS-independent activation is mediated by the competence transcription factor ComK, which binds specifically to sequence motifs consisting of two AT boxes (consensus sequence AAAA-N5-TTTT) located upstream of recA and other late competence genes (Van Sinderen et al., 1995). Activation of recA during competence development seems reasonable since uptake of exogenous DNA is followed by recombinational integration into the chromosome. Thus, expression of the two global regulons, i.e. SOS response and competence development, is linked in B. subtilis (Yasbin et al., 1991, 1992).

Taking into account the divergence concerning different SOS regulons among several Gram-negative bacterial groups, variations of the common pattern can be assumed within Gram-positive bacteria, too. However, except for the recA gene of Bacillus anthracis, which contains a Group I self-splicing intron (Ko et al., 2002), essentially nothing is known about recA expression in representatives of the genus other than B. subtilis. Thus, we looked for recA and its function in DNA repair in Bacillus megaterium. Interestingly, this biotechnologically relevant species (Vary, 1994) has been reported to resist DNA damage much more efficiently than B. subtilis (English & Vary, 1986). As a result of our work, B. megaterium – unlike several other Bacillus species – was found to harbour two recA genes, both being damage-inducible and functional in DNA repair.

METHODS

Growth conditions and transformation procedures. Strains used in this study are listed in Table 1. Cells were cultivated at 37 °C in Luria–Bertani (LB) broth or in minimal medium (Sambrook et al., 1989) with 1% glucose, 0.1 mM CaCl₂, 0.01% yeast extract and 0.02% Casamino acids. For selection of recombinant E. coli clones, ampicillin was used in the following concentrations: 75 μg ml⁻¹ for derivatives of pACYC177 and 100 μg ml⁻¹ for pUC vectors. Protoplasts of B. megaterium were transformed by a PEG-mediated procedure essentially as described by Vorobjeva et al. (1980). Recombinant strains of B. megaterium were selected with either tetracycline (1.25 or 12.5 μg ml⁻¹) or chloramphenicol (2.5 or 25 μg ml⁻¹); the use of low concentrations in combination with the non-permissive temperature of 42 °C served for the identification of single-copy situations due to integration into the chromosome, whereas high concentrations and the permissive temperature of 30 °C were used for the selection of cells with freely replicating plasmids. For SOS induction, cells were treated with mitomycin C (MMC; Fluka Chemie) at a concentration of 0.2 μg ml⁻¹ (for B. megaterium) or 0.1 μg ml⁻¹ (for E. coli).

DNA hybridization, cloning and sequencing techniques. The plasmids used in this study are listed in Table 1. Molecular cloning procedures were carried out essentially as described by Sambrook et al. (1989). Plasmid DNA was purified using Jetstar columns (Genomed). Genomic DNA from B. megaterium was isolated as previously described (Nahrstedt & Meinhardt, 2004). Southern blotting was performed by capillary transfer onto nylon membranes, and hybridizations with digoxigenin (DIG)-labelled DNA probes were carried out at 68 °C. Signals were detected by using the chemiluminescent substrate CDP-Star (Roche Diagnostics). PCR reactions contained 200 μM deoxynucleotides, 10 ng template DNA, 1 pmol of each primer and 1 U of enzyme, i.e. Taq DNA polymerase (Eppendorf), Pwo DNA polymerase (MBI Fermentas), Pfu DNA polymerase (Promega) and Vent DNA polymerase (New England Biolabs). Primers for PCR (Invitrogen) are listed in Table 2. Extractions of DNA fragments from gels were carried out with the Jetquick gel extraction kit (Genomed). Nucleotide sequences were determined either with the IRD800-labelled pUC primers M13revCS–49– and M13unf–43– (MWG Biotech) using the CycleReader Auto DNA sequencing kit (MBI Fermentas) in combination with an automatic LI-COR sequencer, model 4000L (LI-COR Bioscience), or with fluorescent-labelled dideoxynucleotides using the BigDye Terminator v3.1 sequencing kit (Applied Biosystems) in combination with an ABI Prism capillary sequencer, model 3700. For assembly and analysis of the sequences as well as database searches, tools of the Lasergene sequence analysis software (DNASTAR), HUSAR program package (EMBL), and NCBI server (http://www.ncbi.nlm.nih.gov/) were used.

Isolation and sequencing of the B. megaterium recA genes. The recA1 gene was isolated by PCR applying the two degenerate primers 1recA and 2recA (Dwut et al., 1992), by which an internal recA fragment (362 bp) was amplified from chromosomal DNA of wild-type DSM 319. Using this PCR product as a probe, screening of a λ-EMBL4-based library formerly prepared from genomic DNA of B. megaterium DSM 319 (Meinhardt et al., 1994) resulted in identification of a positive clone, from which a 1.8 kb BamHI fragment with the 3′-end of recA1 was obtained. The remaining 5′-end was eventually isolated by an inverse PCR strategy using a 1.7-kb Acl fragment in combination with the outward-firing primers A7 and A8. Isolation of a 0.89-kb Clal fragment provided additional sequence information further upstream. All fragments mentioned were sequenced on both strands, giving rise to the nucleotide sequence of the 3347 bp chromosomal recA1 locus. For isolation of the second recA gene, chromosomal DNA of recA1 mutant MS991 was used for PCR in combination with the same degenerate primers as applied for recA1, again yielding an internal recA fragment (362 bp). Flanking regions were subsequently combined by two HindIII fragments (1-1 and 1.3 kb in size) isolated in shotgun cloning experiments. Sequencing of the overlapping fragments led to an entire nucleotide sequence of 2403 bp including the chromosomal recA2 locus. The sequences of both chromosomal loci have been submitted to the EMBL database under accession numbers AJ155540 (recA1) and AJ155541 (recA2).

Transcript and primer extension analysis of the B. megaterium recA genes. Cells of B. megaterium were grown in minimal medium to an OD₅₄₆ of 1.8. Cultures were then divided into two samples, one of which was supplemented with 0.2 μg MMC ml⁻¹. During further cultivation 5 ml samples were taken at hourly intervals, from which total RNA was isolated using hot phenol as previously described (Nahrstedt & Meinhardt, 2004). For analysis of
heterologous recA transcription in *E. coli*, cells were cultivated in LB supplemented with 75 µg ampicillin ml⁻¹, and RNA was isolated by applying the RNeasy Midi Kit (Qiagen) according to the manufacturer’s recommendations. In each case, RNA samples were additionally supplemented with 40 U RNasin Plus (Promega), and their concentration was determined spectrophotometrically. For Northern blotting, equal amounts of total RNA (20 µg) were separated by electrophoresis through 1 % (w/v) formaldehyde agarose gels with MOPS as the running buffer and subsequently transferred onto nylon membranes by vacuum blotting. After UV cross-linking for 5 min and prehybridization for 1 h, hybridization was carried out overnight at 55 °C. After heating the samples at 65 °C for 10 min, 200 U transcriptase was added in a total volume of 20 µl and incubated at 42 °C for 70 min. Samples were then kept at 95 °C for 5 min, and after digestion with RNase A for 15 min at 37 °C, cDNA was precipitated using 2-propanol and finally dissolved in sequencing-stopping solution. As templates in sequence reactions with the same primers, the following DNA fragments were used: a recA1 promoter - promoter-recA2 fusion

**Construction of promoter fusions and analysis of recA expression.** By applying a single-copy promoter test system (S. Schmidt & F. Meinhardt, unpublished results), which has been developed for a β-galactosidase-deficient mutant of *B. megaterium* DSM 319, strains carrying transcriptional fusions of the bgaM gene and the recA promoters were constructed: a 489 bp fragment including the recA1 promoter was amplified from strain DSM 319 via PCR using Pwo DNA polymerase and primers A-P1 and A-P2; similarly,
Table 2. Oligonucleotides

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence (5'-3')</th>
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</thead>
<tbody>
<tr>
<td>1recA</td>
<td>TTHATYGANGCYGARCYG</td>
</tr>
<tr>
<td>2recA</td>
<td>CCWCCGKWGTGHTYTTCNCG</td>
</tr>
<tr>
<td>A3</td>
<td>CAGGGGAAAGATAGGG</td>
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<td>A6</td>
<td>CAGTCATAGCTGTCG</td>
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<td>A7</td>
<td>GTGCCGTTCTACAGATATCG</td>
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<tr>
<td>A8</td>
<td>CCCACCGCTTGAACTGCACC</td>
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<tr>
<td>A11</td>
<td>GTGACGCTGTATTTTCTTTC</td>
</tr>
<tr>
<td>A12</td>
<td>CAAACAGCGCGGGCTATTCTCAGAT</td>
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</tr>
<tr>
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<td>GTTGACTGCTTGTAGTACATACAGGG</td>
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</tr>
<tr>
<td>A-P2</td>
<td>GATTGTTATACTGTACCAATATAAAGCG</td>
</tr>
<tr>
<td>A-P2-IRD</td>
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</tr>
<tr>
<td>M1</td>
<td>TGCAAAAAACCATTTCAGACGCGC</td>
</tr>
<tr>
<td>M2</td>
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<tr>
<td>M3</td>
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</tr>
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</tr>
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</tr>
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<tr>
<td>M13revCS[-49]</td>
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</tr>
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a 400 bp fragment carrying the recA2 promoter was isolated using the primers M-P1 and M-P2. Both fragments were ligated into the single Smal site of the recA disruption vector ppts (S. Schmidt & F. Meinhardt, unpublished results), respectively. The resulting vectors were then used for integration of the fusion into the E. coli locus, generating the leucine-auxotrophic strains MS031 (carrying the recA1 promoter) and MS032 (with the recA2 promoter). Since bgaM encodes a β-galactosidase (Strey et al., 1999), expression of the recA promoters was analysed by measuring β-galactosidase activities. In parallel with the promoterless control MS023, strains MS031 and MS032 were cultivated in 100 ml minimal medium to an OD_{600} of 1.2. Cultures were then divided into two samples, one of which was supplemented with 0.2 μg MMC mL^{-1}. During 2 h further cultivation, 800 μl samples were taken every 30 min in duplicate, and β-galactosidase activity was assayed using the chromogenic substrate o-nitrophenyl β-D-galactopyranoside (ONPG) as previously described (Strey et al., 1999). Simultaneously, cell density (OD_{600}) was further monitored in order to obtain Miller units (Miller, 1972).

Construction of recA disruption vectors and gene knockout experiments. For targeted gene disruption, derivatives of the temperature-sensitive shuttle vectors pUCTV2 (Wittench & Meinhardt, 1995) and pUCTV3 were applied. The latter carries the bgl gene of Paenibacillus macerans encoding an extracellular (1,3-1,4)-β-glucanase as an additional marker which can be screened by using LB plates containing 0-02% (w/v) lichenin and subsequently overlaying grown colonies with Congo red solution (0-2%, w/v). A 1086 bp deletion spanning the complete ORF of recA1 was generated by applying flank A (amplified with A11/A12) and flank B (A8/A13) (see also Fig. 1). For construction of pDRECA1, the respective deletion cassette was first cloned in pUCBM20, and subsequently ligated into the single EcoRI site of pUCTV3. Similarly, two different cassettes for the disruption of recA2 were developed: in pDRECMcat1, the disruption cassette consists of flank A (amplified with M1/M2) and flank B (M3/M4), creating a deletion of 1373 bp covering nearly the complete recA2 ORF (see also Fig. 3a). pDRECMcat2 carries flank C (M7/M8) and flank B (mentioned above), causing a 337 bp deletion within the 3'-end of recA2. In both cases, the cat (chloramphenicol acetyltransferase) gene of Staphylococcus aureus was inserted in between the respective flanks. In pDRECMcat3, cat was directly integrated into the single HindIII site within the centre of flank C, which was subsequently ligated into pUCTV3. Vector-carrying clones of B. megaterium DSM 319 or MS991 were selected on tetracycline-containing plates at 30 °C. Integrants carrying the entire vector inside the chromosomal locus were selected by growth on plates containing I-25 μg tetracycline mL^{-1} at 42 °C. Single colonies of such candidates were further cultivated on plates without tetracycline at 42 °C. Plasmid-cured clones were identified by loss of tetracycline resistance (and of β-glucanase activity if pUCTV3 derivatives were used).

Construction of recA expression vectors for complementation experiments in E. coli. A 1719 bp fragment carrying the recA1 ORF including its promoter and terminator was amplified via PCR with primers A-P1 and A19 using Vent DNA polymerase. Similarly, a 1888 bp segment carrying the complete recA2 was isolated by applying the primers M-P1 and M6. Both fragments were each ligated into the single Smal site of pACYC177 (in the same orientation relative to the vector’s backbone), resulting in vectors pACYCrecA1 and pACYCrecA2. For construction of pACYCrecA2-1, upstream of a promoterless recA2 (1510 bp; amplified with M9-Smal and M6) which was integrated in pACYC177, a fragment including the recA1 promoter (489 bp; amplified with A-P1 and A-P2) was inserted. The sequences of all products were verified by DNA sequencing.

UV and MMC survival measurements. Strains were cultivated to mid-exponential phase (B. megaterium in minimal medium and recombinant E. coli strains in LB with 75 μg ampicillin mL^{-1}). Cells were then washed in 15 mL NaCl and serially diluted to produce titres suitable for viable counts. For UV survival measurements, cells were plated on LB and irradiated with increasing doses of UV light using a UV-C lamp with an intensity of 1 J m^{-2} s^{-1}. In the case of MMC survival measurements, cells were plated onto LB medium with increasing amounts of MMC. Plates were then incubated overnight in the dark, and relative survival was calculated by relating the number of colonies to those of control plates that had not been irradiated.

RESULTS AND DISCUSSION

B. megaterium harbours two copies of recA

Degenerate oligonucleotides for the amplification of recA sequences have been successfully applied for a number of different bacteria (Duvat et al., 1992; Dybvig et al., 1992). Such a PCR-based approach also facilitated isolation of an internal recA fragment from chromosomal DNA of B. megaterium DSM 319. Subsequent cloning of flanking...
segments eventually led to the identification of a genomic region (3347 bp), in which a complete recA homologue (designated recA1) is located (Fig. 1). For studying its functionality, a deletion experiment was performed as described in Methods, resulting in mutant strain **B. megaterium** MS991 (ΔrecA1). The chromosomal 1086 bp deletion (Fig. 1) spans the complete ORF of recA1 as verified by Southern analysis (see also Fig. 3b). MS991 was then analysed in terms of sensitivity to the DNA-damaging agents MMC and UV light in comparison with the parental strain MS319. As depicted in Fig. 2(a), agents MMC and UV light in comparison with the parental strain could be seen (Fig. 2b). This finding is in striking contrast to results obtained for other bacteria, in which expression of recA (or at least another genetic factor capable of complementing the loss of RecA1 with respect to UV resistance) seemed to be more likely. We therefore used chromosomal DNA of the recA1 deletion mutant MS991 as template in PCR, again applying the degenerate primers already used for recA1 amplification. As a result, another fragment was obtained, the sequence of which proved to be 83% identical to the respective PCR product from recA1. Subsequent isolation and sequencing of **B. megaterium** mutants defective in NER (Nahrstedt & Meinhardt, 2004), the differences in UV sensitivity became clearly evident (Fig. 2b). Such findings correspond to previous studies (English & Vary, 1986), in which **B. megaterium** recA mutants, obtained by random mutagenesis, with increased sensitivity to MMC were hardly more sensitive to UV light than the respective parental strain, but clearly more resistant than uvr mutants. One possible explanation for such lack of UV sensitivity might be an NER mechanism that is not part of an SOS-like system but constitutively expressed. However, this would be in marked contrast to results obtained for other bacteria, in which expression of uvr genes is controlled in an SOS-dependent manner. Moreover, as recent studies in **B. megaterium** revealed the uvrB/A genes to be similarly regulated (i.e. SOS-dependent) regulated (Nahrstedt & Meinhardt, 2004), this explanation appeared to be extremely unlikely. Rather, a second functional recA (or at least another genetic factor capable of complementing the loss of RecA1 with respect to UV resistance) seemed to be more likely. Thus, we therefore used chromosomal DNA of the recA1 deletion mutant MS991 as template in PCR, again applying the degenerate primers already used for recA1 amplification. As a result, another fragment was obtained, the sequence of which proved to be 83% identical to the respective PCR product from recA1. Subsequent isolation and sequencing of

**Fig. 1.** Genetic organization of the recA1-encoding genomic region from **B. megaterium** DSM 319 and mutant strain MS991 (ΔrecA1). ORFs are depicted as arrows and were designated according to their similarities to genes from **B. subtilis** (as a result of FASTA searches in the SubtiList database). ydfA, incomplete ORF (295 nt) with 73% identity (in 295 nt overlap) to ydfA (BG12148), encoding a hypothetical protein similar to arsenic efflux pumps; arsC, complete ORF (423 nt) with 76% identity (in 420 nt overlap) to arsC (BG11304), encoding an arsenate reductase; recA1, complete ORF (1038 nt) with 73.5% identity (in 943 nt overlap) to recA (BG10721), encoding RecA involved in homologous recombination and DNA repair; ywoF, incomplete ORF (774 nt) with 58% identity (in 740 nt overlap) to ywoF (BG12493), encoding a hypothetical protein of unknown function. Transcriptional terminators are sketched as hairpins. Positions of PCR primers are marked as solid arrows. Positions of primer extension are marked by open boxes. The potential ComK box is indicated by four open boxes. The first four predicted amino acids of RecA1 are given in the standard one-letter code.
The flanking regions led to characterization of a chromosomal segment (2403 bp), in which a second recA-homologous gene (in the following referred to as recA2) is situated (Fig. 3a). It is noteworthy that – with the exception of Myxococcus xanthus, a member of the Proteobacteria δ class (Norioka et al., 1995) – there are to our knowledge no reports of other bacteria harbouring two different chromosomally located recA genes. To exclude the possibility that the presence of two recA genes is a strain-specific phenomenon, only to be encountered in DSM 319, two additional B. megaterium strains were tested by Southern hybridization, i.e. DSM 32 (type strain) and DSM 1804. In parallel, DNA of DSM 319 and the recA1 mutant MS991 was probed with a DIG-labelled recA2 PCR product (amplified with 1recA and 2recA). Due to the high degree of identity within this central recA part, this probe was capable of detecting both recA genes in chromosomal DNA from DSM 319 under stringent conditions. As shown in Fig. 3(b), signals corresponding to recA2 of DSM 319 were also obtained for the type strain DSM 32 and for DSM 1804. Furthermore, as in strain DSM 319, signals that could be attributed to recA1-carrying fragments were also obtained. The observed variations in size of such rather large fragments are most likely due to restriction fragment length polymorphism. In contrast, deletion mutant MS991 did not generate any recA1 signals. Accordingly, the presence of two recA copies is highly likely not to be strain specific but can be generally accepted for the species B. megaterium.

Expression of recA1 and recA2 is damage-inducible

As recA genes normally belong to the inducible SOS regulon, they have SOS boxes located within their promoter regions. In B. subtilis, this binding site is termed the DinR box, displaying the consensus sequence 5’-CGAACRNRYGTTCG-3’ (Winterling et al., 1998). By DNA sequence analysis of the regions upstream of both B. megaterium recA genes, similar sequence motifs were identified. Subsequent primer extension analyses demonstrated that transcription is initiated 181 bp (recA1) and 87 bp (recA2), respectively, upstream of the translational start codon (Fig. 4). The determined transcriptional startpoints are in good accordance with the positions of σ^A-dependent Bacillus promoter structures, tentatively suggested from nucleotide data (see Figs 1 and 3a for details). Hence, the putative DinR box of recA2 is located adjacent to and upstream of the −35 region of the respective promoter, which is in agreement with the DinR box described for recA of B. subtilis. For recA1, however, the respective box is situated directly downstream of the transcriptional startpoint. The presence of potential DinR boxes suggests inducible expression of both genes following DNA damage in an SOS-like manner. To further investigate such an induction effect, equal amounts of RNA isolated from B. megaterium cells exposed to MMC were analysed in Northern blots in parallel with those of untreated cultures. As transcripts displaying electrophoretic mobilities of 1·3 kb (recA1) and 1·2 kb (recA2) were obtained (Fig. 5a), both genes were proven to be monocistronically organized. Furthermore, at each reading point (1–4 h after induction) stronger signals for both transcripts were detected in samples from MMC-treated cells, demonstrating induction of transcription concomitant to DNA damage. Moreover, signals obtained for recA2 were strikingly stronger than those of recA1; in particular, already under non-inducing conditions, basal expression of recA2 appeared to be significantly higher. It has to be pointed out that in Fig. 5(a), recA2 transcription was analysed for the recA1 mutant with the possibility of some changing effect on native recA2 expression. Subsequent analyses of recA2 transcripts in wild-type background, however, yielded similar expression patterns (not shown), thus confirming higher basal expression of recA2. In order to confirm these findings, reporter gene analyses were performed as described in Methods. Expression of both recA promoter fusions with and without MMC was monitored by measuring β-galactosidase activities. As depicted in Fig. 5(b), the recA2 promoter appeared to be about sixfold more active than the recA1 promoter under normal conditions (without MMC). In the presence of MMC, both
promoters conferred significantly higher activities at each reading point (0.5–2 h after addition of MMC), supporting induction in an SOS-dependent manner. As for the already higher basal activity, the values obtained for recA2 under inducing conditions are similarly higher than for recA1. However, when comparing relative induction ratios of each fusion, recA1 is slightly more strongly induced than recA2: after 2 h of induction, recA2 activity was elevated 3.8-fold, whereas it was elevated 5.8-fold for recA1. This difference could be due to a more efficient binding of the repressor to the operator site (DinR box) of recA1. Further analysis of the potential DinR boxes applying in vitro studies such as gel mobility shift assays and footprinting may shed light on the differential regulation.

In addition to DinR-box-like elements, sequence analysis of the recA promoters also revealed distinctive AT-rich motifs (see Figs 1 and 3a for details), displaying striking similarities to sequence motifs typically found in promoter regions of late competence genes in B. subtilis (Hamoen et al., 2002). By binding to such AT-rich elements, the competence transcription factor ComK activates expression of late competence genes (including recA) during development of natural competence. The presence of potential ComK boxes in B. megaterium is of special interest because to date this Bacillus species has never been reported to exhibit natural transformability. However, as recent studies in B. megaterium revealed a chromosomal locus encoding a ComEA homologue functional in DNA uptake (Lammers et al., 2004), the finding of ComK-box-like motifs upstream of both recA genes once more points to a potential (or at least a latent potential) of B. megaterium to develop natural competence. Further studies of such promoters with respect to activation independent of DNA damage, and thus suggesting a ComK-like regulation in this species, are in progress.
**recA2 cannot be disrupted in B. megaterium**

The UV survival of recA1 mutant MS991 indicates RecA2 to be functional in complementing loss of RecA1. In contrast, RecA2 does not appear to be able to fully complement the function of RecA1 in repair of DNA damage caused by MMC. In this context, it is of note that UV and MMC affect different RecA functions. Repair of UV damage primarily reflects NER, and thus the role of RecA in SOS induction. The bifunctional alkylating agent MMC predominantly generates interstrand cross-links through reaction with guanine residues. For *B. subtilis* it has been shown that the repair of such two-strand damage involves the concerted action of both RR and NER (Friedman & Yasbin, 1983). However, since RR is the more crucial component in interstrand repair, MMC is routinely used for monitoring RecA as a recombinase. Anyhow, our present survival studies do not provide evidence for different functions of the two proteins (e.g. RecA1 only functioning as a recombinase and RecA2 exclusively being involved in SOS induction). In fact, it appears plausible that the double gene dose present in *B. megaterium* might have a greater impact on survival when RecA acts as a recombinase rather than fulfilling its regulatory function. To further investigate the functionality of RecA2, we tried to inactivate the respective gene via targeted gene disruption. In addition to a deletion approach, the *cat* (chloramphenicol acetyltransferase) gene from *S. aureus* was introduced as an additional marker to enhance screening efficiencies. The general procedure of such a gene replacement using a replicating vector can be divided into two steps. Firstly, the entire vector is integrated into the desired chromosomal locus via one of the two homologous flanks; if the respective flank is entirely located within the ORF this first integration step already results in a gene disruption, while a flank situated outside the ORF leads to its restoration. Subsequent excision of the vector via the promoterless control MS023 with (filled symbols) and without (open symbols) MMC (0·2 μg ml⁻¹). By referring β-galactosidase activity values to the respective cell density (OD₆₅₀) Miller units (MU) were obtained. □, MS023 (AbgaR/bgaM, ΔleuC::bgaM) −MMC; ■, MS023 + MMC; ▽, MS031 (AbgaR/bgaM, ΔleuC::recA1-bgaM) −MMC; ▼, MS031 + MMC; △, MS032 (AbgaR/bgaM, ΔleuC::recA2-bgaM) −MMC; ▲, MS032 + MMC.

**Fig. 4.** Primer extension analysis of recA1 and recA2 in *B. megaterium*. Total RNA isolated from cells of *B. megaterium* (treated with 0·2 μg MMC ml⁻¹ for 4 h) was used for reverse transcription in combination with IRD-labelled primers specific for recA1 and recA2, respectively. Applying the same primers, appropriate DNA fragments were sequenced and analysed in parallel with the extension products on a Li-COR sequencer. TS, transcriptional startpoint.

**Fig. 5.** Expression analyses of recA1 and recA2 in *B. megaterium*. (a) Transcript analyses of the *B. megaterium* recA genes. From cells grown with (+) and without (−) MMC (0·2 μg ml⁻¹), total RNA was isolated, and equal amounts (20 μg) were subjected to Northern analysis; recA2 transcripts were detected in mutant MS991, whereas recA1 transcripts were analysed in wild-type cells using RNA probes specific for recA1 and recA2, respectively. The numbers denote the time (h) after addition of MMC. (b) Promoter fusion studies of the *B. megaterium* recA genes. The promoter-fusion-carrying strains MS031 (recA1-bgaM) and MS032 (recA2-bgaM) were cultivated in parallel with the promoterless control MS023 with (filled symbols) and without (open symbols) MMC (0·2 μg ml⁻¹). From cells grown with (+) and without (−) MMC. (a) Transcript analyses of the *B. megaterium* recA genes. From cells grown with (+) and without (−) MMC (0·2 μg ml⁻¹), total RNA was isolated, and equal amounts (20 μg) were subjected to Northern analysis; recA2 transcripts were detected in mutant MS991, whereas recA1 transcripts were analysed in wild-type cells using RNA probes specific for recA1 and recA2, respectively. The numbers denote the time (h) after addition of MMC. (b) Promoter fusion studies of the *B. megaterium* recA genes. The promoter-fusion-carrying strains MS031 (recA1-bgaM) and MS032 (recA2-bgaM) were cultivated in parallel with the promoterless control MS023 with (filled symbols) and without (open symbols) MMC (0·2 μg ml⁻¹). By referring β-galactosidase activity values to the respective cell density (OD₆₅₀) Miller units (MU) were obtained. □, MS023 (AbgaR/bgaM, ΔleuC::bgaM) −MMC; ■, MS023 + MMC; ▽, MS031 (AbgaR/bgaM, ΔleuC::recA1-bgaM) −MMC; ▼, MS031 + MMC; △, MS032 (AbgaR/bgaM, ΔleuC::recA2-bgaM) −MMC; ▲, MS032 + MMC.
recombinational events (i.e. via flank A and B, respectively) were identified (data not shown); however, from further curings of such integrants only the wild-type lacking resistance to chloramphenicol was obtained. Following a second approach, vector pDRECMcat2 was applied to create a disruption at the 3’-end of recA2. Here, only integration via flank B was found, and again, further curings of these strains resulted exclusively in wild-type cells. In a third disruption strategy applying pDRECMcat3, integration via both flanks would already cause inactivation of the gene, and in fact, neither type of integrants was obtained. Altogether, these results clearly demonstrated that in our hands recA2 cannot be inactivated via targeted gene disruption, although recA1 and a number of other genes (Wittchen & Meinhardt, 1995; Strey et al., 1999; Lee et al., 2001; Nahrstedt & Meinhardt, 2004) have been successfully disrupted in B. megaterium by applying similar approaches. Apart from the fact that in several instances recA mutants were reported to show decreased viability as well as an irregular nucleoid morphology (Sciochetti et al., 2001), in many bacteria such mutants can be obtained. In fact, we previously performed a knockout of the single recA gene from Bacillus licheniformis via a similar deletion strategy (H. Nahrstedt & F. Meinhardt, unpublished results). Hence, in general recA is not considered to be essential for cell viability. To our knowledge, the only other recA gene for which a disruption was achieved only under special circumstances is that of Streptomyces lividans (Muth et al., 1997), in which viable mutant strains were found to lack only short extensions of the RecA C-terminus, and displayed residual RecA activity. Recent studies in S. lividans suggested that an additional mutation (resulting in a sporulation defect) is required to tolerate total recA deficiency, although its role remains obscure (Vierling et al., 2001). Although the observed inability to inactivate recA2 demands further investigation, our present data indicate that this locus might be required for sustaining cell viability in B. megaterium.

recA1 and recA2 complement recA defects in E. coli

It has been previously reported that expression of recA genes may have lethal effects on their cloning hosts, at least on multi-copy vectors; for example, cloning of recA from B. subtilis was found to be critical not only in E. coli, but also in B. subtilis itself (Marrero & Yasbin, 1988). Consistent with such findings, our initial attempts to clone the B. megaterium recA genes faced similar problems (not shown). Since no low-copy plasmids for Bacillus were readily available, we made use of appropriate E. coli vectors. Eventually, low-copy plasmid pACYC177 in combination with the native B. megaterium recA promoters facilitated cloning in a RecA null mutant of E. coli. Due to the high conservation of RecA proteins, such interspecies complementation approaches have been successfully applied not only for several recA genes of Gram-negative bacteria but also for some from Gram-positive species such as Clostridium perfringens (Johnston et al., 1997) and B. anthracis (Ko et al., 2002). The resulting vectors pACYCrecA1 and pACYCrecA2 (for cloning strategies see Methods) were used in parallel with pACYC177 (as the control) for transformation of the RecA null mutant E. coli JM109. Subsequently, recA transcription was confirmed by Northern analyses with the specific RNA probes (see also Figs 1 and 3a), yielding transcripts with sizes of 1-3 kb (recA1) and 1-2 kb (recA2), which demonstrated that both promoters are functional in E. coli. However, although equal amounts of RNA were applied, the recA2 signals always appeared to be stronger compared to the ones of recA1, suggesting a higher expression level for recA2 (not shown). The two plasmid-carrying strains were then analysed with respect to single-filament growth (in the presence of 0·1 μg MMC ml\(^{-1}\) for SOS induction) and by measuring survival either following UV irradiation or in the presence of MMC. Single-filament growth is a typical SOS phenomenon resulting from inhibition of cell division in the event of DNA damage (Huisman & D'Ari, 1981) since SulA, a component of the SOS response in E. coli, prevents formation of the FtsZ ring. As induction of sulA depends on RecA, the phenotype of single-filament growth should not be visible in a RecA null mutant unless it is complemented. As shown in Fig. 6(a), complementation with plasmid pACYCrecA2 resulted in filamentation, while for the growth of the pACYCrecA1-harbouring strain this phenotype was hardly observable, with only minor deviations in cell morphology compared to the control strain. Thus, either the gene product of recA1 is less active in SOS induction, or the amount of heterologous protein is not sufficient for eliciting such a response in E. coli. Besides single-filament growth, RecA complementation should also increase survival of a RecA null mutant after UV irradiation. The data in Fig. 6(b) show that UV survival of both recA strains was indeed enhanced. However, survivability of the pACYCrecA2 strain was significantly higher than that of the pACYCrecA1 strain. Analogous studies performed with MMC yielded similar results (Fig. 6c); both genes enhanced survival in the presence of MMC, but the effect of recA2 was more significant than that of recA1. Altogether, the results demonstrate that both B. megaterium RecAs can mediate DNA repair in E. coli, as shown by the use of UV light as well as MMC. Strikingly, however, the complementation effects of the two RecAs were quite different in strength. RecA2 turned out to be much more effective, while the impact of RecA1 was rather poor. This discrepancy becomes particularly evident when filamentous growth is examined. One possible explanation for these results could be that the transcription levels of the two genes are different. Similar to the results in B. megaterium, the Northern analysis in E. coli suggested a higher expression level of recA2 compared to recA1. When the effect of recA2 under control of the recA1 promoter was subsequently checked by the use of vector pACYCrecA2-P1 (see also Methods), the respective strain displayed the same phenotypes as obtained for the pACYCrecA1-carrying strain, i.e. hardly any filamentation was observable, and
long-known recA bacillus halodurans genes of members. (a) For SOS induction, 0.1 μg Mmc ml⁻¹ was added to overnight cultures grown in LB with 75 μg ampicillin ml⁻¹; after 2 h of further cultivation, single-filament growth was monitored under a microscope. (b) Survival after UV irradiation. (c) Survival in the presence of MMC. □, JM109(pACYC177); ▼, JM109(pACYCrecA1); ▲, JM109(pACYCrecA2).

Fig. 6. Complementation studies of recA1 and recA2 in recombinant strains of E. coli JM109. The curves presented are based on mean values obtained from six independent experiments. (a) For SOS induction, 0.1 μg Mmc ml⁻¹ was added to overnight cultures grown in LB with 75 μg ampicillin ml⁻¹; after 2 h of further cultivation, single-filament growth was monitored under a microscope. (b) Survival after UV irradiation. (c) Survival in the presence of MMC. □, JM109(pACYC177); ▼, JM109(pACYCrecA1); ▲, JM109(pACYCrecA2).

survival after UV irradiation or in the presence of MMC was clearly lower compared to the pACYCrecA2 strain (data not shown). These results indicate that the different complementation effects of the two B. megaterium RecAs are most likely due to different expression levels of the respective genes in E. coli rather than to different functionalities of the respective gene products.

Sequence and structure comparisons suggest recA2 to be the original recA

RecA-encoding genes are highly conserved, and thus can be used as a phylogenetic marker for bacterial classification (Eisen, 1995; Karlin et al., 1995). In addition to the relatively long-known recA sequence of B. subtilis and the respective genes of Bacillus halodurans, B. anthracis and B. cereus, which were previously published as part of their complete genome sequences, we recently isolated and sequenced the recA genes of Bacillus amyloliquefaciens (GenBank/EMBL accession no. AJ515542) and B. licheniformis (GenBank/EMBL accession no. AJ511368). Except for recA of B. anthracis, which contains an intron (Ko et al., 2002), all other known recA genes of Bacillus species look very similar. With a length of 1038 bp, both B. megaterium genes are average in size compared to those of other Bacillus species. For their deduced gene products (345 aa each) similar molecular masses (37.7 kDa for RecA1 and 37.4 kDa for RecA2) as well as isoelectric points (5.4 for RecA1 and 5.0 for RecA2) can be calculated. Based on studies of the E. coli RecA (352 aa), the primary amino acid sequence is generally divided into three sections (Bianco et al., 1998): an N-terminal domain (aa 1–33), the central ‘core’ region (aa 33–240), and the C-terminal domain (aa 241–352).

Highly conserved subdomains of the ‘core’ region contain residues which constitute the ATP-binding domain, i.e. the two Walker motifs A and B (aa 66–73 and 140–14, respectively), and the DNA-binding loops L1 (aa 157–164) and L2 (aa 195–209). Neither of the B. megaterium recA gene products shows significant deviations within these domains (not shown). Altogether, at the amino acid level the degree of conservation stretches basically across the entire sequence with the exception of the highly acidic C-terminus, in which RecA proteins are typically less conserved. To obtain some information about the origin and possible evolution of the two B. megaterium recA genes, pairwise global alignments of each gene with those of other Bacillus species were performed using the program GAP of the HUSAR package (EMBL). Here, recA2 reveals the highest degrees of identity at the nucleotide level to the recA genes of B. cereus (79.7%), B. subtilis (77.9%), B. amyloliquefaciens (75-2%), B. halodurans and B. licheniformis (75-1% each), whereas its relatedness to recA1 is only 74.8%. In contrast, the identity values obtained in the respective recA1 alignments were significantly lower. In fact, recA1 appears to be most identical to recA2, followed by the genes of B. cereus (71.8%), B. amyloliquefaciens (70-1%), B. subtilis (69-5%), B. licheniformis (68-4%) and B. halodurans (68-2%). Furthermore, the surrounding genetic organization of recA1 strongly differs from those of other Bacillus species. In the latter, recA is typically flanked upstream by cinA (for competence damage inducible protein) and downstream (in the case of B. subtilis and B. amyloliquefaciens) by ppx (for penicillin-binding protein) and ymdA (for hypothetical protein of unknown function) or directly by ymdA (as encountered in B. cereus, B. anthracis and B. licheniformis). In contrast to recA1, the recA2 locus is in good agreement with this rather conserved genetic structure (see Fig. 3). Altogether, these findings suggest recA2 to be the original recA gene in B. megaterium. The origin of recA1, however, appears to be less certain: whether this gene has evolved by acquisition from another organism via horizontal gene transfer or due to a simple duplication of the primal recA2 remains obscure. No striking differences in the respective G+C contents (37 mol% for recA1 and 40 mol% for recA2) or in codon usage were observed. Nevertheless, the fact that the regions surrounding the two genes are different indicates that their origin is unlikely to be attributable to a recent single chromosomal duplication. So far, the only other bacterium for which two independent unlinked copies of chromosomally encoded
recA genes (designated recA1 and recA2) have been described is Myxococcus xanthus (Norioka et al., 1995). Interestingly, also in the case of M. xanthus disruption of only recA1 was achieved, while all attempts to generate recA2 mutants failed. Additionally, although both genes were able to complement UV sensitivity in E. coli, RecA1 appeared to be less functional than RecA2. Unlike the two B. megaterium RecAs (which share an identity of 81.7%), the genes of M. xanthus are different in length and the respective gene products have only 67.0% identity to each other. In view of the surprisingly low degree of identity, Karlin et al. (1995) proposed that one of the genes (probably recA1) was acquired via horizontal gene transfer rather than both genes having arisen by an endogenous duplication event. Also in contrast to B. megaterium, only one of the M. xanthus genes (recA2) was shown to be damage-inducible. Consistently, a LexA-binding sequence, which is unique for the genus Myxococcus, was identified only upstream of recA2, whereas recA1 exhibits a degenerate copy of this motif (Campoy et al., 2003). As one possible role of the two M. xanthus recA genes, Karlin et al. (1995) suggested that the presence of two genes might enable greater RecA expression, mitigating problems of this topsoil bacterium with UV irradiation. For B. megaterium, this hypothesis can virtually be ruled out since the loss of one of the two RecA proteins did not result in a reduced UV sensitivity (Fig. 2b). However, it is still conceivable that a greater amount of RecA might increase the cells’ capacity for RR. English & Vary (1986) found strains of B. megaterium to be consistently more resistant to DNA damage than those of B. subtilis. Recent studies revealed B. licheniformis also to be more sensitive to DNA damage than B. megaterium (H. Nahrstedt & F. Meinhardt, unpublished results). The presence of two recA genes in the latter organism might thus play a role in maintaining its greater resistance to DNA damage.

Conclusions

B. megaterium contains two functional recA copies, designated recA1 and recA2. Involvement of recA1 in DNA repair was evidenced by a recA1 mutant which displayed increased sensitivity to MMC. The lack of UV sensitivity of that particular mutant, however, indicated that recA2 could complement loss of recA1 with respect to UV-mediated DNA damage. Since all efforts to disrupt recA2 were unsuccessful, further functional studies were performed by complementation experiments in E. coli. Both genes were able to complement recA defects to a certain degree, with recA1 appearing less effective than recA2. Consistently, single-filament formation was induced by recA2 only. Such differences are most likely due to higher expression of recA2 in E. coli. Accordingly, similar expression patterns were observed in B. megaterium, in which transcription of both genes was inducible by DNA damage. This finding accords well with the presence of potential DinR boxes located within the promoters, suggesting negative transcriptional control by a DinR homologue as for the uvrBA locus of B. megaterium, upstream of which such a DinR-box-like motif was found as well (Nahrstedt & Meinhardt, 2004). Since the gene encoding the SOS repressor DinR was recently identified (H. Nahrstedt & F. Meinhardt, unpublished results), a regulon similar to the SOS systems in B. subtilis is present in B. megaterium. However, in marked contrast, B. megaterium possesses two functional recA genes, which presumably enhances efficiency of this highly conserved DNA repair mechanism.

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