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INVOLVEMENT OF *bolA* AND *rpoS* GENES IN OXYGEN UPTAKE (RESPIROMETRY) BY *E. coli* K-12 MG1655 UNDER VARIOUS STRESS CONDITIONS

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Abstract - Differences in respiratory activity of *Escherichia coli under* various stress-induced environments has shown the ability of *rpoS* and *bolA* genes to respond in several stress conditions and adaptation, leading to an increase rate of oxygen uptake. The results have shown the rate of respiratory activity (defined in mg O_2/g bacteria min^{-1}) changed under various stress-induced conditions, i.e. heat shock, cold shock and oxidative stress. Sudden shifts from optimal growth conditions to stress-induced environments led to an increase of the oxygen uptake rate of *E. coli* in presence of *rpoS* and *bolA* genes.

INTRODUCTION

Flexibility of gene expression in bacteria permits their survival in varied environments. The genetic adaptation of bacteria through systematized gene expression is not only important, but also clinically relevant in their ability to respond under various stress environments (Adnan *et al.*2010; Latasa *et al.* 2006). Stress-induced responses enable their survival under more severe conditions, thus enhancing resistance and/or virulence (Costerton *et al.*, 1987).

The study of the cell envelope (i.e., cytoplasmic membrane, outer membrane and periplasm) protein expression in response to environmental stresses has been described as the extra cytoplasmic stress response (ESR) (Rowley *et al.* 2006). The ESR due to environmental changes and stresses has been studied in many bacterial pathogens, but there is no investigation on respiratory activity of bacteria under various stress environment conditions in relation with *bolA* and *rpoS* genes. Perturbations in either the external or internal environments such as heat and cold shock, pH fluctuations or nutrient starvation, must be communicated to the cytoplasm so that gene expression and post-translational responses can be modified to ensure the survival of organism (Hengge -Aronis, 1996).

When subjected to stress, bacteria respond by metabolizing intracellular substrates. This endogenous respiration presumably supplies energy to maintain cell viability and substrates to repair damage caused by auto degrading enzymes (Boylen and Ensign, 1970). Temperature change serves as a good signal to regulate gene expression in *E. coli* and other bacteria. *E. coli* is likely to encounter shifts to either low or high temperatures either for short term or long term gain during their life cycle (White-Ziegler *et al.* 2008). Stress response genes are induced whenever a cell needs to adapt and survive under adverse growth conditions (Vieira *et al.* 2004). Morphogene *bolA* in *E. coli* is one

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such example. It was first thought to play a role in adaptation to stationary growth phase (Lange and Hengge-Aronis, 1991; Aldea, *et al.*1989). However its function is still not fully understood and is not confined to the stationary phase. In fact its expression might be induced by different forms of stresses, such as heat shock, pH stress or cold shockwhich result in high level of expression of *bol*A mRNA (Lange and Hengge-Aronis, 1991; Aldea, *et al.* 1989). It also has a major effect on the bacterial envelope and therefore is probably involved in cellular protection under adverse growth conditions (Santos *et al.* 1999).

Respiratory activity, measured by oxygen uptake rate due to glucose oxidation, has already been used to assess the potential of antimicrobial agents (Simões *et al.* 2005). However, no reports are available concerning the use of this technique in studying the gene expression and its involvement in respiration or metabolic activity. This study used *rpoS* + / *rpoS*- (defective in the stress regulator sigma S) and *bolA*+/*bolA*-strains to study the respiratory activity of *E. coli* with different induced stress environments.

The subjects of endogenous respiration, the utilization of endo-cellular reserves and starvation survival have been dealt before (Boylen and Ensign, 1970). In this study, the effects of a variety of stresses including oxidative stress have been investigated asthey might alter the cell envelope, increasingthe susceptibility of *E. coli* to peroxidise bactericidal action on respiration of *E.coli* in presence and absence of *rpo*S and *bol*A genes.

MATERIALS AND METHODS

Bacterial strains and growth conditions

E. coli K-12 MG1655 wild type (WT) and two mutant strains (Δ) used in this study were kindly provided by National Institute of Genetics, Japan. The mutants were *E. coli* K-12 MG1655 *rpoS* mutant (*Δ rpoS*) and *E. coli* K-12MG1655 *bolA* mutant (*Δ bolA*). Cells were grown in TSB (Tryptic Soy Broth) medium for 18 hours at 37 °C and under agitation (130 rpm). Samples were taken atOD_{600} = 1.0 which was considered as exponential growth phase, whilst OD_{600} = 2.2 was considered to be stationary growth phase.

Stress response experiment
Heat shock, cold shock, pH stress and H_2O_2 stress

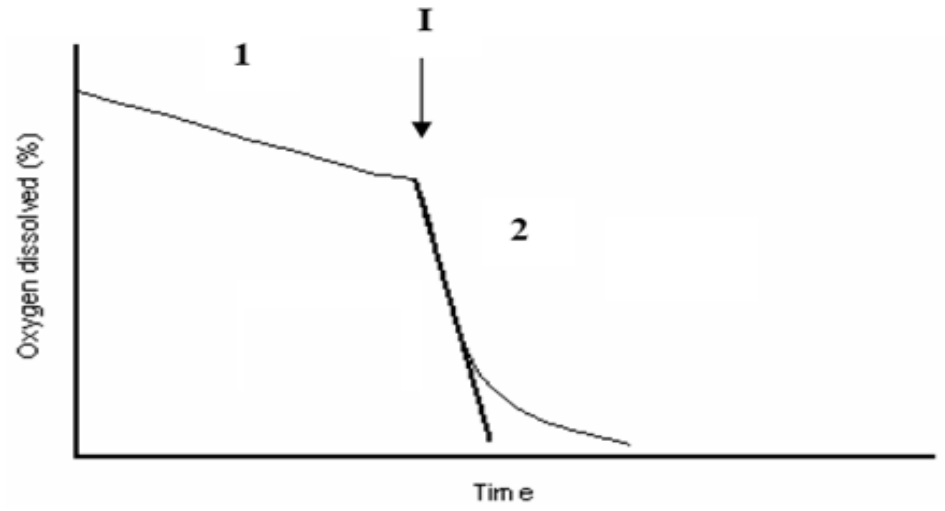
Aliquots of 0.1 mL of *E. coli* K-12 MG1655 culture (WT, *Δ bolA* and *Δ rpoS*) were withdrawn at 2 min intervals and plated out directly on TSA plates to determine the viable cellnumbers. Percentage survival was defined as the percentage change in the CFU counts per mL obtained after inoculation into TSB medium and incubation for 15 mins following asudden shift from optimal growth conditions, i.e., heat shock temperatures (42 and 46 °C), cold shock temperatures (5 and 20 °C) and different concentrations of H_2O_2 (3, 4 and 5 mM). Cells were washed three times in sterile distilled water. Cells were finally resuspended into 50 mL of distilled water and a volume of 5 mL of the cell suspension was pipetted the into respirometry cell. The respirometry cell was fitted in the respirometry chamber and desired temperature was adjusted with the sample for 15 minutes to check the sudden change in respiratory activity of *E. coli*. For oxidative stress condition, the vessel of the biological oxygen monitor (BOM) was controlled at 37 ºC ± 1ºC and H_2O_2 with final concentrations of 3, 4 and 5 mM was added for 15 min and respiring samples was monitored for 3 to 10 minutes.

Assessment of bacterial respiratory activity

The rate of O_2 consumption by stressed cells was measured by a polarographic oxygen sensor (model 53, Yellow Springs Instrument Co., Inc.) using a published procedure (Pereira *et al.*2002; Thomas and Aune, 1978). For each respirometry assay, 5 mL of bacterial cultures were placed in the temperature-controlled vessel of the BOM (T= 37 ºC ±1ºC). These parameters were varied for mimicking stress-induced conditions.The vessels contained dissolved oxygen (DO) probes connected to a DO meter. Once inside the vessels, the bacterial cultures were aerated for 30 min to ensure oxygen saturation. After reaching 100% saturation, the relative rates of O_2 consumption were determined from the linear portion of the continuous recording of O_2 consumption over 3 to 10 min and the decrease in oxygen concentration was monitored over time. To determine the oxygen uptake rate due to substrate oxidation, a small volume (12.5 µL) of a glucose solution (5 mg/L) was injected into each vessel. The parameter was expressed in mg of O_2 consumed per g of bacteria per time (mg O_2/g bacteria min^{-1}).

Biological mass quantification (Determining dry weight)

The dry mass of the biological samples was



1- Oxygen consumption due to endogenous metabolism
I- Nutrient addition
2- Oxygen consumption due total metabolism (endogenous and exogenous)

Fig. 1 Typical profile of oxygen uptake in an assay of respiratory activity* according to Stewart *et al*. (1994).
*The initial linear decrease observed (1) corresponds to the endogenous respiration rate. To determine the oxygen uptake due to substrate oxidation, a small volume (12.5µL) of a glucose solution (5 g/L) was injected within each vessel (point I), (2) corresponds to the total respiration rate.

Table 1. Determining the dry weight of the homogenised bacterial suspensions. The results were expressed in gram of biomass per litre (g/biomass/L).

Sample	Sample volume (mL)	Initial weight (g)	Final weight (g)	Total solid weight g/ (Final-Initial)	Total dry biomass g/ biomass/L
WT at 37 °C	4	1.212	1.219	0.007	1.825
bolA at 37 °C	4	1.304	1.375	0.071	17.725
rpoS at 37 °C	4	1.456	1.525	0.069	17.175
WT at 42 °C	4	1.383	1.449	0.067	16.625
bolA at 42 °C	4	1.655	1.659	0.004	0.95
rpoS at 42 °C	4	1.678	1.758	0.081	20.125
WT at 46 °C	4	1.452	1.521	0.069	17.3
bolA at 46 °C	4	1.525	1.528	0.004	0.875
rpoS at 46 °C	4	1.438	1.504	0.066	16.425
WT at 20 °C	4	1.455	1.530	0.075	18.775
bolA at 20 °C	4	1.801	1.877	0.076	19.075
rpoS at 20 °C	4	1.355	1.397	0.042	10.45
WT at 5 °C	4	1.318	1.365	0.047	11.75
bolA at 5 °C	4	1.343	1.411	0.068	16.975
rpoS at 5 °C	4	1.633	1.689	0.057	14.125
WT at 3 mM	4	2.629	2.633	0.004	1
bolA at 3 mM	4	2.955	3.106	0.151	37.75
rpoS at 3 mM	4	2.852	2.955	0.103	25.75
WT at 4 mM	4	3.028	3.032	0.004	1
bolA at 4 mM	4	2.735	2.843	0.108	27
rpoS at 4 mM	4	3.019	3.138	0.119	29.75
WT at 5 mM	4	2.737	2.889	0.152	38
bolA at 5 mM	4	2.828	3.010	0.182	45.5
rpoS at 5 mM	4	3.005	3.153	0.148	37

Table 2. Determining respiratory activity of the *E. coli* wild type and mutant strains under various stress-induced environments using dry weight of the homogenised bacterial suspensions from Table 1. Respiratory activity (RA) was expressed in mg O2/g biomass min^{-1}.

Sample	Exogenous RA ($\%O_2$/sec)	Total RA ($\%O_2$/sec)	Exogenous RA (mg O_2 /L/sec) Total-Endo	Exogenous RA (mg O_2/g Total-Endo	Total dry biomass (g/biomass/L)	RA (mg O_2/ biomass g /min)
WT at 37 °C	0.0072	0.3612	0.3540	0.0326	1.825	1.0706
bolA at 37 °C	0.0032	0.3341	0.3309	0.0304	17.725	0.1030
rpoS at 37 °C	0.0051	0.3374	0.3323	0.0306	17.175	0.1068
WT at 42 °C	0.0235	0.5077	0.4841	0.0445	16.625	0.1607
bolA at 42 °C	0.0036	0.3644	0.3607	0.0332	0.95	2.0960
rpoS at 42 °C	0.0135	0.4094	0.3959	0.0364	20.125	0.1086
WT at 46 °C	0.0528	0.5487	0.4959	0.0456	17.3	0.1582
bolA at 46 °C	0.0091	0.4077	0.3986	0.0367	0.875	2.5144
rpoS at 46 °C	0.0387	0.4509	0.4121	0.0379	16.425	0.1385
WT at 20 °C	0.0152	0.1060	0.0908	0.0084	18.775	0.0267
bolA at 20 °C	0.0012	0.1086	0.1067	0.0098	19.075	0.0309
rpoS at 20 °C	0.0046	0.0984	0.0938	0.0086	10.45	0.0495
WT at 5 °C	0.0406	0.0101	0.0304	0.0028	11.75	0.0143
bolA at 5 °C	0.0096	0.0023	0.0073	0.0007	16.975	0.0024
rpoS at 5 °C	0.0217	0.0102	0.0114	0.0011	14.125	0.0045
WT at 3 mM	0.0053	0.3814	0.3760	0.0346	1	2.0757
bolA at 3 mM	0.0040	0.1244	0.1204	0.0111	37.75	0.0176
rpoS at 3 mM	0.0165	0.3345	0.3181	0.0293	25.75	0.0682
WT at 4 mM	0.0125	0.4032	0.3907	0.0359	1	2.1564
bolA at 4 mM	0.0019	0.0804	0.0785	0.0072	27	0.0160
rpoS at 4 mM	0.0051	0.3338	0.3286	0.0302	29.75	0.0610
WT at 5 mM	0.0045	0.3858	0.3812	0.0351	38	0.0554
bolA at 5 mM	0.0157	0.0757	0.0601	0.0055	45.5	0.0073
rpoS at 5 mM	0.0016	0.2544	0.2529	0.0233	37	0.0377

assessed by the determination of the homogenised bacterial suspensions, accordingto the APHA, AWWA, WPCF Standard (2490 A-D) Methods (1989). The results were expressed in gram of biomass per litre (g/biomass/L) (Table 1).

RESULTS

The aim of this investigation was to compare the rates of endogenous and exogenous respiration of *E. coli* K-12 MG1655 suspended cultures (Table 2) subjected to stress-induced conditions. Figure 2 shows the effect of various shock conditions i.e., heatshock temperatures (42 and 46 °C), cold shock temperatures (5 and 20 °C) and different concentrations of H_2O_2(3, 4, and 5 mM) on respiratory activity (RA) of *E.coli* wild type (WT), *bolA (bolA*$^+$*/rpoS*$^-$*)* and *rpoS (rpoS*$^+$*/bolA*$^-$*)*. The results show that, at 37 °C and in presence of both *bolA*$^+$ and *rpoS*$^+$ gene i.e. WT at 37 ºC,RA is higher when compared to that obtained in the absence of both genes. This shows the importance of both genes in the respiratory activity of *E. coli*. Under heat shock conditions, *bolA* responds well as compared to *rpoS*, while cold shock temperatures are found to be unsuitable for *E. coli* to respire. From the results, it appears that there is a higherrate of respiration of *E. coli* under heat shock in the presence of *bolA* gene. Interestingly, on the other hand only wild type can respond to oxidative stress, which shows that both *rpoS* and its coordinated expression with *bolA* are required to respond under hydrogen peroxide stress, and this varies at different concentration of H_2O_2(Fig 2). The use of the respirometry in relation with *rpoS* and *bolA* genes showed that, when bacteria were exposed to various stresses, metabolic activity seemed to be varied.

DISCUSSION

Respirometry is defined as the respiratory activity of a bacterial population by measuring the consumption of oxygen by a known amount of bacterial cells which occurred in a specific period of

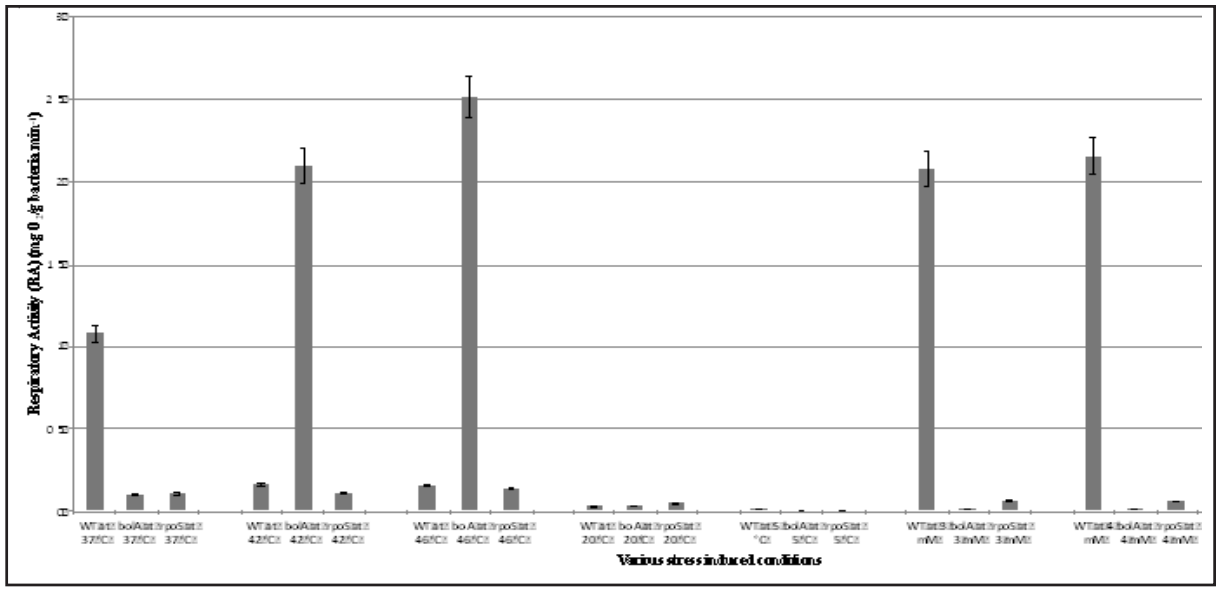


Fig. 2 Bar chart showing respiratory activity (RA) of E. coli in presence of *rpoS** and *bolA*** gene under various stress-induced conditions.
**rpoS*: *rpoS*+/*bolA*- ***bolA*: *bolA*+/*rpoS*-

time (Stewart *et al.*1994). The bacterial respiratory activity was evaluated by the measurement of the oxygen uptake rate due to glucose oxidation. Since cells exhibit metabolic activity, the value of the respiratory activity (exogenous and endogenous) per gram of cells varies under different stress conditions. Our data show that, overall metabolic activity, i.e. oxidation of glucose in presence and absence or *rpo*S and *bol*A genes varies from one stress condition to other.

This study employed the well established and genetically tractable microorganism, *E. coli* to rapidly identify the significance of *rpo*S and *bol*A genes inoxygen uptake rate. The results of this study have shown marked changes in the respiratory activity of *E. coli* employing either heat shock, cold shock or oxidative stress. The current evidence suggests that *E. coli* respiratory activity varies from stress to stress and respond significantly. This study reports on the kinetics of stress environment and its correlation with metabolic activity of bacteria with the main emphasis on *rpoS* and *bolA* genes.

In a previous work short-term respirometry proved to be a rapid, reliable, economic and easy methodology that can be used to evaluate respiratory activity in various stress conditions (Simões *et al.* 2003). The scrupulous physiology of bacterial cells helps to explain both their extraordinary phenotypic and genotypic properties when compared with other bacteria or in varied environments. Planktonic cells presented more differential features including elongated shape, more activity and a higher content of proteins and polysaccharides per cell (Simões *et al.* 2003).

This novel study, employing respirometry has demonstrated that *bolA* can respond to external environments in absence of *rpoS*. Also it appears that *bolA* is expressed under unfavourable conditions (i.e., stress and stationary phase). The expression of *bolA* is under the transcriptional control of σS (encoded by *rpoS*). The presence or absence of σS has an impact on *bolA* and mutation in *bolA* results in less uptake of oxygen in various stress induced environments. This indicates that sS might act through *bolA* (Adnan *et al.* 2011). The overall study concludes the importance of *bolA* gene than *rpoS* in respiratory activity of *E. coli*, under heat shock condition, where consumption of oxygen and oxidisation of glucose is at faster rate.

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