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Presence of the Bacterial Hemoglobin Gene (*vgb*) Enhances Culturability of a Recombinant *Escherichia coli* αDH5 Strain

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The research was conducted to investigate the culturability of the *E. coli* αDH5 (Wt) by using two different plasmids; *Vitreoscilla* hemoglobin gene (*vgb*), which was cloned into plasmid pUC8 and transformed into *E. coli* αDH5 (strain VHb) as well as the plasmid pUC8 that was similarly transformed into the same strain (strain pUC8). The culturability (expressed by the relative survival cells under all starvation conditions) was increased significantly in the order of pUC8, Wt and VHb. Similarly the total proteins of the strains were increased significantly in the order of pUC8, Wt and VHb. It seems that the first increase in culturability is related to the role of bacterial hemoglobin gene (*vgb*), irrespective of the plasmid size. However, the second increase in total proteins, could be more attributed to the function of bacterial hemoglobin gene (*vgb*), for enhancing the starvation-induced proteins rather than to the total cellular protein.

Key words: *E. coli, vgb,* culturability, plasmid, bacterial hemoglobin

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Introduction

The development of viable but nonculturable cells (VBNC) has been widely investigated in many organisms (Oliver *et al*., 1993 and Paludane-muller *et al*., 1996). Recently, a lot of information became available regarding the effect of individual nutrient starvation, on the maintenance of several bacterial cultures (Huisman and Kolter, 1994). In *Vibrio vulnificus*, the use of viable accounts at 24°C starvation of either one of the following elements: carbon(C), nitrogen(N), phosphorus(P) when present singly or simultaneously altogether (multiple effect) have shown similar patterns (Paludane-muller *et al*., 1996). Also in *E. coli* (Jenkins *et al*., 1988) a significant and overlapping responses to the individual starvation conditions were similarly found. Additionally, extracellular products of bacteria have been shown to regulate wide variety of microbial processes, fore example homoserine lactone has been proposed as a starvation signal in *E. coli* (Huisman and Kolter, 1994; Matin, 1991). Nevertheless, extracellular factors produced during starvation have no effect on the culturability of cells incubated at low temperature (Wolf and Oliver, 1992; Weichart *et al*., 1992).

In particular, some investigations have indicated that there is an inverse correlation between the size of a plasmid harbored by a bacterial strain and the growth of that strain (*et al*., 1989; Liu *et al*., 1995). Also it is known that the presence of a recombinant plasmid can alter the levels of certain metabolites (Axe and Bailey, 1987) and that the presence of a plasmid vector can increase the oxygen demand of cells which harbor it and may result in general alteration of cell metabolism (Khosravi *et al*., 1990). Other studies have shown that, in the presence of a plasmid burden metabolism occurred on host bacteria, which enhanced as the size of the plasmid increases.

It is well known that the hemoglobin of Gram-negative obligate aerobe, *Vitreoscilla* (VHb) is the best-characterized bacterial hemoglobin (Kallio *et al*., 1994). The VHb gene (*vgb*) has been isolated, sequenced and cloned in many species of bacteria, the new heterologous hosts of *vgb* were shown to enhance cellular growth, viability, ATP production and production of valuable proteins (Liu *et al*., 1994; Kallio *et al*., 1994). The hemoglobin gene enables cells to grow in a microaerophilic environment by serving as an oxygen-binding protein (Joshi and Dikshit, 1994). Also it was shown that the cell size in strains without the hemoglobin gene decrease during the late log phase in response to starvation (Khosravi *et al*., 1990). In contrast, strains that bearing *vgb* maintained a fairly constant cell size (White, 1995). It was realized that, the presence of VHb alleviate this stress and allows the size of the *vgb*-bearing cells to remain constant (Kallio *et al*., 1994).

The aim of this research work was to investigate the effect/s of *vgb* on the culturability alterations caused by a plasmid or in another meaning to assess the ability of bacterial hemoglobin gene (*vgb*) for compensating the heavy duty caused by a plasmid. To the best of my knowledge the effect of bacterial hemoglobin (*vgb*/VHb) on the culturability of *E. coli* has not been investigated before.

Materials and Methods

Bacterial strains and plasmids: The strains used were *Escherichia coli* αDH5 as parental cell (denoted Wt). Second strain was the *E. coli* αDH5 transformed with plasmid pUC8 denoted by pUC8 and was obtained from the Illinois Institute of Technology, Chicago, IL, USA. Third strain was the same, Wt transformed with Plasmid pUC8:16 (Liu *et al*., 1994) contains *Vitreoscilla* fragment of 1.4 kb which has been cloned into pUC8. (Dikshit and Webster, 1988) and this fragment was engineered to have *vgb*. The third strain was denoted by VHb.

Culture Conditions: Broth medium was used for growth (Mitra *et al*., 1975) and contained the following salts gram per liter: 1g NH_4Cl, 1g NaCl, 1g KCl, 0.2g $MgSO_4$ and 0.1g $(NH_4)_2SO_4$ in K_2HPO_4 5 $\times 10^{-5}$M. Tris-HCl was added gradually with mixing up to a final concentration of 0.05M, pH was maintained at 7.2 and glucose was added to a final concentration of 0.5%. Medium for the transformed strains also contained ampicillin (Ap) at 100μg ml^{-1} Cells of overnight cultures grown in a GFL Model 3032 shaker fisher model 129 at 37°C, 125 rpm in synthetic or synthetic-Ap of the untransformed and transformed strains, respectively, were inoculatated (1:50) into 50ml of starved-fresh synthetic or synthetic-Ap respectively. These media were modified to contain single defined inorganic N and P sources (9.25mM NH_4Cl; 10.32mM K_2HPO_4) and 0.2% (w/v) glucose as a single carbon source as described by Paludane-muller *et al*. (1996). Transferred washed growing cells to the medium lacking carbon (SM-C) or nitrogen (SM-N) or phosphorus (SM-P) or C, N and P simultaneous (SM-CNP) attained starvation conditions. The experiments were performed without shaking at room temperatures (23 ± 2 °C).

Plasmid stability: Cells were harvested at 2, 4, 6, 8, 16, 24, and 32 days of all starvation conditions and their Plasmid stability was monitored at each time point. 25 colonies from randomly selected viable cells were cultured into LB agar plates containing 100μg ml^{-1}AP and left to grow overnight. The number of colonies growing indicated the stability of the Plasmid (Lenski *et al*., 1988).

Hemoglobin determination: The presence of *Vitreoscilla* hemoglobin and it's concentration were determined by the CO-difference spectrum method according to Liu and Webster (1974). Cell cultures used were grown in synthetic medium to stationary phase.

Determination of c.f.u.: To test the culturability of *E. coli* αDH5 (Wt, pUC8 and VHb), samples were taken at the indicated times and diluted in the respective starvation medium. Plate counts (number of viable cells ml^{-1}) were performed on LB agar and LB-Ap agar respectively. Plates were incubated for 24hr. at 37°C before the evaluation of c.f.u. Consequent to extended incubations for a total of 96 hr no longer colony development could be watched (Paludane- muller *et al*., 1996).

Prestarvation at different incubation temperatures: Cells of the three strains were grown at 37°C overnight in synthetic broth medium (SM) transferred to fresh medium at a dilution of 1:50 and grown overnight incubated at 20, 30, 37 and 45°C. The cells were grown to med-exponential phase OD = 0.25-0.5. The cultures were divided into 4 samples, each was harvested and washed (8000 rpm, 15 °C , 15 min, Sorval RC5 B plus centrifuge, SS 34 rotor) in either SM-N, SM-P, SM-C or SM-CNP, and the cells resuspended in vol. 1 of the respective starvation medium. Subsamples of these suspensions were starved for 72hrs. at respective temperatures.

Protein determination: Protein was determined by the method of Lowry *et al*. (1951) with bovine serum albumin as a standard.

Results and Discussion

Culturability of the three strains (Wt, pUC8 and VHb) was followed up to 32 day (Table 1 and 2). To bear out that, the respective nutrients, limit the growth of these strains, the growth lost by starvation was restored by adding back the deficient nutritional source to a subsamples of the experiment after 72hrs. of starvation, and the cultures incubated at 37°C with shaking. After overnight incubations, the amended samples showed an increase in c.f.u. of 20, 16 and 28 folds as compared with the counts of C, N and P-starved cultures, respectively. However, the actual cultures showed 5-10% decrease in c.f.u., assuring that the starving cultures were growth-limited for the omitted substrate. Additionally, the simultaneous addition of the three nutrients resulted ri growth, whereas, the addition of single and combination of two nutrients did not show any increase in c.f.u.

Also, the results showed that bacterial hemoglobin (VHb) is expressed in *vgb*-containing *E. coli* cells grown in the synthetic medium and under certain starvation conditions by using CO-difference spectra (Fig. 1), colony colors and AP resisting of the cells. Additionally the VHb strains showed more viability and culturability of their cells as compared with strains lacking *vgb* (parental cell and pUC8- containing cells) (Table 1). A few scientists reported that there is an inverse correlation between the size of a plasmid harbored by a bacterial strain and the growth of that strain (Zund and Lebek, 1980; Cheah *et al*., 1987; Ryan *et al*., 1989). While those have shown that, in the presence of a plasmid burden metabolism occurred on host bacteria, which elevated as the size of the plasmid increases. Strains harboring plasmids are more sensitive to lower oxygen levels than plasmid-free strains by the criterion of cell growth (Khosravi *et al*., 1990). This agreed with the results reported here, by which the survival of *E. coli* strain pUC8 under the starvation condition showed a little lower than that of the strain Wt. The strain VHb's survival has been shown to be 1.5-2 folds as compared with the strains Wt and pUC8 after 32 days of incubations (Table 1).

The possible explanations why the strain VHb showed this advantage, are that, in strain VHb, the bacterial hemoglobin gene can compensate for the effects of plasmid burden Joshi and Dikshit (1994) have shown that about 40% of the hemoglobin protein is in the periplasmic space, and this localization is suited it's function to transfer oxygen to the terminal oxidase under hypoxic condition through the facilitated diffusion. The VHb may, also, carry oxygen directly to enhance viability by delivering oxygen to terminal oxidases and consequently increasing ATP production (Chen and Bailey 1994; Kallio *et al*., 1994). Another possible explanation, by enhancing oxygen delivery to enzymes which may expressed during starvation and keep cells culturable a compared with non-*vgb* strains. Additionally Liu *et al*. (1994) reported that as the *vgb* is transformed into heterologous bacterial host, it can enhance growth and production of valuable biochemicals. The results, may confirm that *vgb* seem to reflect the cell's strategy to increase oxidative phosphorylation as energy requirement increase (Khosravi *et al*., 1990). This also emphasize that with the current limited amount of nutrition, the potential VHb effects should be evaluated on a case-by-case basis. Monitoring the survival of *E. coli*, non–*vgb* strains (Wt and pUC8) especially, their culturability after prestarvation may does not imply that they did not survive, but they may be viable but nonculturable.

Plasmid Stability: Plasmid stability was determined at each time point. At times 2, 4, 8, 16 and 24 days, the data revealed that pUC8 and VHB exhibited 100% plasmid stability; after 24 day, however pUC8 exhibited plasmid instability and at the 32 day time point only 55% of the colonies had

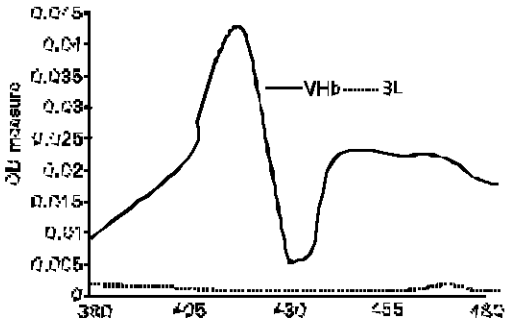

Fig. 1: Co-difference spectrum of whole cells (36 mg mL^{-1}) of *E. coli* strain VHB grown in LB medium to late loge phase. The x-axis is nanometers intervals and the y-axis represents the OD measure. Bacterial hemoglobin (VHB) is determined by observing the peaks in this type of spectra at 419nm and a groove at 436 nm. BL means baseline.

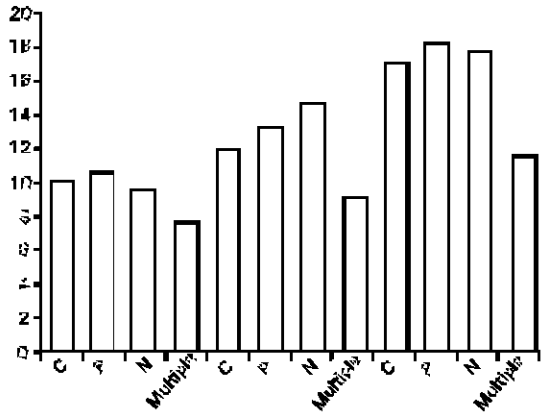

Fig. 2: Correlation of protein content of *E. coli* with two different size of plasmid including pUC8 and VHb (bacterial hemoglobin gene "*vgb*" containing pUC8 and the parental cells which have no plasmid (Wt).

Table 1: Proportional survival of different *E. coli* strains at room temperature during starvation.

Time (d)	Survival % of initial cfu of *E. coli* strain Wt			
day	C	P	N	multiple*
2	90	96	90	88
4	80	82	75	69
8	45	50	48	33
16	15	23	17	18
32	8	8.6	6.5	6
	Survival % of initial cfu of *E. coli* strain pUC8			
2	89	90	88	80
4	73	71	75	66
8	40	69	48	26
16	14	18	13	15
32	6	5	4	3.5
	Survival % of initial cfu of *E. coli* strain Vhb			
2	89	90	88	80
4	86	80	77	69
8	49	69	58	39
16	30	27	23	22
32	12	9.4	8.5	6.8

*: Starvation for Carbon(C), Nitrogen(N) and Phosphorus (P) simultaneously

Table 2: Effect of incubation temperatures (*) on the culturability of the prestarved *E. coli* strains Wt, pUC8 and Vhb.

Survival (% of initial c.f.u.)				
Temperature (°C)	Starvation type	Wt	pUC8	VHb
45	C	7	8	13
	P	5	10.5	15
	N	6	5	14.5
	Multiple	0.5	2	5.5
37	C	48	62	76
	P	68	82	89
	N	48	74	85
	Mutiple	28	46	63
30	C	77	70	79
	P	87	88	94
	N	73	68	90
	Mutiple	37	42	76
20	C	61	60.5	71
	P	87	90	92
	N	72	69	84
	Mutiple	33	36	63

*10°C incubations was also tried several times but the incubation times used could not be enough for the cells to grow.

plasmids. The instability in this strain was confirmed further to determine if it involved either or both strains, pUC8 and VHb. (Table 1)

Effect of incubation temperatures on the culturability of the *E. coli* strain Wt, PUC8 and VHb: The effect of incubation temperatures on the culturability of the three strains was studied by four temperature (20, 30, 37 and 45°C). The incubation times chosen was 72hr. In the three strains the results (Table 2) were paralleled with the results in Table 1, by which the strain VHb has been shown to has higher culturability as compared with the that of non-*vgb* strains on the c.f.u. basis. However, at incubation temperature (30°C), the viability of the VHb was the highest. This showed that, may be 30°C temperature is the optimal incubation temperature, for activity of starvation-induced proteins and the VHb at that temperature also enhances the production of these proteins that prolonged the cells culturability. The 20°C it may needs more incubation times because of the unsuitable temperature to the enzymes and proteins involved in bacterial culturability sustenance. However, 45°C mostly is an uncomfortably hot temperature that might inhibit the synthesis of proteins/enzymes involved in the prolongation of cell's culturability.

Protein concentration: The total amount of protein was determined to see the effect of respective starvation and the *vgb* on the total protein. The starvation condition and the *vgb* dramatically affected the total protein content (Fig. 1 and 2). The total protein increase significantly in the order pUC8, Wt and VHb and the culturability expressed by the relative survival cells also increase significantly in the order pUC8, Wt and VHb irrespective to plasmid size. This increase, support the possible role of the *vgb*, by which it enhances the starvation–induced proteins but not the total proteins. Demodena *et al.* (1993), reported that modification of cell response by VHb give an evidence for association of it with the cell metabolism and it's role in cell growth and increasing valuable products.

From this investigation, concluded that omitting any of these nutritional sources (C, P, N and multiple) generally showed the same pattern. However, irrespective to either starvation conditions or plasmid size, the results indicate that the effects of *vgb*/VHb on the culturability of cells appeared to be advantage. Additionally, this increase in culturability enhanced by VHb may also reflected in the response of physiological behavioral alterations that enabled transformed cells compensating for the heavy duty presented by plasmids.

References

Axe, D. D. and J. E. Bailey, 1987. Application of P nuclear magnetic resonance spectroscopy to investigate plasmid effects on *Escherichia coli* metabolism. Biotechnol. Lett., 9: 83-88.

Cheah, U. E., W. Weigand and B. S. Stark, 1987. Effect of recombinant plasmid size on cellular processes in *Escherichia coli*. Plasmid, 18:127-134.

Chen. R., J. E. Bailey, 1994. Energetic effect of *Vitreoscilla* hemoglobin (VHb) expression in *Escherichia coli*: An on-line P NMR and saturation transfer study. Biotechnol. Prog., 10: 360-364.

Demodena, J. A., S. J. Gutierrez, F. Velasco, R. A Fernendes, J. L Fachini, D. E. Galazo, J. F. Hughes, J. F. Martin, 1993. The production of cephalosporin C by *Acremonium Chrysogen um* is improved by the intracellular expression of a bacterial hemoglobin. Bio/Technology, 11: 926-929

Dikshit, K. L., D. A. Webster, 1988. Cloning characterization and expression of the bacterial globin gene from *Vitreoscilla* in *Escherichia coli*. Gene, 70: 377-386.

Huisman, G. W. and R. Kolter, 1994 Sensing starvation: a homoserine lactone-dependent signalling pathway in *Escherichia coli*. Science, 265: 537-539.

Jenkins, D. E., J. E. Schhultz and A. Matin, 1988. Starvation-induced cross protection against heat or H2O2 challenge in Escherichia coli. J Bacteriol, 170: 3910-3914.

Joshi, M. and K. L. Dikshit, 1994. Oxygen dependent regulation of *Vitreoscilla* globin gene: Evidence for positive regulation by FNR. Biochem . Biophys. Res., Comm., 202: 535-542.

Kallio, P. T., D. J. Kim, P.S. Tsai, J. E. Bailey, 1994. Intracellular expression of *Vitreoscilla* hemoglobin alters *Escherichia coli* energy metabolism under oxygen-limited conditions. Eur. J. Biochem., 219: 201-208.

Khosravi, M., D. A. Webster and B. C. Stark, 1990. Presence of the bacterial hemoglobin gene improves alpha amylase production of a recombinant *E. coli* strain. Plasmid, 24: 190-194 .

Lenski, R. E. and T. T. Nguyen, 1988. Stability of recombinant DNA and its effects on fitness. Trends Biotechnol. Ecol. Evol., 6: 51-53.

Liu, S. C., D. A. Webster and B. C. Stark, 1995. Cloning and expression of the *Vitreoscilla* hemoglobin gene in *Pseudomonas*: Effects on cell growth. Appl. Microbiol. Biotechnol., 44: 419-424.

Liu, S. Y., D. A. Webster, 1974. Spectral characteristics and interconversion of the reduced, oxidized, and oxygenated forms of purified cytochrome O. J. Biol. Chem., 249: 4261-4266.

Liu, S. C., D. A. Webster, B. C. Stark, 1994. Sequence of the region downstream of the *Vitreoscilla* hemoglobin gene *vgb* is not part of a multigene operon. Appl Microbiol Biotechnol., 42: 304-308

Lowry, O. H., N. Rosebrough, Al. Farr and R. Randall, 1951. Protein measurement with the Folin phenol reagents. J. Biol. Chem., 193: 267-275.

Matin, A., 1991. The molecular basis of carbon-starvation-induced general resistance in *Escherichia coli*. Mol Microbiol., 5: 3-10.

Mitra, R. S., R. H. Gray, B. Chin and I. A. Bernstein, 1975. Molecular mechanisms of accomodation in *Escherichia coli* to toxic levels of Can. J. Bact., 121: 1180-8.

Oliver, J. D., 1993. Formatiom of viable but nonculturable cells. In starvation in Bacteria, pp. 239-272. Edited by S. Kjelleberg. New York and London: plenum press.

Paludane-Mûller, C., D. Weichart, D. McDougald and S kjelleberg, 1996. Analysis of starvation conditions that allow for prolonged culturability of *Vibrio vulnificus* at low temperature Microbiol., 142: 1675-1684.

Ryan, W., S. J. Parulekar and B. C. Stark, 1989. Expression of beta-lactamase by recombinant *Eschorichia coli* strains containing plasmids of different sizes-Effects of pH, phosphate, and dissolved oxygen. Biotechnol. Bioeng., 34: 309-319.

Weichart, D., J. D. Oliver and S. Kjelleberg, 1992. Low temperature induced non-culturability and killing of Vibrio vulnificus. FEMS Microbiol. Lett., 100: 205-210.

White, D., 1995. The physiology and biochemistry of prokaryotes. Oxford University Press, New York.

Wolf, P. W. and J. D. Oliver, 1992. Temperature effects on the viable but nonculturable state of *Vibrio vulnificus*. FEMS Microbiol. Lett., 100: 205-210.

Woese, C. R., W. G. Weisburg, B. J. Paster, C. M. Hahn, R. S. Tanner, N. R. Kreig, H. P. Koops, H. Harms and E Stackebrandt, 1984. The Physiology of Purple Bacteria: the Beta Subdivision. Sys. Appl. Microbiol., 8: 706-717

Zund, P. and G. Lebek, 1980. Generation time prolonging R plasmids: correlation between increases in the generation time of *Escherichia coli*. Caused by R Plasmid and their molecular size. Plasmid, 3: 65-69.

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