Industry

THE EFFECT OF MICROWAVE ON THE CELLULAR DIFFERENTIATION *BACILLUS SUBTILIS* YB 886 AND REC DERIVATIVES YB 886 A4

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ABSTRACT

This study was carried out to investigate effects of microwave irradiation on cell differentiation and SOS repair system in Bacillus subtilis rec+ YB 886 and rec derivatives YB 886 rec A4. Amount of a specific protein that shynthesed during DNA damage by SOS repair system and binding to din C promoter region increased by microwave irradiation in rec+ bacteria. More increasing were determined in amount of the specific protein during Ultraviolet treatment. If amount of DNA, protein and RNA are taken into consideration, it has observed that amount of DNA decreased (P>0.05) but not statically significant, amount of protein decreased (p<0.05) and a little increasing, (P>0.05) in amount of RNA may be due to structural deformation.

Introduction

SOS like DNA repair systems in *Bacillus subtilis* (gram +) have been widely reported (17). The SOS regulatory system of bacteria controls the cellular response to conditions that damage DNA or inhibits DNA replication (7, 23). When cells are treated with agents that induce the response, such as UV irradiation or mutagens, on inducing signal produced, which rec protease is activated (3) to a form that can increase the rate of *lex A* repressor cleavage. During cleavage, *lex A* inactivated and the SOS genes are activated. Accordingly, the rate of *rec A* dependent cleavage controls the state of the SOS system (8). Although cleavage of *lex A* requires an activated form of *rec A* in-vitro at neutral pH, these repressors cleave themselves at high pH (9). This behavior is called self-processing reactions. The locations of cleavage site and active site in *lex A* protein have been established by a combination of genetic and biochemical analyses and by comparisons with the amino acid sequences of other cleavable proteins (10). The mechanisms and regulation of inducible DNA repair systems have been extensively characterized in the bacterium *Esherichia coli*. The best understood of these is the SOS regulatory system, which comprises about 20 unlinked genes that are coordinately induced by a variety of agents that cause DNA damage (23, 7). Induction of these damage inducible (din), or SOS genes result in the pleitropic SOS response characterized by increased DNA repair capacity, increased mutagenesis, and filementation (11). As in *E.coli*, a variety of DNA damaging agents induce cellular functions in *B. subtilis* as increase DNA repair capacity, increase mutagenesis, and inhibition of cell division (11, 16, 22). The introduction by DNA damage of *B.subtilis* din genes are dependent on a functional rec A protein. A palindromic consensus sequence, $GAACN_4GTTC$, is located within promoter region of several distinct *B.subtilis* din genes: the locations of these sequences, coupled with deletion analyses, suggest

that it could serve as a SOS repressor-binding site (12).

Microorganisms are affected by thermal and nonthermal effects of microwaves. There are some reports suggesting that nonthermal microwave affects are due to the energy required to produce various types of molecular transformation and alterations (4). On the other hand, low intensity microwaves do not have sufficient energy to break chemical bonds directly (5, 21) implying that there must be alternative mechanisms of interaction between cm waves and biological molecules such as DNA. Moreover, in microorganisms by microwave irradiation were reported to alter the permeability of cell walls and cell membranes, and generated ionic currents in the vicinity of cell membranes (2), and enzyme activity, protein synthesis and some metabolic phenomena would change to dependent on excited of cell macromolecules (2, 15). Finally, some investigators suggested that the nonthermal exposure of *E.coli* to pulsed microwaves at 10 mW/cm^2 produced the repairable DNA damage (18).

This study was carried out to study cell differentiation by microwave in rec- and rec+ *B.subtilis.*

Materials and Methods

Bacterial culture and lysis: Wild type strain *Bacillus subtilis* YB886 (met B5, trpC2xin, 1SPβ-) and rec derivatives YB1015 (YB886 rec A4) were obtained from Dr. Charles M. Lowett, Williams College, O'Gara Lab. Williamstown, Massachusetts. Bacteria were grown on Luria Bertoni (LB) medium (10 g. tryptone, 5g. yeast extract and 10 g. NaCl for 1 liter) at 37°C and harvested in optical density at 600 nm: 0.8 (late log phase) centrifuged at 10.000 X g. for 15 minutes. Pelleted cells were resuspended in 5ml of lysis buffer (2 mM tris pH 7.5, 10% sucrose, 1mM dithioreitol, 0.1 mM EDTA) per liter of bacterial culture. After addition of PMSF (phenylmetylsulfonyl fluoride) to 0.6 mg/ml. Then the pellets were added with 2 mg/ml lysozyme, and incubated on ice for 30 minutes, sonicated 1 minute and incubated at 37 °C for 15 minutes. Debris was removed by centrifugation at 10.000 g. for 45 minutes at 4°C. Protein concentration was determined relative to a bovine serum albumin (BSA) standard curve by using the lowry's method (13).

Preparation of *din C* promoter region: *din C* promoter regions (GAACN_4GTTC) were syntheses with a pharmacia nucleotide syntheser, then amplified by polymerase chain reaction (PCR) using synthetic oligonucleotide primer (1μM) with Perkin-Elmer thermal cycler.

Measurement of binding to *din C* promoter regions: Crude extracts were incubated with *din C* promoter region (30 μg/ml) for 30 minutes at 25°C in incubation buffer containing HEPES- NaOH pH 7.9, 4 mM Tris Cl. pH 7.9, 12 % glycerol, 60 mM KCl, 1 mM EDTA, 1 mM Dithioreitol and 0.3 mg/ml BSA (12, 1). After 10 minutes, the incubation mixture was added 2 ml. of 0.5 M ammonium acetate in preparing ethanol, and incubated -20°C for 40 minutes. Then mixture was centrifuged at 20.000 r.p.m. for 30 minutes at 4°C. The pellets were resolved TE buffer. The optical density in 260 and 280 nm of pellets resolved in TE was determined by UV spectrophotometer (Unicam, 8625, UV/VIS). The ratio, 260/280, being decreased indicates that the amount of protein bounded to DNA is increasing (14)

Chemicals: Chemical sources were as follow; PCR materials, tris-Cl, KCl, EDTA, sucrose, tris-base, NaCl, lysozyme, PMSF, HEPES, ammonium acetate from Sigma, Taq polymerize from New-England Biolabs and other chemical material from Merck.

Microwave Exposure: In these experiments, we used 2450 MHz microwaves in 55 W. (İmperial V- 8505T). The device modifielt that supplied nonthermal conditions was attack to circulate cold water by

chryostate. Thus , The experiments carried out at 37°C (± 1 °C) The heat generated during the applications was controlling with circulating cold water that controlled with thermostat. Irradiation of bacteria was performed at 37-38°C for 1hour during 3 days. Irradiated bacteria were employed in experiments immediately.

Bacterial DNA and Total RNA isolations: Bacterial chromosomal DNA was isolated by modification of the phenol extraction procedure (19). The cells were harvested by centrifugation, washed twice in 10 ml saline-EDTA buffer (0.15 M NaCl containing EDTA pH 8.00) and suspended in 5 ml of saline-EDTA containing 1 mg of lysozyme per ml. After 30 min of incubation in shaker at 37°C sodium laurlyl sulfate (SLS) and sarkosyl NL97 were added to a final concentration of 1% and the mixture was incubated at 55 °C for 30 min. Twenty ml of Tris-SLS buffer (0.1 M Tris containing 1% SLS and 0.1 M NaCl, pH 9.00) and 50 ml of redistilled phenol saturated with Tris-SLS buffer were added, and the lysate was shaken at room temperature for 20 min. After centrifugation at 3.000 g at 20°C for 5 min, the aqueous layer collected and finally centrifuged at 18.000 g at 20°C for 10 min. The aqueous DNA solution was decanted and the phenol extraction was repeated twice.

Total RNA was isolated according to Assubell et al (1). Gram+ *B.subtilis* cell wall was broken by sonication, detergent lyse the membranes (SDS), and protease digestion was used to degrade cellular protein. Organic extraction and precipitation yield total nucleic acids. DNA is removed enzymatically and the RNA is repurified. Nucleic acid content determined according to optical density (14).

Quick lysis colony screening was prepared for a quick examination of the nucleic acid in bacterial cells modified according to Hardy (6). Bacteria were lysed in 0.1 ml. lysis solution (per 10 ml 100 mg SDS, 0.1mg bromphenol blue, and 0.8 ml. glycerol). Using a plastic toot-pick scoop up the cells from a length of 0.5-1 cm. drop in the corresponding glass tube. Then mix vigorously, keep at room temperature for at least 10 min., place at 70°C for a few minutes before loading gel (0.8% Agarose). After the run, DNA and RNA were stained 30 min with ethidium bromur (5µg/ml), and the bands were visualized by UV light and photographed by MP4 camera.

The Determination of rec- and rec+: Bacteria were grown to over-night at 32°C, then bacteria were transferred to 42°C. Bacteria that grown to over night at 42°C, subjected to U.V. irradiation for 10, 20, 30 second. The continuous growth bacteria sets were evaluated as rec+; uncontinuous growth bacteria sets were evaluated as rec - (14).

Results and Discussion

Microwave exposure studies on *B.subtilis* YB 886 (Rec+) and YB 886 A4 (Rec-) showed that the amount of DNA was decreased, however not statically significant, in both bacteria. DNA amount decreased more in rec- bacteria (**Table**). Microwave radiation effect on the bacteria also caused a decrease in the amount of protein. The DNA decrease in rec-was found statistically significant compares to rec+, and maximum decrease was observed in rec- bacteria. When the amount of RNA in Table are examined, it would bee seen that total amount of RNA increase for rec- and rec+ bacteria, but not statistically significant. The ethidium bromur was more bonded to migration band in electrophoresis gel for the bacteria exposed to microwave during quick colony screening experiments compared to control bacteria (**Fig. 1** and **Fig. 2**).

Differentiation in ratio, 260/280, of binding protein were tested by UV exposure on *din C* promoter region in the studies carried out on crude extract. The ratio of (260/280) was found 1.49 for rec+ and 1.9 for rec- bacteria exposed to UV expo-

TABLE

The statistical variation of cell number, the amounts of DNA, RNA and protein in microwave exposed bacteria and controls, and the ratio change at 260/280 due to binding protein to *din C* promoter region

	Control-DNA	Control-RNA	Control-Protein	Control-Cell amount	UV treatment 260/280	Microwave treatment 260/280
B.subtilis YB 886 (Rec+)	P>0.05	P>0.05	P<0.05	P>0.05	1.49	1.72
B.subtilis YB 886 A4 (Rec-)	P>0.05	P>0.05	P<0.05	P>0.05	1.9	1.92

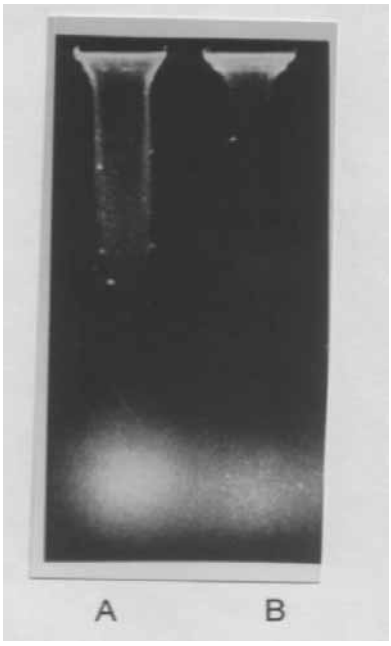

Fig. 1. Electrophoretic migration of nucleic acids of microwave exposed B.subtilis YB 886 (Rec+) using quick colony screening experiments (A. Irritated Microwave; B. Control).

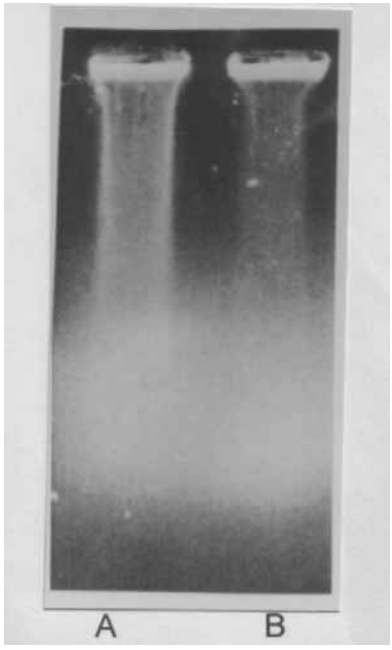

Fig. 2. Electrophoretic migration of nucleic acids of microwave exposed B.subtilis YB 886 (Rec+) quick colony screening experiments (A. Irritated Microwave; B. Control).

sure. Pure synthetic *din C* promoter region of 260/280 was measured as 2.005, and used as control. The ratio, 260/280 was found to be 1.72 and 1.92 for rec+ and rec-, respectively after microwave exposure treatments. These experiments were repeated many times to test reliability of these results.

If we consider binding of proteins to *din C* promoter region during DNA damage stage, the replecting the ratio, 260/280 which is smaller in rec. bacteria than rec.- indicates that microwave treatment affects DNA harmfully. The amount of binding protein having been less in comparison with UV may be originated from microwave nonionisation (4). DNA damage may result from transformation of nucleotides in normal tautomeric form to rare imino form or seconder chemical bonds transformed to resonance form by microwave effects (20). This case may arise due to differentiation of cell membrane permeability, excited of molecule electrons or inhibition of en-

zymes by microwave exposure (2, 15). The quick colony screening experiment carried out for determining whether DNA and RNA were effected by microwave exposure showed that broken H bonds in t-RNA and deformation of mRNA's coil may induce more divergence of RNA in electrophoretic gel. Migration capacity of nucleic acids in electrophoretic gel are decreased by deformation of coil structure of them (5, 21). The RNA molecules that lost his natural conformation may be caused by abnormalities in regulation of protein synthesis in cell.

Amount of RNA were found a little greater in experiments but not statically significant. This situation may arise from breaking H bonds and deformation of coil structure of RNA and it has been stated (1, 14) that nucleic acids with single coils absorb UV. radiation more. at 280 nm. Also, the results obtained from quick colony screening experiments support this idea. Less deformation in conformation of RNA in rec+ bacteria possibly resulted from SOS response is triggered in the presence of DNA damage in rec+ bacteria. The results from this experiment shows that amount of DNA, total RNA, and generation of rec+ and rec- bacteria are virtually equal exempt the proteins that bind to *din c* promoter region. The reason of this similarity may be due to inhibition of cell division and replication of DNA by SOS response in rec+ bacteria (17, 11, 16, 22). Differentiation in amount of DNA, total RNA and growth in rec- bacteria may be resulted from the effect of microwave irradiation instead of SOS response. This is an evidence of this case; while rec- bacteria died, the rec+ bacteria were continuing to grow during U.V. experiments. It has been stated that growth of rec- bacteria rather decreased when they are exposed to microwave irradiation for a long time (23, 3, 18).

The result of this study reveal that the SOS regulatory system responds that microwave irradiation affects on bacteria. As stated in previous studies, microwave irradiation effects bacteria by breaking weak chemical bonds in DNA, RNA and proteins, excited electrons, changing O_2 to O form and getting water molecules in resonance (9, 2, 8, 15). SOS regulatory system reduced microwave effects on cell components for rec+ bacteria

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