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Full Length Research Paper

Bacillus halodurans RecA-DNA binding and RecA-mediated cleavage enhancing at alkaliphilic pH *in vitro*

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In *Escherichia coli*, RecA protein catalyzes DNA pairing and strand exchange activities essential for genetic recombination. This is critical for normal cellular function under conditions that lead to altered DNA metabolism and DNA damage. The RecA proteins of *E. coli* and *Bacillus halodurans* both can bind to DNA and catalyze the specific proteolytic cleavage of LexA and lambda repressor which induces SOS response. At neutral pH self-cleavage of LexA depends exclusively on its binding to RecA filament, while at elevated pH (~10) it autodigests in the absence of RecA. We have shown in this work that the RecA-mediated cleavage and the binding of RecA to DNA promoted by *B. halodurans* are similar to those promoted by *E. coli* RecA, excepted that in the case of *B. halodurans* the rate of the cleavage reactions is increased at alkaline pH and that NaCl favors the binding of RecA to DNA. The results lead to two hypotheses for the pathway for RecA-mediated cleavage, in which we first suppose that the internal pH of the bacteria is neutral. Thus LexA cannot undergo autodigestion, the genes involving in DNA repair and replication are not transcribed, regulating the growth of the cell. The second hypothesis is that the external environment and the internal pH of the bacteria are alkaline; here also the bacteria may have developed strategies to maintain LexA not inactivated. These observations suggest that the LexA autodigestion in *B. halodurans* at high pH may be regulated at the transcriptional level and that *B. halodurans* may be haloalkaliphile bacterium.

Key words: DNA binding, haloalkaliphile, autodigestion.

INTRODUCTION

Bacillus halodurans is a haloalkaliphilic, aerobic or anaerobic *Bacillus* strain widely found in extremely alkaline saline environments, such as the Rift Valley lakes of East Africa and the western soda lakes of the United States (Horikoshi, 1999; Takami et al., 2000). Studies have been done to elucidate the mechanisms of adaptation to alkaline environments. Industrial applications of alkaliphilic microbes like *B. halodurans* have been also investigated (Horikoshi, 1999). This *Bacillus* produces many industrially useful alkaliphilic enzymes that have brought great advantages to industry (Horikoshi, 1999). Thus, it is clear that alkaliphilic *Bacillus* are quite important and interesting not only academically but also industrially. Generally, alkaliphilic *Bacillus* strains cannot grow or grow poorly under neutral pH conditions, but well at pH > 9.5. Adaptability to extreme environment especially to highly alkaline environments can be the result of a transcriptional regulation of stress response genes. In *Escherichia coli*, RecA plays a critical role by coordinating a comprehensive defense mechanism in what is referred to as the SOS response (Radman, 1975). For a growing number of bacteria, the SOS response has been recognized as a critical component of the response to environmental stress (Friedberg et al., 2006; Takami et al., 2000). It is an important mechanism which allows bacterial cells to maintain genome integrity. It is also induced by conditions which cause massive DNA damage or inhibit accurate DNA duplication or replication (Radman, 1975; Sassanfar and Roberts, 1990).

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Two major proteins are involved in SOS regulation, LexA a negative regulator (repressor) and RecA a positive regulator (co-protease). RecA catalyzes DNA pairing and strand exchange activities essential for genetic recombination (Roca and Cox, 1997). This is critical for normal cellular function under conditions that lead to altered DNA metabolism and DNA damage. RecA produces by specific activities, filaments on a single-stranded DNA depending on the type of DNA damage, and plays an important role in the DNA damage response repair mechanism (Friedberg et al., 1995; Kowalczykowski, 1991; Roca and Cox, 1997; Walker, 1984).

LexA binds to RecA nucleofilament in response to DNA damage or to inhibition of DNA replication. The binding of LexA to RecA catalyzes their specific proteolytic cleavage which induces SOS response (Friedberg et al., 1995; Little, 1984; Luo et al., 2001). At neutral pH self-cleavage of LexA depends exclusively on its binding to RecA filament, while at elevated pH (~10) it autodigests in the absence of RecA (Little, 1984). This cleavage inactivates the LexA repressor from binding to the SOS operator sequences and results in the transcription of the SOS genes, helping the cell to manage the DNA break (Cirz et al., 2006; Little and Mount, 1982; Matic et al., 2004; McKenzie et al., 2000; Paes da Rocha et al., 2008). This process promotes the cell survival and stimulates DNA repair *via* recombination, excision repair and mutagenesis, and by up-regulating inhibition of cell growth (Ennis et al., 1989; Little and Mount, 1982; Witkin, 1976). The genes that constitute the SOS-like processes have been conserved in a wide variety of bacteria including *B. halodurans* (Au et al., 2005; Cirz et al., 2006; Courcelle et al., 2001; Goranov et al., 2006; Miller and Kokjohn, 1990; Roca and Cox, 1990).

B. halodurans is useful everywhere in the world. It can be academically and industrially important and interesting. It can be used as pollution indicator and for remediation of alkaliphilic medium in Africa. *B. halodurans* is a bacterium growing in alkaline environments and produces many industrially useful alkaliphilic enzymes. These enzymes are widely used as additives to laundry detergents, to bleach pulp in the process of paper-making. Since the autodigestion of LexA can also occur at alkaline pH (~10) in the absence of RecA, we sought to understand the mechanism of RecA-mediated cleavage in this bacterium strain. To achieve this goal, we cloned, and purified the RecA, the full length $LexA_{1-207}$, a truncated version of LexA consisting of residues 66-207 of *B. halodurans* and the hypercleavable lambda repressor $cI_{101-229}DM$ (Ndjonka and Bell, 2006). Here further we compare the RecA-mediated cleavage of LexA, of the hypercleavable $cI_{101-229}DM$, and also the binding of RecA to DNA at neutral and alkaline pH. Furthermore we crystallized the *B. halodurans* RecA, the crystals diffracted at low resolution (2.5 Å), and we were not able to solve the structure.

MATERIALS AND METHODS

Protein expression and purification

The complete ORF of RecA was PCR-amplified from *B. halodurans* C-125 genomic DNA and ligated into an NdeI/BamHI digested pET-9a expression plasmid. The resulting plasmid is engineered to allow the expression of an untagged version of the recombinant RecA protein. The integrity of the insert and the in-frame insertion into the plasmid were verified by DNA sequencing. The recombinant plasmid was transformed and overexpressed into the arabinose-inducible BL21-AI strain of *Escherichia coli* (Invitrogen). Cell cultures were grown in 1 L Luria Broth at 37°C and induced at OD_{600} 0.5 with 0.2% arabinose. RecA was purified as described previously (Lusetti et al., 2003) with some modifications. After cell lysis by sonication in 50 ml sucrose lysis buffer (25% (w/v) sucrose, 250 mM Tris-HCl, pH 7.5), 30 ml of 25 mM EDTA was added to the cocktail. After centrifugation at 18,000 x g for 30 min, polyethyleneimine pH 7.5 was added to the supernatant to a final concentration of 0.5% for precipitation of DNA. The proteins were stirred 30 min at 4°C and centrifuged at 12,000 x g for 20 min. The pellet was washed with 50 ml of buffer R (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 10% glycerol) and 50 mM ammonium sulfate, centrifuged at 12,000 g for 20 min, and was extracted with buffer R supplemented with 300 mM ammonium sulfate. After centrifugation at 12,000 g for 20 min, the protein in the supernatant was precipitated at 0.37 g/ml of solid ammonium sulfate and slowly stirred for 30 min at 4°C. The resulting pellet after centrifugation was washed twice with buffer R and 2.1 M ammonium sulfate. Finally the pellet was resuspended in 50 ml buffer R plus 100 mM KCl and dialysed overnight against the same buffer.

The proteins were loaded onto a 25 ml HiTrap Q HP column (GE Healtcare) and eluted with a linear gradient of 0.1 M-1M KCl. The collected fractions were dialysed overnight in buffer A containing 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5% glycerol and 100 mM NaCl. The proteins were further purified onto a 25 ml HiTrap Heparin column (GE Healtcare), and Mono Q (GE Healtcare). The pure proteins were dialyzed into 20 mM Tris-HCl, pH 7.5, 1 mM DTT and 100 mM NaCl.

The dialyzed protein was concentrated to 81 mg/ml, aliquoted and stored at –80°C. RecA protein concentration was determined spectrophotometrically using an extinction coefficient calculated from its amino acid sequence by O.D. at 280 nm. The homogeneity of the enzyme preparation was analyzed by Glycine-SDS-PAGE (12.5% gel). The proteins were visualized by Coomassie Brilliant Blue staining.

The lambda repressor protein fragments $cI_{101-229}DM$, which contains the A152T, and P158T mutations, were constructed, expressed and purified as described previously (Ndjonka and Bell, 2006). We refer to this construct as $cI_{101-229}DM$, where the DM stands for double mutant.

The *B. halodurans* LexA was cloned into the plasmid pET14b, expressed and purified in the same method as described for the lambda repressor protein (Ndjonka and Bell, 2006). This plasmid expresses full length 207 amino acids and residues 66-207 of LexA with an N-terminal 6His tag. The protein was expressed from BL21 (DE3) cells grown at 37°C, induced at 1 mM IPTG (Research Products International), and purified by Ni-affinity chromatography (Qiagen). Pooled fractions from the nickel column were digested at 22°C with 50 units of thrombin for 24 h to remove the His-tag and dialyzed into 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM DTT, concentrated to ~36 mg/ml, and frozen at -80°C in small aliquots. Protein concentration was determined by O.D. at 280 nm using the extinction coefficient calculated from the amino acid sequence. All of the LexA and lambda repressor proteins used in this study contain the extra N-terminal GSHM sequence from the expression vector.

Crystallization of *B. halodurans* RecA

B. halodurans RecA was crystallized at 10 mg/ml by hanging drop vapor diffusion at 22°C where the reservoir solution consisted of 24% glycerol, 0.05 M Imidazole pH 6.5, 0.1 M Calcium Acetate. For X-ray data collection, crystals were transferred to the solution above with glycerol increased to 30%, and frozen in liquid nitrogen. The crystal diffract by 2.5 Å. X-ray diffraction data were collected at –180°C using a Rigaku RU300 rotating anode generator and a RAXIS-IV++ image plate detector. Images were processed with CrystalClear software (Molecular Structure Corporation). Crystals belong to space group P61 with one monomer per asymmetric unit. The structure of *B. halodurans* RecA was not solved

DNA binding assay

The RecA binding reaction was performed using a gel shift assay using [γ-^{32}P]dATP (GE Healtcare). RecA was incubated with 0.05 pmol ^{32}P labelled DNA 48-mer TGG (and cold 48-mer TGG DNA at 1 µM final concentration) at 37°C for 30 min in 50 µl of buffer containing 20 mM Tris-HCl (pH 7.4) or 20 mM CHES (pH 9.5), 1 mM DTT, 10 mM $MgCl_2$, 20 mM KCl, presence or not of NaCl (150 mM), 1 mM EDTA, 1 mg/ml BSA, 5% glycerol, 0.5 mM ATPγS. The reaction was terminated by adding 3x loading buffer and loaded onto a 1.2% agarose gel. The gel was subjected to electrophoresis in 0.5x Tris borate-EDTA buffer supplemented with 3 mM $MgCl_2$. Electrophoresis was conducted at room temperature at a constant 20 V for 4 h.

After electrophoresis, gels were dried onto filter paper under vacuum and were exposed in a cassette to a Kodak film. The level of DNA binding, the radioactivity in the free, uncomplexed substrate and that in the shifted forms was quantified using a Storm 860 PhosphorImager (Amersham Biosciences) and Image Quant 5.2 software (Molecular Dynamics). The percentage of protein binding to DNA was calculated from the ratio of the net intensities of the bands corresponding to complex protein-DNA and unbound DNA. Each reaction was carried out in duplicate.

RecA-mediated cleavage

RecA-mediated cleavage reactions were performed as described by Ndjonka and Bell (2006). A typical cleavage reaction was set up to contain 20 mM Tris–HCl (pH 7.4) or 20 mM CHES (pH 9.5), 50 mM NaCl, 30 µM (nucleotides) of 15-mer GTG-repeating oligonucleotide (Integrated DNA Technologies), 1 mM ADP (Sigma-Aldrich), 2 mM aluminum nitrate, 10 mM NaF, 2 mM $MgCl_2$, and 10 µM RecA. This mixture was incubated at 37°C for 20 min at which point 10 µM of lambda repressor or LexA proteins were added to the above mixture and incubated for another 20 min at 37°C. At the indicated time point aliquots were removed and the reaction was terminated by addition of 0.25 volumes of 5× SDS-PAGE loading buffer and immediately heating to 95°C for 5 min. The proteins were separated onto a 15% SDS (w/v) polyacrylamide gels, and visualized by Coomassie brilliant staining. The bands for cleaved and uncleaved lambda repressor or LexA were quantified by scanning densitometry, and used to determine the % cleavage.

The percent cleavage was calculated as indicated by Ndjonka and Bell (2006). Each reaction was carried out in triplicate.

RESULTS AND DISCUSSION

The study was carried out to examine the impact of the pH and the NaCl on the cleavage rate and on the binding of RecA to DNA. In the absence of NaCl the binding of *B. halodurans* RecA to DNA is reduced. At alkaline pH the *B. halodurans* RecA-mediated cleavage of LexA and lambda repressor is increased, while at neutral pH, it is reduced.

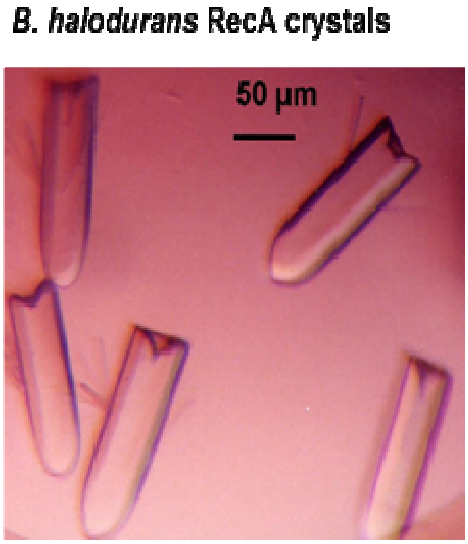

Figure 1. Crystals of the *B. halodurans* RecA. The protein crystallized at 10 mg/ml by hanging drop vapor diffusion at 22°C where the reservoir solution consisted of 24% glycerol, 0.05 M Imidazole pH 6.5, 0.1 M calcium acetate. The crystals were >200 µm big and diffracted at 2.5 Å.

We first crystallized the *B. halodurans* RecA protein. The crystals are shown in Figure 1. Secondly we studied the effects of NaCl and pH (neutral and alkaline) on the binding of *B. halodurans* RecA to DNA. Simplified schematics of the DNA substrates used in DNA binding experiments are provided in Figure 2. Thirdly we studied the *E. coli* RecA-mediated cleavage reaction of the hypercleavable lambda repressor $cI_{101-229}$DM at neutral pH. The introduction of the two inducible mutations (DM) significantly increases the cleavage rate (data not shown). Finally we studied the *B. halodurans* RecA-mediated cleavage reaction of the *B. halodurans* truncated $LexA_{66-207}$, full length $LexA_{1-207}$ and the hypercleavable lambda repressor $cI_{101-229}$DM at neutral and alkaline pH then we compared the different results.

Crystals of *B. halodurans* RecA

B. halodurans RecA protein was crystallized at 10 mg/ml by hanging drop vapor diffusion. The protein crystallizes at 22°C in solution containing 24% glycerol, 0.05 M Imidazole pH 6.5, 0.1 M Calcium Acetate. The crystals were >200 µm in size and shown in Figure 1. The crystal diffracted by 2.5 Å, but the structure was not solved.

Binding of RecA to DNA

The Gel shift assay of RecA was performed in the pre-

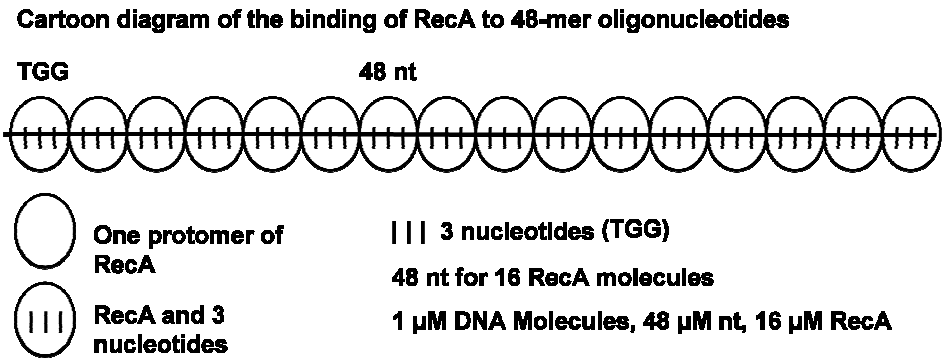

Figure 2. Cartoon diagram of the binding of RecA to 48-mer oligonucleotides. The TGG-repeating oligonucleotide is 48-mer and that one protomer of RecA is bound to three nucleotides of ssDNA, 16 protomers of RecA are bound to one 48-mer TGG-repeating oligonucleotide. That means 1 µM DNA; TGG-repeating oligonucleotide will need 16 µM RecA for a full binding of RecA to DNA.

sence of ATPγS, ^{32}P labelled DNA 48-mer TGG-repeating oligonucleotide. The triplet-repeating nature of the sequence is due to the fact that in the nucleoprotein filament, one protomer of RecA is bound to three nucleotides of ssDNA as shown in Figure 2. Since the TGG-repeating oligonucleotide is 48-mer and that one protomer of RecA is bound to three nucleotides of ssDNA, 16 protomers of RecA are supposed to bind one 48-mer TGG-repeating oligonucleotide (Figure 2). That means 1 µM 48-mer TGG-repeating oligonucleotide will need 16 µM RecA for a full binding of RecA to DNA. As shown in Figure 3a, notice that the addition of NaCl to the reaction slightly favors the binding of RecA to DNA at alkaline pH. The binding is increased by 1.4-fold in the presence of NaCl at alkaline pH. It has been shown that *Bacillus halodurans* C-125 requires Na^+ for growth under alkaline conditions (Takami et al., 2000). Also Na^+ was found in the crystal structures of *E. coli* RecA in the compressed form (Xing and Bell, 2004). This fact can explain why NaCl is needed to increase the binding of RecA to DNA. Since growth is related to DNA replication.

Figure 3b shows RecA and DNA binding at neutral pH. At this pH 7.4, the entire DNA is not bound to RecA at the concentration 16 µM in the presence or absence of NaCl. This result suggests that the NaCl has no effects on the binding of *B. halodurans* RecA to DNA at neutral pH. The results show that neutral pH affect negatively the binding of *B. halodurans* RecA to DNA. At this neutral pH, *E. coli* RecA binds completely to DNA and NaCl has no effect on the binding (data not shown). This result can be explained by the fact that *E. coli* grows at neutral pH. Figure 3c shows RecA and DNA binding at alkaline pH. At this pH 9.5, in the absence of NaCl, there are unbound DNA remaining in the solution at 16 µM RecA. In the presence of NaCl, the entire DNA is bound to 16 µM RecA. This result suggests that at alkaline pH, NaCl affects positively the binding of *B. halodurans* RecA to DNA by increasing the rate of binding.

Design of repressor and characterization of *B. halodurans* RecA-mediated cleavage

RecA-mediated cleavage reactions were performed in the presence of 15-mer GTG-repeating oligonucleotide, ADP-AlF_4. $LexA_{1-207}$ and truncated version of LexA consisting of removing residues 1-65 at the N-terminal DNA binding domain have been constructed and cloned. Removing the N-terminal domain of both LexA and λcI repressor was intended to destabilize the dimer formation, since it is the monomeric form of LexA or lambda that can bind to RecA and undergo self-cleavage and enhanced cleavage. The effects of RecA-mediated cleavage of LexA and lambda were compared at two different pH points as shown in Figure 4a. The time courses for the RecA-mediated cleavage reaction are quantitated in Figure 4a. The fraction of the LexA or lambda repressor cleaved is actually increased with pH, and the yield of final products is increased by 1.5- and 1.3-fold after 20 min of reaction in the case of RecA-$LexA_{1-207}$ and RecA-$cI_{101-229}$DM mediated cleavage at alkaline pH respectively. As seen in this Figure 4a, deletion of the N-terminal domain fragment of LexA and lambda repressor did not enhance RecA-mediated cleavage at neutral pH. At this pH 7.4, 37% of both repressors remain uncleaved, while at pH 9.5, $LexA_{1-207}$ is cleaved considerably faster than the same fragment at pH 7.4 (data not shown). Surprisingly the full length $LexA_{1-207}$ (98% cleavage after 20 min, Figures 4a, c) and the hypercleavable $cI_{101-229}$DM (85% cleavage after 20 min, Figures 4a, e) are cleaved significantly faster at pH 9.5 than the truncated $LexA_{66-207}$ (67% cleavage after 20 min, Figures 4a, b) and the hypercleavable $cI_{101-229}$DM (63% cleavage after 20 m, Figures 4a, d) at neutral pH. This result is different with the one shown by *E. coli* RecA-mediated cleavage of $cI_{101-229}$DM where the N-terminal deletion of cI enhances cleavage (Ndjonka and Bell, 2006). These results suggest that *B. halodurans* RecA-mediated cleavage is

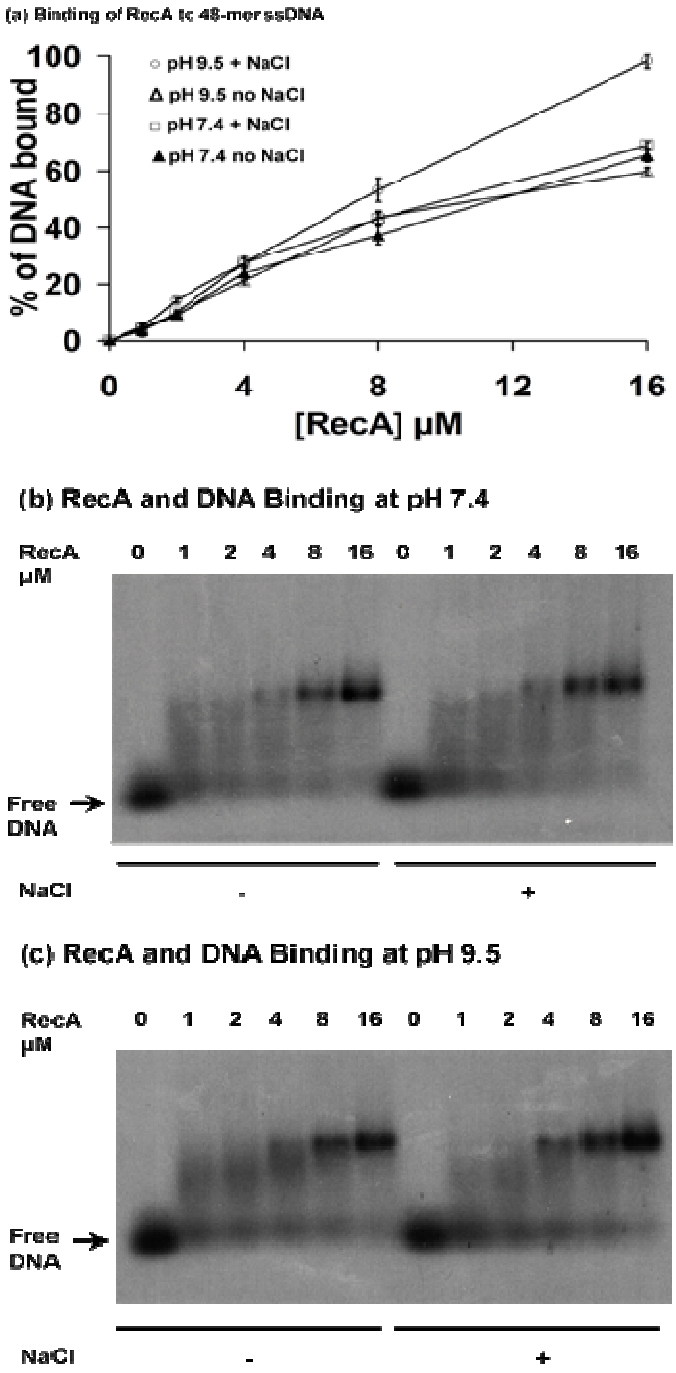

Figure 3. Gel shift assay of RecA in the presence of ATPγS, ^{32}P labelled DNA 48-mer TGG-repeating oligonucleotide. (a). binding of RecA to 48-mer ssDNA. Notice that the addition of NaCl to the reaction is helpful for the full binding of RecA to DNA at alkaline pH. (b) RecA and DNA binding at neutral pH. At this pH 7.4, the entire DNA is not bound to RecA at the concentration 16 µM in the presence or absence of NaCl. (c). RecA and DNA binding at alkaline pH. At this pH 9.5, in the absence of NaCl, the entire DNA is not bound to RecA at the concentration 16 µM. Free DNA is still in the solution. In the presence of NaCl, the entire DNA is bound to RecA at the concentration 16 µM. The presence of NaCl increases the binding of DNA to RecA. The triplet-repeating nature of the sequence is due to the fact that in the nucleoprotein filament, one protomer of RecA is bound to three nucleotides of ssDNA.

not only specific to *B. halodurans* LexA, but requires also alkaline pH. Removing the N-terminal domain of both LexA and λcI repressor was intended to destabilize the dimer formation as shown by Ndjonka and Bell (2006), since it is the monomeric form of LexA or lambda that can bind to RecA and undergo self-cleavage and enhanced cleavage (Cohen et al., 1981; Phizicky and Roberts, 1980). That was the same objective by introducing the inducible mutations in the hypercleavable lambda repressor $cI_{101-229}DM$ (Gimble and Sauer, 1989; Sauer et al., 1982). But the gel in Figures 4b, c, d, e shows unexpected results compared to the one reported (Gimble and Sauer, 1989; Ndjonka and Bell, 2006).

These results can be explain by the fact that *in vivo*, in response to DNA break or inhibition of DNA replication, RecA is activated and stimulate the cleavage activity of LexA, which regulates the expression of DNA repair genes (Friedberg et al., 1995; Little, 1984; Luo et al., 2001). While in *E. coli*, at neutral pH self-cleavage of LexA depend exclusively on its binding to RecA filament, at elevated pH (~10) it autodigests in the absence of RecA (Little, 1984). Taking in account the fact that *B. halodurans* is an alkaliphilic *Bacillus* strains, his LexA is probably auto-digested all the time, therefore inactivating the LexA from binding to the SOS operator sequences, and allowing genes involving in DNA repair and replication to be transcribed without regulation. This may be not the case and may be explained by two hypotheses. (a) One hypothesis is that the *B. halodurans* has developed structures in his cell wall to survive to alkaline environments by regulating the pH or a cytoplasmic pH regulation (Hamamoto et al., 1994; Krulwich et al., 1998; Takami et al., 2000). This first possibility supposes that the internal pH of the bacteria may be neutral. Thus, LexA cannot undergo autodigestion. The growth of the cells is then regulated. This may explain the slower RecA-mediated cleavage of LexA and lambda repressor we observed at neutral pH. (b) The second hypothesis is that the external environment and the internal pH of the bacteria are alkaline; here also the bacteria may have developed strategies to maintain LexA not inactivated. Adaptability to extreme environment especially to highly alkaline environments can result from the transcriptional regulation of stress response genes (Takami et al., 2000). The SOS response is known to be an important mechanism which allows bacterial cells to maintain genome integrity. These explanations can explicit the regulation of the SOS response and the RecA-mediated cleavage of LexA at alkaline pH.

The present study has demonstrated that using SDS gel and gel shift assay, both the RecA-mediated cleavage and the binding of RecA to DNA of *B. halodurans* are increased *in vitro* at alkaline pH. Interestingly the results of these studies also indicate that NaCl is needed to favor the binding of RecA to DNA at alkaline pH (9.5). *B. halodurans* may be by this fact a haloalkaliphilic bacterium. It would be interesting to explore the auto-digestion of LexA *B. halodurans* at high pH and to solve

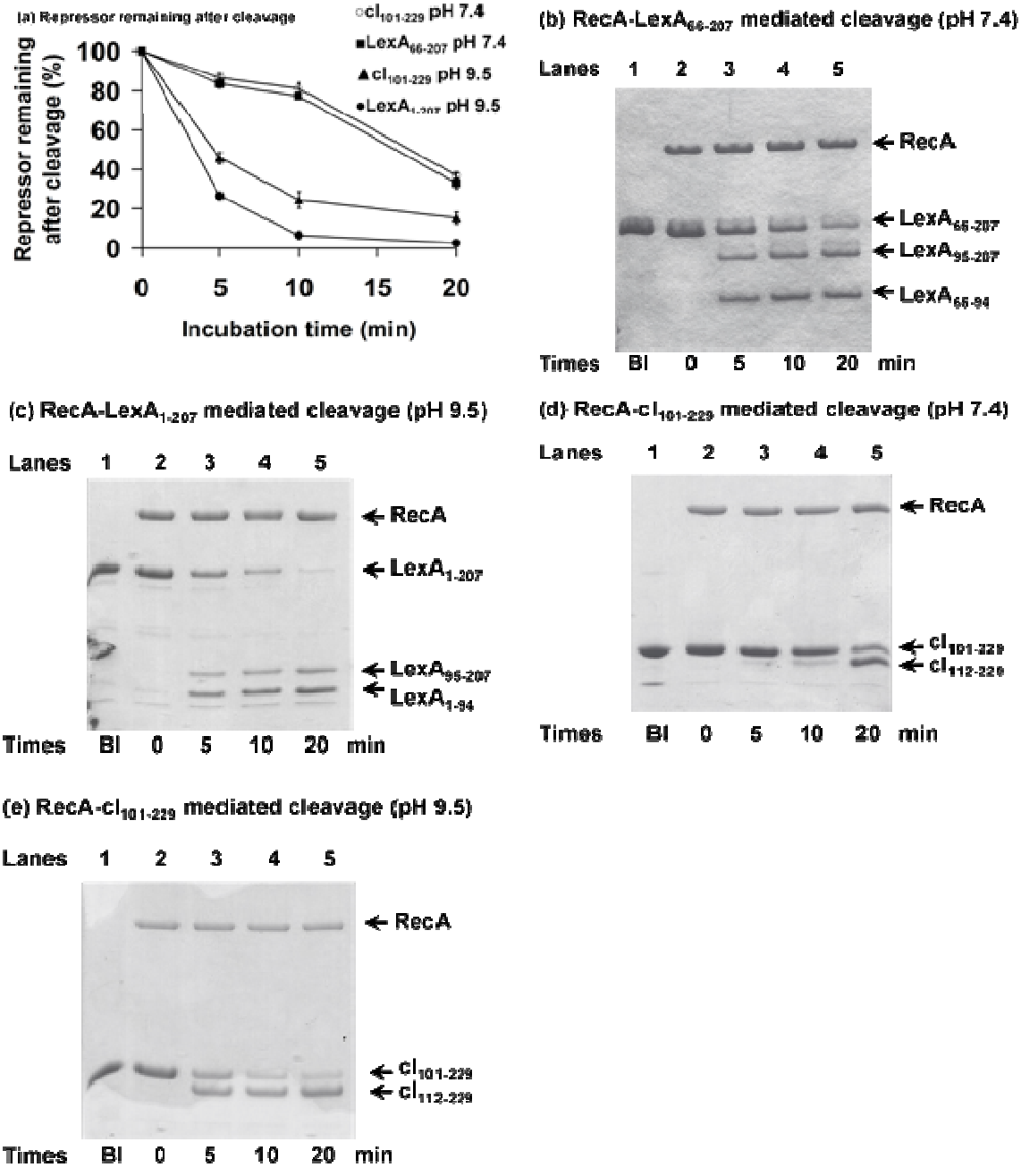

Figure 4. Biochemical characterization of LexA and cI repressor in the presence of ADP-AlF_4 and 15-mer GTG-repeating oligonucleotide. (a) Repressor rate remaining after cleavage. Notice that at pH 9.5, the cleavage rate of LexA and cI are significantly increased compared to the cleavage rate at pH 7.4. The cleavage rate of cI fragment is slightly slower than LexA, this may be attribute to the cross cleavage. (b) RecA-mediated cleavage of truncated $LexA_{66-207}$ fragment at pH 7.4. (c) RecA-mediated cleavage of truncated $cI_{101-229}$DM fragment at pH 7.4. At this pH 7.4 the introduction of inducible mutation in $cI_{101-229}$DM did not increase the cleavage rate. $cI_{101-229}$DM and $LexA_{66-207}$ are cleaved at the same rate as shown in (a). The removal of the N-terminal DNA binding domain of LexA did not increase the cleavage rate. (d) RecA-mediated cleavage $LexA_{1-207}$ fragment at pH 9.5. (e) RecA-mediated cleavage of truncated $cI_{101-229}$DM fragment at pH 9.5. Notice that the two repressors cleave almost at the same rate.

the structure of the RecA of this bacterium strain. Since haloalkaliphilic bacteria are exclusively distributed in two regions in the world (the Rift Valley lakes of East Africa and the western soda lakes of the United States) more attention must be taken on these bacteria.

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