

Differential Response and Signal Transduction Due to Cold Shock and Oxidative Stress in *Bacillus simplex* TWW-04

¹O.M. Gomaa and ²O.A. Momtaz

¹National Center for Radiation Research and Technology (NCRRT), P.O. Box 29, Nasr City, Cairo, Egypt

²Agricultural Genetic Engineering Research Institute (AGERI), 9 Gama St. Giza, Cairo, Egypt

Abstract: The accumulation of stress proteins in the cytoplasm, periplasm or membrane of bacteria is considered an initial signal which induces the expression of genes involved in defense, recovery and stress acclimation. Whole cell protein profile for *Bacillus simplex* TWW-04 showed a gradual increase in expressed proteins by increasing the hydrogen peroxide concentration, this was different from the heat shock proteins induced at 60°C and were closer to the cold-shock proteins expressed at 4°C, a marked increase in band number and intensity varied by the increase in hydrogen peroxide concentrations. The bacterial count and total protein content showed a decrease in cold-shock cultures, hydrogen peroxide (150 mM) and gamma (0.5 KGy) exposed cultures after 4 h. The colony forming ability for cultures exposed to cold-shock, hydrogen peroxide and gamma radiation showed no change after 4 h, but after 16 h of incubation, only the hydrogen peroxide culture failed to show any colony forming ability. The growth pattern followed by absorbance at O.D₆₀₀ after stress exposure showed different response for each culture. The level of membrane lipid unsaturation was measured through FTIR, the spectra showed a close pattern for both the cold-shock and oxidative stress exposed cultures at the characteristic wave number for C=C. It is therefore concluded that the response of *Bacillus simplex* TWW-04 during oxidative stress is close to that exhibited during cold-shock; the role is similar in protecting the cell membrane from damage during hydroxyl attack by increasing the ratio of unsaturated fatty acids to saturated fatty acids in the membrane lipids.

Key words: *Bacillus simplex* . oxidative stress . protein profile . stress response . lipid desaturation

INTRODUCTION

Biological membranes are essential for the cell integrity, providing a barrier between the inside and outside environments for the cell [1]. These barriers act as support to different proteins which are involved in an array of different cell functions such as energy transduction, signal transduction, solute transport, DNA replication, cell-cell recognition, protein targeting and trafficking [2]. A number of studies suggest that membranes can sense extreme environmental changes such as cold-shock, heat stress or the presence of oxidants in the media [3-5].

Bacteria respond to such extreme changes in environmental conditions by inducing unique groups of proteins, each according to the type of stress, there are also another set of commonly synthesized proteins, regardless of the type of stress induced. Approximately 30 common proteins shared between vegetative proteins, stress and starvation in *Bacillus subtilis* [6]. Another common adaptation response among all

bacteria is the adjustment of membrane lipid composition at low temperatures [7], this change is also reported due to oxidative stress [8]. *Bacillus simplex* TWW-04 was studied for its tolerance to hydrogen peroxide and low temperature, its adaptation was due to the production of a number of counteracting enzymes [9], the study of the cell membrane showed that its fatty acid composition was affected under oxidative stress conditions [10]. It was assumed that the membrane lipoproteins are the major component responsible for loss of colony forming ability. Previous studies show that membranes have the ability to sense external changes and as a consequence, changes occur in its physical state and microdomain organization, sending signals which activate transcription [3, 7]. Therefore, it seemed likely that bacteria sensing the environmental changes could start sending signals for transcription of different counteracting enzymes via an initial change in the membrane's biophysical state. The pattern is assumed to be close to that for cold-shock response since both oxidative stress (via the addition of hydrogen

peroxide) and cold-shock are considered initial signal transducers in bacteria [8]. The increase of unsaturated fatty acids in the cell membrane is a general response in some thermotolerant strains exposed to one or a combination of stresses especially oxidative stress [11]. Besides adaptation to external stress, the alteration in membrane fatty acid desaturation is thought also to be for maintenance of membrane functionality at low temperature [12].

In the present study, *Bacillus simplex* TWW-04 is exposed to sublethal oxidative stress and compared to cold-shock in order to compare the regulation response of this strain to both stresses. The protein profile, protein content, colony count, colony forming ability and growth pattern for the different cultures are studied. The degree of unsaturation for both cold-shock and oxidative stress of membrane fatty acids under the same conditions is examined by FTIR.

MATERIALS AND METHODS

Microorganism and culture conditions: *Bacillus simplex* TWW-04 obtained from a previous study [13] was used to inoculate LB media, static cultures were incubated at 30°C for 24 h. Two % of preculture inoculum (vol/vol) was used to inoculate flasks containing 50 ml LB media, H₂O₂ (30%) was added in different concentrations (50, 100 and 150 mM) after sterilization. Cultures were incubated for 120 minutes at 30°C and centrifuged rapidly to stop the action of hydroxyl radicals formed. For +ve control, cultures were exposed to cold shock, cultures were transferred to 4°C. For -ve control, cultures were exposed to 60°C. For culture growth under different stresses, the cultures O.D was measured at 600 nm for 60, 120, 240 and 300 min.

Irradiation process: Gamma irradiation was used in order to study another form of sublethal oxidative stress and its effect on the bacterial cells. A 50 ml sample of the mid-exponential liquid culture was subjected to gamma radiation at National Center for Radiation Research & Technology (NCRRT) using ⁶⁰Co source with dose rate 1.85 Gy/sec. The irradiation dose was 0.5 KGy.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE): Whole cell cultures were centrifuged at 3000 rpm for 15 minutes after each treatment, Phosphate Buffer Saline (PBS) (pH 7.2) was used for protein extraction. SDS-PAGE was performed for whole cell protein extracts at room temperature with 10% gels and Tris-glycine buffer (pH 8) at 125 V for 90 minutes to obtain the molecular weight of the enzymes,

protein bands were stained by 0.05% coomassie brilliant blue R-250. Molecular weights were compared to a low/medium protein marker (Bio-Rad). Protein gel documentation was performed using Alphatec 2200 software for protein band analysis.

Colony Forming Ability (CFA), colony count and total protein: For the detection of colony forming ability, 10 µl was spotted using a sterile tip onto LB agar plates at the chosen time. Photos were taken after 24 hr incubation at 30°C.

To measure the number of colonies, 100 µl aliquots of 10⁻⁹ time diluted cultures were spread onto LB-agar plates and incubated for 24 h at 30°C.

Protein concentrations were determined by the method of Lowry *et al.* [13] using bovine serum albumin as a standard using Shimadzu UV 2100 spectrophotometer.

FTIR analysis of membrane lipids: The analysis was performed on cultures exposed to cold-shock and hydrogen peroxide and were compared to that of a control culture. Cultures were centrifuged at 3000 rpm for 15 minutes to collect the cells, resuspended in phosphate buffer pH 7.2, ultra centrifuged (Sorvall ultra 80) at 100,000 rpm at 4°C for 10 minutes, the supernatant decanted and the pellets freeze-dried using Modulyo, Edwards Freeze-Drier, the dried pellets were homogenized and prepared for analysis using the FTIR (Unicam mattsom 1000).

RESULTS AND DISCUSSION

Microorganisms adapt to different environmental stresses, primarily by producing a number of stress proteins, some are general and others are specified according to the type of stress [6]. In facing stress, *Bacillus* cells have developed complex adaptational network, inside of which the induction of general or unspecific stress proteins seems crucial to its survival [14]. A set of about 50 proteins, which are induced by many environmental stimuli, is one of the most drastic responses of the *B. subtilis* cell to the transition from a growing to a non-growing state [15]. Accumulation of heat stress proteins (HSPs), which are termed chaperones, or the appearance of cold-shock proteins enable the cells to accommodate to such variant temperatures which could halt growth and damage many physiological parameters. In the current study, it is evident that the resulting protein profile reflects a change in protein synthesis related to the different stresses induced. Visual examination of Fig. 1 shows the SDS-PAGE for the whole cell proteins exposed to different types of stress, the increase in band intensity shows an increase in protein expression in cells with the

Table 1: Represents the data obtained from the analysis of the gel, data show the number of peaks, area and molecular weight for each distinct band

Lane	Peak	Dist	Width	Height	Area	%	MW
M	1 mws	73	14	213	3132	10.9	192.77
M	2 mws	92	5	226	1282	4.5	117.90
M	3 mws	134	19	241	4700	16.3	99.26
M	4 mws	231	26	242	6350	22.0	54.15
M	5 mws	295	22	241	5376	18.7	37.78
M	6 mws	330	12	242	2964	10.3	29.46
M	7 mws	436	19	225	4083	14.2	20.20
M	8 mws	453	6	77	913	3.2	7.44
-ve control	1	134	28	207	5844	44.0	116.42
-ve control	2	231	36	208	7443	56.0	79.26
+ve control	1	131	20	218	4303	27.8	117.57
+ve control	2	231	52	225	11165	72.2	79.26
1	1	131	22	199	4606	29.9	117.57
1	2	231	53	202	10775	70.1	79.26
2	1	118	13	191	2369	5.6	122.55
2	2	150	29	185	5454	12.9	110.29
2	3	340	163	232	34294	81.4	37.49
3	1	124	19	216	4043	23.7	120.26
3	2	147	12	201	2610	15.3	111.44
3	3	177	7	192	1429	8.4	99.95
3	4	224	41	213	8969	52.6	81.94

(M) Denotes for the marker (low/medium), (-ve control)heat shock, (+ve control) cold-shock, (1) hydrogen peroxide 50 mM, (2) hydrogen peroxide 100 mM, (3) hydrogen peroxide 150 mM

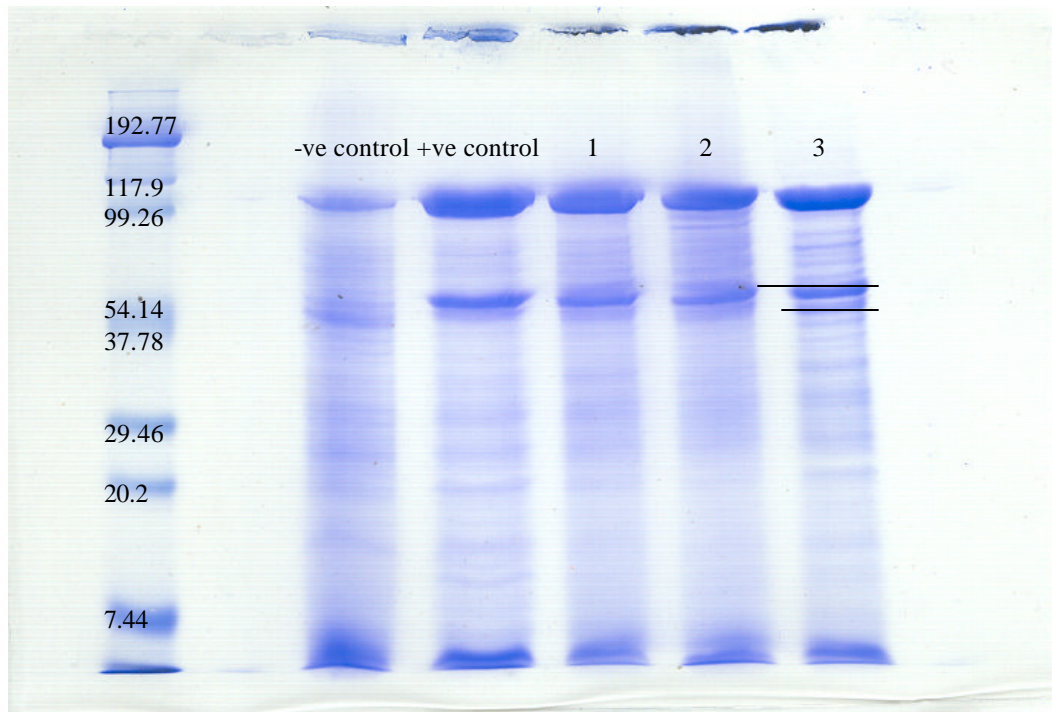


Fig. 1: SDS-PAGE for total cell protein of *Bacillus simplex* TW-04 exposed to 60°C (-ve control), 4°C (+ve control), hydrogen peroxide 50 mM (1), 100 mM (2) and 150 mM (3)

increase in hydrogen peroxide concentration but doesn't show a pattern resembling HSPs but rather close to the +ve control which is the cold-shock induced proteins. This is logical as this strain was known to tolerate to grow at extremely low temperatures and unable to grow

at 40°C [16]. The computer analysis of the gel using Alphatec 2200 reveals in depth data. The number of bands and their areas differ by the stress induced; also there is an increase which is relevant to the concentration of hydrogen peroxide indicating a

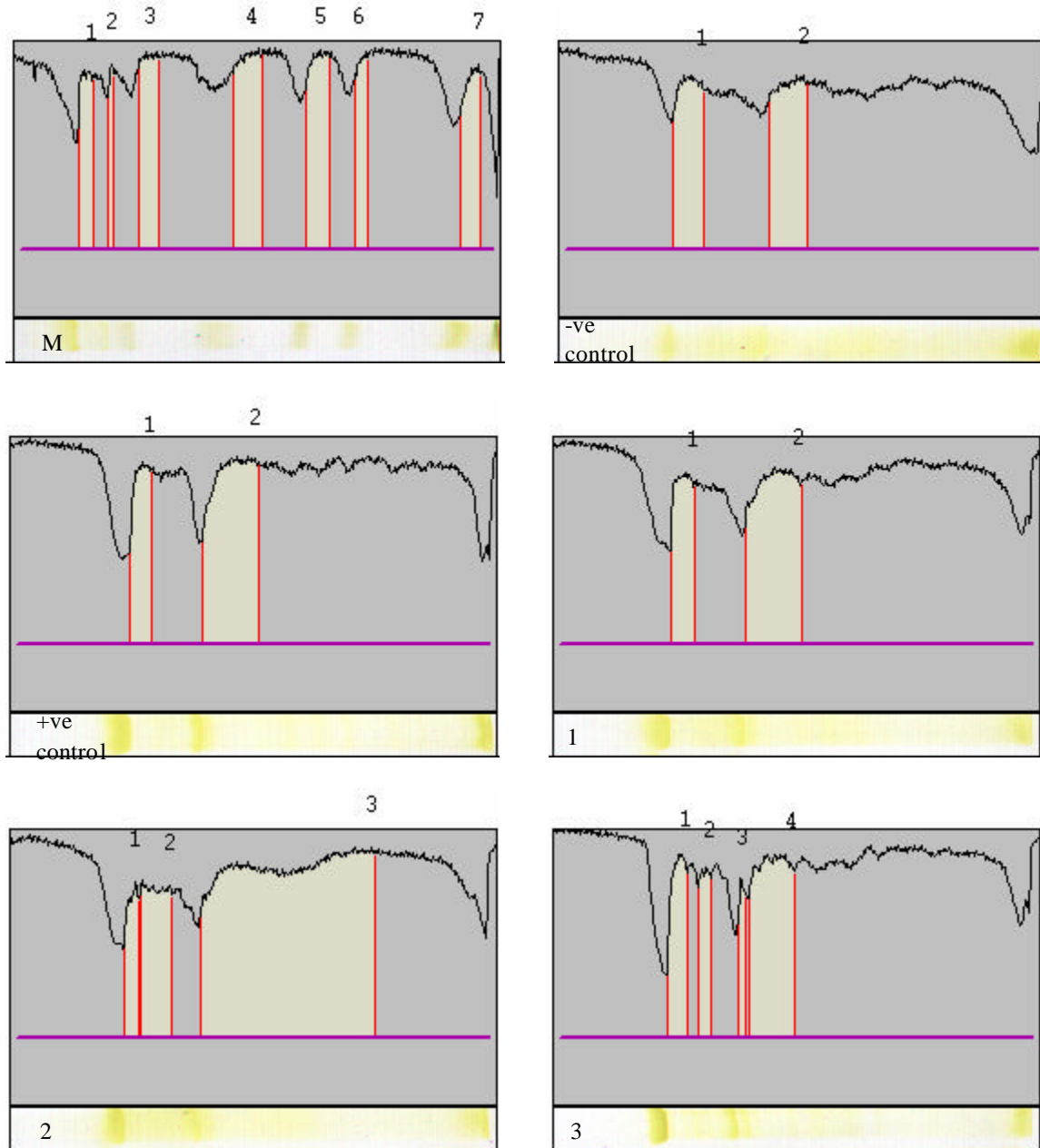
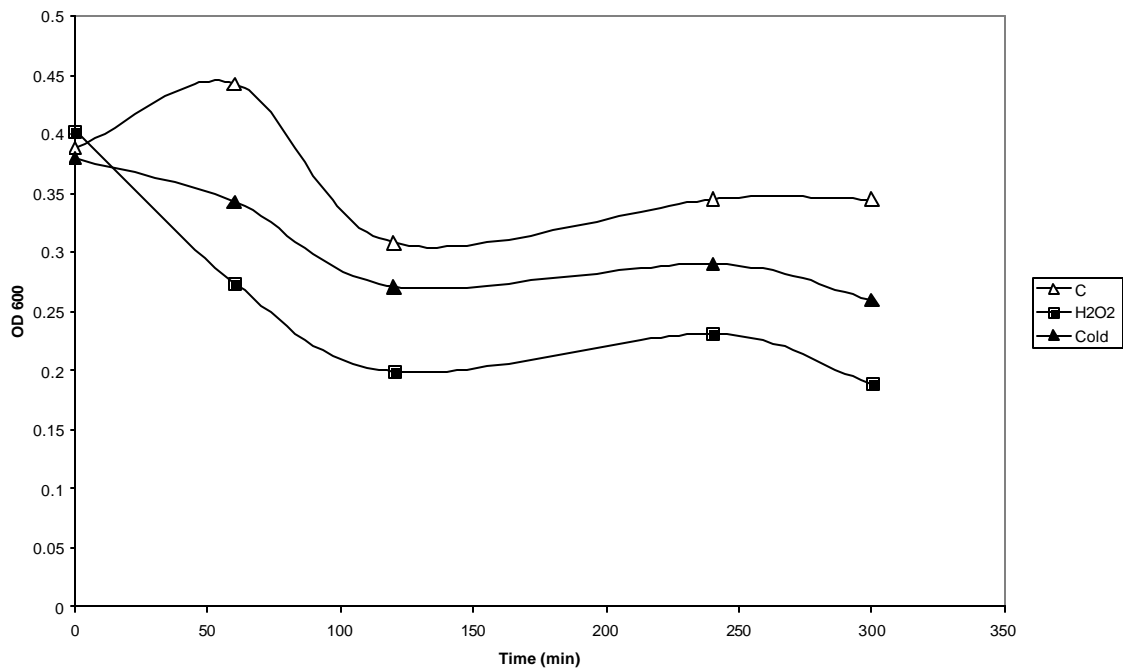


Fig. 2: Analysis of the obtained bands by the protein analysis software for the number and area for each peak obtained for every lane (M) marker, -ve control, +ve control, (1) hydrogen peroxide 50 mM, (2) hydrogen peroxide 100 mM, (3) hydrogen peroxide 150 mM

Table 2: CFU and protein content for cells subjected to different treatments in comparison to control cells after 4 h

Treatment	CFU ml ⁻¹	Protein (mg ml ⁻¹)
Control	8x10 ⁷	25.70
Cold-shock	23x10 ⁵	22.13
Hydrogen peroxide	12x10 ³	16.65
Gamma-irradiation	18x10 ³	19.20

relatively increased level of protein synthesis which is relevant to the level of stress induction (Fig. 2). Table 1 represents these changes. It is evident that there are two distinctive bands obtained at MW 117 and 79 KDa in -ve, +ve control and culture exposed to 50 mM hydrogen peroxide, the areas of which were the same for cold-shock and 50 mM hydrogen peroxide cultures. Another band appeared when the culture was exposed to 100 mM and a fourth band appeared at 150 mM



ig. 3: Growth of *Bacillus simplex* TWW-04 in LB media with no stress induced (Δ), after cold-shock at 4°C (\blacktriangle) and after the addition of 150 mM hydrogen peroxide (\blacksquare). The cultures were grown on LB at 30°C until OD₆₀₀ of 0.38-0.4 was reached before inducing cold-shock or oxidative stress

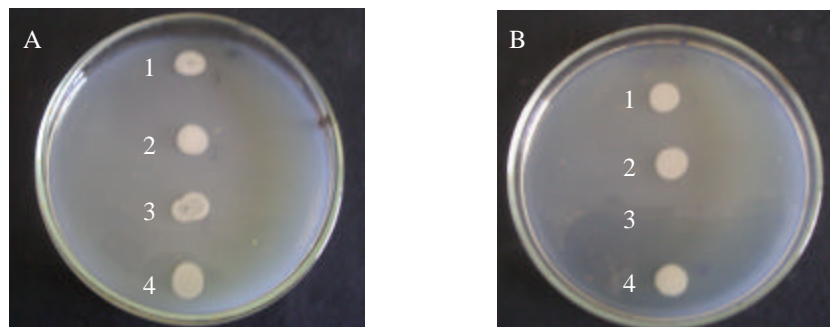


Fig. 4: LB plates representing the colony forming ability for the different cultures after exposure to the stress for 4 hours (A) and 16 hours (B). The samples represent (1) control culture, (2) cold-shock, (3) oxidative stress by the addition of 150 mM hydrogen peroxide, (4) 0.5 KGy gamma radiation

hydrogen peroxide. The table shows the distinct variation in the bands intensity. It is not strange that the pattern of protein synthesis might be similar when cultures are exposed to two different abiotic stresses, there are some regulons which are generally induced in bacteria exposed to parquat, heat, hydrogen peroxide and salt stress in *Bacillus subtilis*, sometimes overlapping occurs among two or more stress conditions, the same occurred when cells were tested for starvation [6].

To assess the level of response of cells to the stresses, a number of parameters were tested. The colony count of the cultures subjected to low doses of

hydrogen peroxide, gamma radiation or cold-shock revealed an obvious decrease in colony forming units after 4 hours of incubation, except for hydrogen peroxide exposed cultures, all cultures showed maintenance for their colony forming ability even after 16 hours of exposure (Fig. 3 and Table 2). This experiment was conducted to ensure the visual response of the cell viability under the different stresses used in this study, the results are in agreement with previous studies for the same strain [5, 16].

The whole cell protein levels (Table 2) were affected by the oxidative stress, indicating an interaction with proteins which could be attributed to an

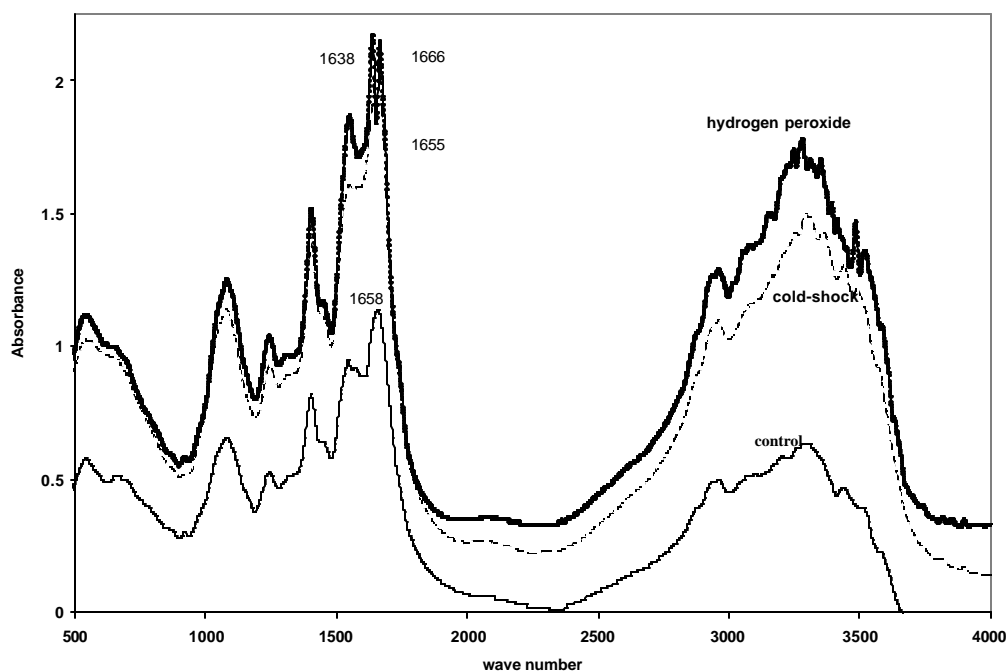


Fig. 5: FTIR for control cultures (no stress), cultures exposed to cold-shock and cultures exposed to oxidative stress by the addition of hydrogen peroxide (150 mM)

Table 3: Wave number and intensity for the peaks of cold-shock and hydrogen peroxide exposed cultures compared to the control

Culture	Wave number	Intensity
Control	1658.92	1.14
	-	-
Cold-shock	1088.34	0.66
	1655.68	2.07
Hydrogen peroxide	-	-
	1080.14	1.14
	1638.86	2.17
	1666.77	2.15
	1080.76	1.25

interaction between hydroxyl radicals formed via the addition of hydrogen peroxide and gamma radiation which interacts directly with the cells. Cold-shock cultures showed a slight decrease in total protein concentrations. Although the three cultures showed similar growth pattern, yet they didn't exhibit the same growth rate, hydrogen peroxide exposed cultures experienced the slower growth, while cold-shock cultures were intermediate between control cultures and hydrogen peroxide (Fig. 4). The results are in accordance with Weber *et al.* [12] and Gomaa and Momtaz [16]. All previous experiments didn't precisely reveal the exact similarity between cell response to cold-shock and oxidative stress. Therefore, it was essential to study a relevant parameter which could explain the resistance of *Bacillus simplex* TWW-04 to

both cold-shock and oxidative stress and highlight the cell response to these stresses. It is known that low temperature causes changes in membrane lipid composition, these changes could be alteration of fatty acid types, altered lipid classes, changes in lipid to protein ratios or increase in fatty acyl unsaturation [17].

Figure 5 represents the spectrum using FTIR of cell membrane to show the degree of lipid unsaturation exhibited at approximately 1650 (characteristic for the C=C), there is an obvious shift in the wave number for the cultures exposed to stress compared to the control culture. Also, the intensity of unsaturation increases by the exposure to both cold shock and oxidative stress, compared to the control sample (2.07 and 2.17 for the stress exposed cultures compared to 1.14 for the control culture), there is splitting in the peak at 1638.86 and 1666.77 for the hydrogen peroxide exposed culture with an increase in the intensity. There is an obvious stretching in the C-C bond at approximately 1080 from 1.14 for the cold-shock culture and 1.25 for the hydrogen peroxide exposed culture compared to 0.66 for the control culture (Table 3).

The degree of fatty acyl desaturation of membrane lipids is considered to be a critical determinant in membrane fluidity [18], this membrane fluidity is a trigger for membrane remodeling and aids in bacterial tolerance to heat in *E. coli* [4]. The relation between fatty acyl desaturation and bacterial adaptation was reported for a number of strains exposed to heat, salinity, cold or oxidative damage [4, 11, 18, 19]. This

implies that membrane lipids play an essential role in microbial adaptation under different environmental conditions.

It is our conclusion that *Bacillus simplex* TWW-04 responds to oxidative stress through tight regulation of the lipid composition of the cell in a similar mechanism to low thermal adaptation, this mechanism is believed to be designed to ameliorate the external changes on the physical state of the cell membrane through desaturation of fatty acids of their membrane lipids. Cold shock adaptation was found to be through *des* gene encoding $\Delta 5$ desaturase, this enzyme is responsible for increasing the ratio of unsaturated fatty acids (UFA) to saturated fatty acids (SFA) [20], it is present constitutively in all prokaryotes and eukaryotes in response to decrease in temperature. It was also reported that the acyl chain desaturase was proved to be an integral membrane protein in yeast [18], this proves that the first reaction of microbial cells upon exposure to stress is a change in the degree of acyl chain desaturation of membrane lipids, this occurs before the cell sends signals to produce a set of specific proteins to counteract the damage exerted.

It is therefore speculated that the *Bacillus* under study might use the same approach since it tolerates cold-shocks as well as high concentrations of hydrogen peroxide. The identification of the gene responsible for this adaptation is currently under investigation in our laboratory.

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