Role of Heat Shock in Survival of Escherichia Coli and its Small Colony Variant IH3 against Acid Stress.

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Abstract

The mechanism of killing of bacterial cells by low pH is not known. We studied the survival of Escherichia coli at pH 3.0 and in apple cider, which represents organic acid stress. All studies involve the wild type (WT) E. coli K-12 strain HfrH3000, and IH3, a highly acid resistant small colony variant derived from the WT, and were done in Luria-Bertanim (LB) medium at 37ºC with log phase cells. The death of these strains occurs in two distinct phases- a rapid early phase followed by a slower death phase suggesting two different mechanisms of cell damage. The evidence indicates that an increase in membrane permeability to small molecules and increased aggregation of cytoplasmic and membrane proteins contribute to acid-induced death in WT. We have also shown that there is a profound increase in lipopolysaccharide (LPS) release from the outer membrane and lowering of intracellular pH in WT cells with incubation in acidic LB or apple cider. IH3 shows much higher survival in low pH, less membrane damage, lower LPS release, lower protein aggregation and better ability to maintain its intracellular pH on exposure to low pH. Moderately heat shocking the WT cells prior to acid exposure resulted in considerable protection against cell death, membrane damage and cellular protein aggregation but pre-heat shocked IH3 does not show extra protection against acid stress. The data indicate that acid kills the WT cells by multiple mechanisms, and that IH3 resists acid-killing due to a strong LPS-outer membrane interaction.

Keywords: acid stress; acid resistance; small colony variant; membrane permeability.

1. Introduction

When exposed to environmental stress (including acid stress), bacteria increase synthesis of acid shock proteins, other general stress proteins and heat shock proteins usually in response to the accumulation of misfolded and denatured proteins [13], which allows them to contend with the adverse environment. Many of these stress-induced proteins are chaperones and proteases. Their biological role is to protect cellular proteins against the toxic effects generated by exposure to stress [20]. The chaperones function to eliminate misfolded proteins by (i) unfolding these proteins and subsequently promoting proper folding and (ii) targeting unfolded proteins for proteolysis [12, 15]. While exposure to acid challenges pH homeostasis, the pH difference across the plasma cell membrane (ΔpH) nevertheless contributes cell energy in the form of proton potential or proton motive force (Δp). But low pH also amplifies the uptake of membrane-permeant acids that dissipate the proton potential [14]. Thus, low pH is expected to induce a combination of positive and negative responses.

Permeant acids pass through the bacterial membrane and dissociate in the cytoplasm, causing accumulation of protons causing depression of internal pH (pHi), and anions. Growth inhibition occurs as a result of both lower internal pH and the differential ability of anions to inhibit metabolism [18]. There are a number of log-phase acid tolerance response systems that will protect acid-adapted bacterial cells at low pH, including the alternate sigma factor σs, encoded by *rpoS* and Fur (for organic acid stress) and PhoPQ (for organic acid stress). But the alternative sigma factor σ^S is a key regulator of many different stress responses in unadapted *S. enterica* and *E. coli*, and is regulated at the levels of transcription, translation, and protein stability in a manner peripherally similar to the acid tolerance response[11].

Post-translational control of σ^S levels is also regulated by DnaK levels, which in turn is controlled by the concentration of aggregated protein levels in the cell [25]. The data of Audia et al. [2] indicate that in log phase, unadapted cells, increasing σ^S production (by exposure to low pH) are sufficient to increase protein half-life. When the cells are exposed to low pH, these regulators help to increase synthesis of acid shock proteins (ASP), which include some periplasmic proteins [7, 11]. Some of these ASPs probably function to protect cells against entry of protons.

Two types of small colony variants (SCV) have been studied. One type, studied in recent years by Proctor and his associates [27, 5] are primarily *Staphylococcus aureus* that are isolated by their resistance to aminoglycoside antibiotics such as gentamicin. Isolates of this SCV colonies are frequently found to be menodine or hemin auxotrophs [5, 26]. The evidence indicates that these mutants have a deficience in electron transport and this can for the aminoglycoside resistance as well as other phenotypes of this SCV class.

A second class of SCVs has been studied, mostly decades ago, with E. coli [8, 9, 16]. These have been isolated by exposure to metal ions or organic compounds. These have been studied in much less detail than the other type. Mutant IH3 is the first SCV isolated by acid resistance. It appears to belong to the second group, as it is sensitive to kanamycin and gentamicin (Hirshfield and Aravantinou unpublished results).

In this study we attempt to elucidate the response of unadapted log phase wild type *E.coli* HfrH3000 and its acid resistant small colony variant IH3 upon exposure inorganic (pH 3.0) or organic acid (apple cider) stress, and the effect of prior sub-lethal heat shock on their acid –stress survival. The possible causes of acid resistance of the small colony variant IH3 were also investigated. The results show that pre-heat shock affords marked protection of the WT strain against acid killing, but there was little further protection of the mutant IH3. With respect to the mutant's considerable resistance to direct acid shock, it appears that an important factor is its ability to maintain lower membrane permeability in low pH which may be due to an enhanced interaction between the lipopolysaccharide (LPS) and the outer membrane.

2. Materials & Methods

2.1 Bacterial strains

E.coli K-12 strain HfrH3000 was obtained from the *E.coli* Genetic Stock Center, Yale University. It was kept as a stock culture in glycerol at –70°C and maintained in Luria-Bertani (LB) medium at 4°C and then transferred to LB agar plates for short term usage. IH3 was isolated by screening log phase HfrH3000 for acid resistant mutants after exposure to pH 3 for 1 hour at 37°C. An aliquot of survivors was regrown to log phase in LB and repeated for 3 rounds to enrich for acid resistant mutants [17].

2.2 Cell growth and media

In all experiments cells were grown aerobically in LB medium to log phase at 37°C in a gyrotory shaker incubator (New Brunswick) rotating at 200 rpm. Growth of the cells was monitored at 580 nm with a Milton Roy spectrophotometer. LB broth was prepared according to Miller [23]. pH 3 LB medium was prepared by adding 6N HCl to LB and apple cider (pH 3.7) was purchased commercially. The pH was determined with an Orion Research Digital pH meter, model 611.

2.3 Survival Assay

150µl of log phase cultures (HfrH300 or IH3) were inoculated in to 10 ml of LB medium in growth flasks. The cultures were grown to log phase (A_{580}=0.25-0.3for WT and 0.13-0.16 for IH3) as monitored spectrophotometrically. This is equivalent to about $1x10^8$ cells/ml for each strain. 100ul of these cultures were then inoculated into test tubes containing either 900µl of pH 3.0 LB medium or 900µl of apple cider (pH 3.7). These tubes were incubated in a stationary water bath at 37°C up to 2 hours (with or without heat shock) and counted for viability. Heat shocked samples were kept at 41°C for 30 minutes (after reaching log phase at 37°C) prior to exposure to low pH. Viability count- After incubation at low pH, two, 100µl aliquots were removed, serially diluted into 0.85% saline, and spread on LB agar plates and incubated at 37°C for 16-24 hours to obtain a viability count. The dilution plan was devised in order to ensure that all cultures were at a similar density to start with, ca. 1.0×10^7 cells per ml.

2.4 Detection of membrane leakage

The possibility of membrane damage was further checked by propidium iodide based staining which detects cells having damaged membranes [4]. In this kit, membrane-permeant SYTO9 and non-permeant propidium iodide were mixed in equal volumes and used for staining the cells after a 1-hour treatment with LB pH3 or apple cider. The fluorescent probes were excited with 470nm blue light and the fluorescence observed using a Nikon E100 fluorescence microscope (manufacturer's instructions www.probes.invitrogen.com/handbook). Membrane-permeant SYTO9 binds to DNA of intact cells and fluoresces green upon excitation, and non-permeant propidium iodide binds to DNA of membrane compromised bacteria and fluoresces red upon excitation.

2.5 Isolation and measurement of aggregated [^{35}S]-L-Met- proteins

Log phase cell proteins were radiolabelled with [^{35}S]-L-Methionine at a specific radioactivity of 50 µCi/ml for15 minutes and washed repeatedly with LB medium to remove all non-specific radioactivity attached to the cells. The radiolabelled cells were subjected to low pH challenge (LB pH 3.0 or apple cider, with or without heat shock) for various lengths of time. The cultures were used for isolation of the aggregated proteins using the method of Tomoyasu et al. [32]. After the cells were initially lysed by sonication, aliquots were used for determining radioactivity counts per minute in a Tri-Carb scintillation counter to estimate the total protein (soluble plus aggregated) for each sample. After isolation of the aggregated protein fraction, the aggregated proteins were estimated again by determining radioactivity counts per minute. The percentage of aggregated proteins was calculated from the ratio of aggregated proteins to total proteins.

2.6 Detection of membrane and cytoplasmic protein aggregation by ANS staining

The degree of protein aggregation occurring at the membrane was examined by staining cells with 100µM of the apolar fluorescent dye 1-anilino-8-naphthalene sulfonate (ANS) using the method of Rauceo et al. [28] with modifications. 10 ml of log phase cultures were treated respectively with acid LB (pH 3) or with apple cider for 60 minutes. Then the cultures were centrifuged, resuspended in 0.85% saline and mixed with ANS to give a final concentration of 100 µM in each of the samples. 5µl of the stained samples were used for viewing the cells with aggregated proteins using a fluorescence microscope (Nikon Instruments Inc.). The samples were excited with light of 390nm wavelength and photographs were taken after 7 minutes of exposure.

2.7 Measurement of intracellular pH

Cells were grown to log phase and centrifuged at 5000 X g for 10 minutes to harvest the cells, which were then washed in HEPES + 5mM EDTA (pH 8). Then the cells were loaded with 1µM CFDASE (fluorescent probe) according to the procedure described by Breeuwer et al. [6]. The CFDASE loaded cells were then resuspended in acid LB (pH 3) or apple cider and incubated for 1 hr at 37°C. Then the cells were washed and diluted to a concentration of 10^7 cells per ml in a 3 ml glass cuvette and placed in the spectrofluorometer (Perkin-Elmer LS45), and the fluorescence intensities were measured at excitation wavelengths 490nm and 440nm as described. Calibration of pHi: Calibration curves for *E. coli* HfrH3000 and IH3 were determined in buffers with pH values ranging from 4 to 10 as described by Breeuwer et al. [6].

2.8 Measurement of [^{14}C]-D-Gal-LPS release from the outer membrane

The method of Alakomi et al [1] was used with slight modifications. *E. coli* HfrH3000 and IH3 cells were grown in LB at 37°C with shaking (200 rpm.) to the log phase and supplemented with [^{14}C]-D-galactose (0·1 µCi/ml) in order to label their LPS. Labelling with [^{14}C]-D-galactose was performed for 5 min at 37°C with shaking (200 rpm). The cells were collected by centrifugation (10000 X g for 10 min at 25°C), and washed with 10 mM Tris/HCl buffer (pH 7·2) at room temperature. The cells were resuspended in the LB medium and then centrifuged at 5000Xg for 10 minutes to harvest the cells. Both the heat shocked and the non - heat shocked cells were treated with LB (pH 7), acid LB (pH 3) or apple cider respectively for 1 hour at 37°C. Aliquots (2x100 µl) were taken for total (cells + supernatant) radioactivity measurements and the remaining cells were centrifuged (15000 X g) at room temperature. After centrifugation, two 100 µl samples from the cell-free supernatants were taken for radioactivity measurements. The amount of radioactivity in the cell-free supernatant was taken as the measure of liberated LPS and the percentage value for LPS release was calculated by comparison to the radioactivity of a similar volume of the acid treated bacterial suspension.

3. Results & Discussion

3.1 Survival Assay

To establish the pattern of survival and cell death of *E.coli* over time on exposure to low pH, we plotted the percentage of cells surviving after exposure to acidic pH or apple cider for increasing time periods. We also compared the survival rate of non- (control) and pre-heat shocked cells on exposure to different lengths of acid shock to see if this treatment can provide protection against acid mediated killing. There are two distinct phases in the cell survival pattern - a rapid cell death in the first 15-20 minutes of low pH exposure, and then a gradual but distinct slower rate of death. This implicates two different death mechanisms in the two phases. IH3 shows a 3 log higher survival in pH 3 and 2.5 logs in apple cider compared to WT without prior heat shock (Fig.1). Upon exposure to heat shock, the WT cells show very good protection (over 4 logs higher survival) against acid shock (Fig. 1A and B) but in *IH3* cells, the protective response is minimal (Fig. 1C and D), especially in apple cider .Other researchers have also demonstrated that exponential-phase cells show a high initial rate of viability loss, and a similar biphasic cell death curve [19]. The data obtained by Tetteh et al., indicate that exposure of non - acid adapted *S. flexneri* cells to a mild heat shock, enhances their survival and ability to grow in acidic conditions [31]. Also, Wang et al. studied strains of *E. coli* O157:H7, a serious food pathogen and *E. coli* K-12 for their ability to survive at pH 2·5 at 37°C. The survival of heat-shocked (10 min at 48°C) cells was about 10–100 times greater compared with untreated cells depending on the strain. Chloramphenicol prevented the heat shock-induced acid tolerance, indicating the requirement for newly synthesized protein(s) [34].

3.2 Detection of Membrane lysis

We tried to identify the location of lethal damage caused by acidic pH, and one reason for cell death could be severe membrane damage causing cell proteins to spill out. To investigate this, the cellular proteins of log phase cells were radiolabelled with [^{35}S]-L-Methionine before challenging with low pH. Then the cells were separated from the medium by filtration through bacterial filter discs (pore size 0.22 micron), and then tested for the presence of the radioactive proteins in the medium. No significant increase in radioactive counts was seen in the filtrate (medium) after incubating in either acidic LB or apple cider, eliminating the probability of massive membrane lysis [4]. Gross cell damage was not observed by microscopy either (results not shown).

3.3 Detection of Membrane leakage

Another possibility that may lead to loss of viability is finer leaks in the membrane allowing smaller molecules to enter/leak from the cell, and this was investigated visually by subjecting the cells to differential staining with membrane-permeant SYTO9 and non-permeant propidium iodide (PI) before and after acid challenge. Cells that have an intact membrane fluoresce green due to binding of SYTO9 to DNA, whereas those having a damaged membrane will let propidium iodide leak into the cell and preferentially bind to DNA and quench the green fluorescence of SYTO9, thus giving red fluorescence.

Upon treatment with acid (pH 3) most WT cells fluoresce red, but with apple cider the number of cells that fluoresce red is a little lower compared to that with LB pH 3. Yellow fluorescence implies mildly damaged cells while red indicates severe damage. IH3 shows lower PI penetration at pH 7, pH 3 and in apple cider. Upon heat shock pre-treatment, there is considerable protection against acid killing and much reduced penetration of PI into the WT cells. In the mutant there is very little change in fluorescence levels with heat shock and subsequent acid exposure (Fig. 2). These results are consistent with that of the survival assay. The results of the study of Jordan et al. also showed that a strong correlation exists between increased acid tolerance and decreased permeability of the cell envelope to protons. This result implies that acid-resistant populations (either an acid-adapted exponential-phase population or a stationary-phase population) have an improved ability to maintain pHi [19].

Although it is not yet clear whether this change in permeability is due to an exclusion of protons from the cell or an active efflux of protons from the cell, it seems plausible to suggest that this change in proton flux may directly contribute to the elevated levels of acid tolerance observed. There is very little information available in the literature about the pre- heat shock mediated membrane changes that lead to protection against acid shock, but the work of Tourdot-Maréchal et al., indicates that heat (42°C) or acid (pH 3.2) shocks decreased the membrane anisotropy values (fluidizing effects), thus increasing the membrane rigidity [33]. In this work, the velocities of membrane fluidity variation with non-adapted or heat-adapted cells were compared. The rate of membrane fluidity variation of heat shock adapted cells was 5-fold lower than the rate calculated with instantaneously stressed non-adapted cells. This data sheds light on our findings as the prior exposure to heat shock adapts the WT cells lowering the membrane fluidity in low pH, thus offering protection against the acid mediated membrane fluidization, which in turn leads to decreased membrane permeability [33].

3.4 Measurement of aggregated proteins

In addition to membrane damage by acid, another possible target would be denaturation and aggregation of cellular proteins. The effect of low pH on the internal proteins were assayed by radiolabelling all cellular proteins and then treating the cells with LB pH 3 or apple cider both with or without subjecting the cells to prior heat shock. In non - heat shocked conditions, there is more acid - mediated internal protein aggregation in WT cells compared to IH3 (Fig. 3A and B), which is consistent with more killing and higher membrane leakage with acid-treated WT. A significant increase in protein aggregation is seen on exposure to acid LB (pH 3.0) or apple cider for 10 min while even higher aggregation is seen on 60 min exposure showing that low pH increases aggregation of cell proteins. The pre-heat-shocked WT cells showed a large reduction in internal protein aggregation on acid challenge (Fig. 3A), but the mutant IH3 shows a very small reduction (Fig. 3B). This poor reduction in protein aggregation in IH3 may reflect the relatively ineffective heat shock response shown by this strain and correlates with the poor heat shock response of IH3 seen in the survival assay. Thus IH3 is refractory to the protective effects of prior heat shock.

The protein aggregation data is consistent with the better survival of pre-heat shocked WT. This could mean that the protection afforded to heat shocked WT cells in the survival assay occurrs because of the lack of internal protein aggregation, i.e., the heat shock induces heat shock proteins which prevent/reduce the massive denaturation of internal proteins seen on exposure to low pH, probably by helping to fold them back to their active configuration. Further investigation into the cause and nature of low pH - mediated protein aggregation and its exact effect on cell death is needed. Two reasons for acid - mediated cell death could be that the internal pH of the cell cytoplasm decreases due to entry of protons from the highly acidic external environment and/or the protein folding machinery of the cell is compromised due to a decline in intracellular pH.

3.5 Detection of membrane and cytoplasmic protein aggregation

ANS is fluorescent only in hydrophobic environments, and commonly used to monitor secondary structural changes during protein folding and unfolding [3]. Upon exposure of WT and IH3 to pH 3 or apple cider the measurement of surface fluorescence as a measure of membrane protein aggregation parallels cytoplasmic protein aggregation. Increased ANS surface fluorescence accompanies an increase in aggregation. Acid shocked cells show particularly high ANS fluorescence intensities due to the exposure of hydrophobic core regions that are inaccessible to the dye in the native structure of the proteins present in control cells [29]. Additionally, some of the fluorescence seen in WT and IH3 could be due to acid-mediated increase in membrane permeability allowing ANS access to cytoplasmic proteins.

In the non-heat shocked WT cells, exposure to acid in LB (pH 3) and apple cider showed a marked increase in fluorescence compared to the pH 7 control. IH3 shows much lower fluorescence levels than WT indicating lesser aggregation/hydrophobicity. The extent of ANS binding to the cell membrane is diminished considerably when WT cells are pre-heat shocked prior to low pH exposure showing a decrease in cytoplasmic and membrane protein denaturation at low pH. At pH 7, none of the cells show strong ANS fluorescence even though phase contrast micrographs of the same fields show the presence of many cells (Fig. 4). Some increase in ANS binding (fluorescence) is also seen in the pre-heat shocked control cells probably because exposure of the cell surface to higher temperature itself causes some conformational changes in the membrane components.

3.6 Measurement of intracellular pH

Acid stress has a direct effect on the proton motive force and therefore may result in instantaneous changes in cell membrane potential [35]. Inevitably, this will significantly affect the transport of all other major nutrients taken in cotransport with H^+ [21]. Regulation of the pHi is therefore a fundamental requirement for the survival and viability of bacteria. The intracellular pH of both pre-heat shocked and non-heat shocked cells subjected to low pH treatments (LB pH3 or apple cider) was measured using the pH sensitive fluorescent probe CFDASE. The results show that the pHi drops a 10- to a 100- fold lower in WT cells than IH3 without the application of heat shock. In the pre-heat shocked WT cells significant protection is seen against pHi decline on exposure to LB pH3 or apple cider. But pre-heat shocked IH3 cells do not show any such protection against lowering of pHi in response to acid stress as in this case the pHi decrease is almost the same as that seen in non-heat shocked cells (Table 1). The initial intracellular pH value (7.7) coincided well with the available literature from studies done on pHi measurements in *E. coli* and other gram-negative bacteria [6, 11]. The decrease in pHi seen in non-heat shocked WT cells and the better maintenance of pHi seen in IH3 coincides well with the survival assay pattern and membrane permeability changes that were observed in our earlier experiments.

This data is supported by the findings of Jordan et al., who showed that exponential-phase cells accumulated protons at a high rate, when exposed to low pH [19].

3.7 Measurement of release of LPS

LPS release from the outer membrane of *E. coli* cells in response to low pH exposure was measured to estimate the extent of outer membrane damage, and also to see if LPS release could be instrumental in the increased membrane permeability seen before in WT cells. The effect of lactic acid and HCl on the outer membrane permeability of *Salmonella enterica* serovar Typhimurium has already been studied and the data obtained from this work indicated that considerable proportions of lipopolysaccharide were liberated by these acids [1]. When WT cells were subjected to LB pH 3 or apple cider, there was a 10- to 1-3 fold increase in LPS release compared to the control, whereas with IH3 this was limited to a 6- to 8- fold increase (Table 2). Hence, there is more acid mediated loss of LPS in WT than IH3 (without heat shock). This data also suggests that a component of acid damage is disrupting the LPS layer and this is consistent with the data obtained by Alakomi et al. [1]. Upon pre-heat shocking WT cells before acid exposure, the LPS loss was reduced to a 1.5- to 2- fold effect, whereas IH3 was again less responsive to heat shock and had a 2.6- to 3.7- fold increase in LPS release compared to the control (Table 2). Thus with pre-heat shock treatment, the WT loses less LPS than IH3.

This indicates that outer membrane damage in the form of increased LPS release could be one of the causes of the low pH induced membrane leakage seen before and probably contribute to acid induced cell death. This also indicates that the outer membrane of IH3 is less susceptible to acid mediated damage than that of HfrH3000, and this probably also helps to protect against entry of H+ and subsequent lowering of intracellular pH. Studies by Daugelavicius et al. have shown that changes in envelope permeability to H+ are influenced by sublethal acid stress (pH 4.5) in exponential phase *E. coli* grown in rich media. This is higher in cells exposed to acidic pH compared to those grown in pH 7.4 [10]. Changes in membrane permeability could result from changes in cell surface and membrane properties developed during the adaptation process and contribute to survival after a strong acid stress. In these experiments, we have first established an initially rapid loss of cell viability followed by a gradually slower death phase on exposure to acidic pH and apple cider suggesting two different mechanisms of cell death. This damage may result for various reasons, namely if low pH causes cell lysis, membrane damage, affect protein folding or even all of these together.

We have not seen any significant cell lysis, but there is a distinct increase in membrane permeability, as shown by propidium iodide staining, and aggregation of cellular proteins (as indicated by cytoplasmic protein aggregation and ANS staining) on exposure to acidic pH. Our data also indicate that the LPS-outer membrane barrier plays an important role in these permeability changes. Protons diffuse into the cells through the compromised permeability barriers as indicated by the consequent drop in intracellular pH, which could lead to protein denaturation/aggregation. Heat shocking the cells prior to acid exposure, results in considerable protection against cell death and protein aggregation in the WT, but not in IH3. Lastly, experiments with the small colony variant IH3 suggests that its resistance to acid killing may occur because of decreased permeability to protons. This may result, at least partially, through a stronger LPS-outer membrane interaction.

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Tables

Table 1: The effect of external pH (pHo) on the intracellular pH (pHi) values pre-heat shocked and non heat shocked cells.

pHo	pHi			
	Non heat shocked		Pre-heat shocked	
	HfrH3000	IH3	HfrH3000	IH3
LB pH 7	7.7	7.7	7.7	7.7
LB pH 3	5.2	7.4	6.7	7.0
Apple cider	6.1	7.5	7.0	7.1

Table 2: The effect of external pH on the release of LPS from the outer membrane.

		Release of LPS (%)		
	Strains	LB pH 7	LB pH 3	Apple cider
Non heat shocked	HfrH3000	3.1 ± 0.2	39 ± 0.5	33 ± 0.3
	IH3	2.8 ± 0.4	17.5 ± 1	15.7 ± 0.5
Pre-heat shocked	HfrH3000	5.4 ± 0.4	10 ± 1	8.6 ± 0.8
	IH3	5.1 ± 0.4	17.7 ± 2	13.5 ± 1

Figures

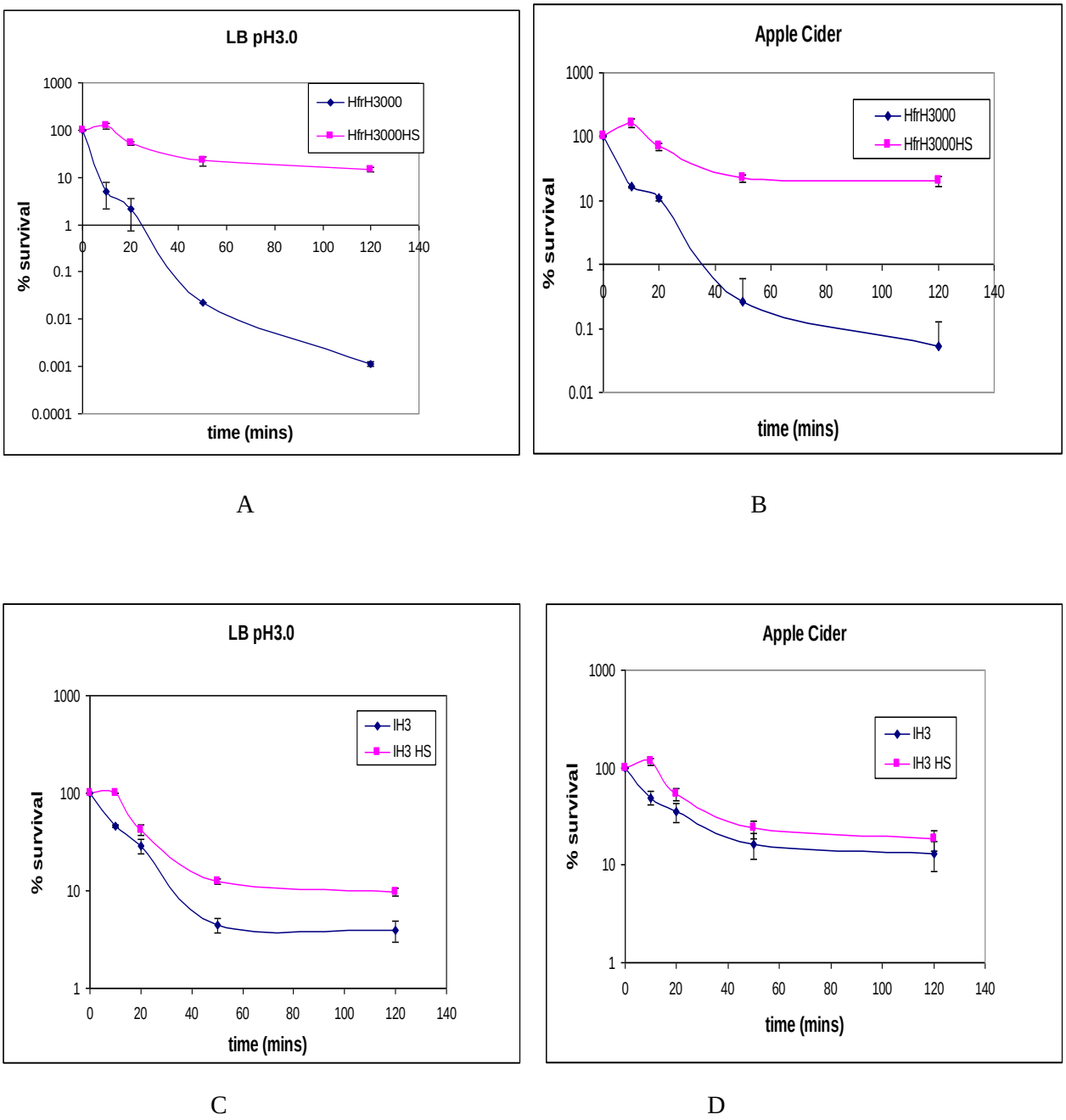


Fig.1. Survival assay plots of non-heat shocked and pre-heat shocked HfrH3000 and IH3 cells showing the effect of heat shock on survival of the cells at low pH. HS= pre-heat shocked cells.

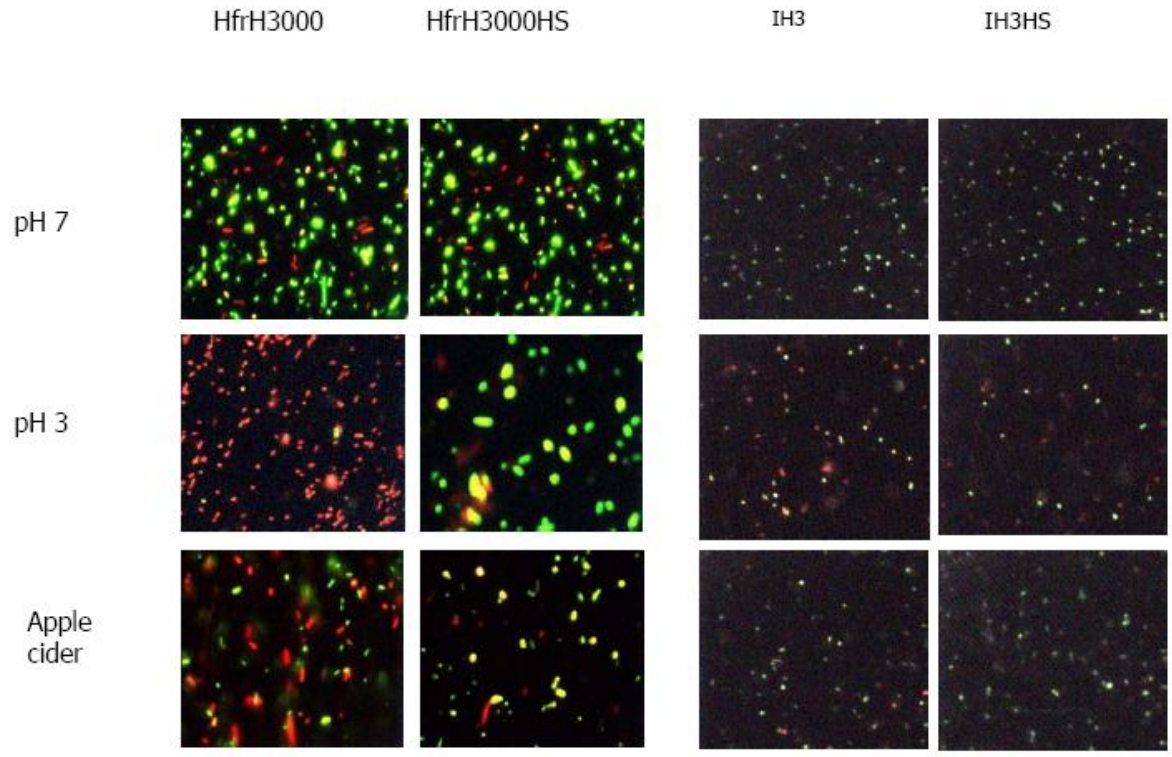
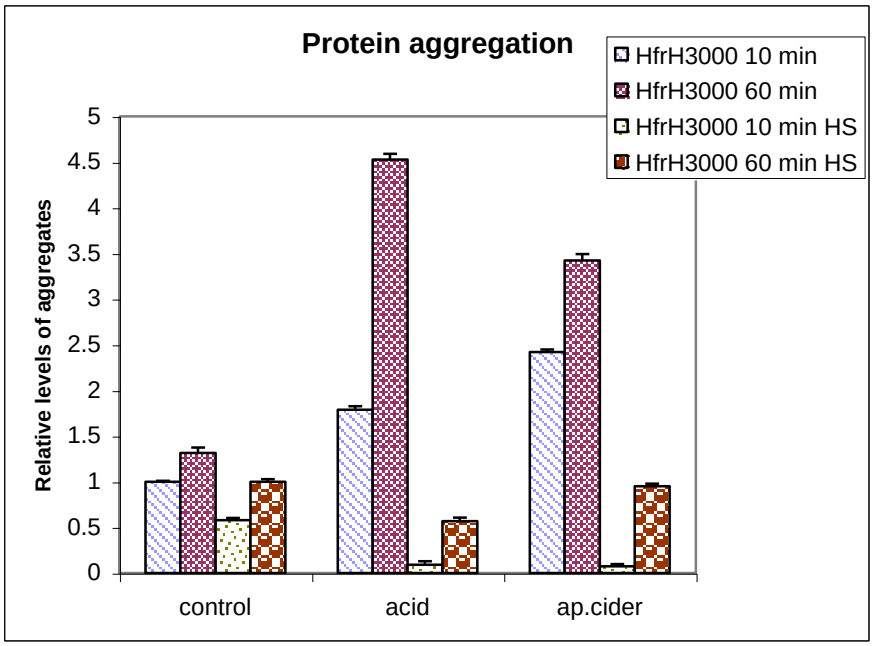
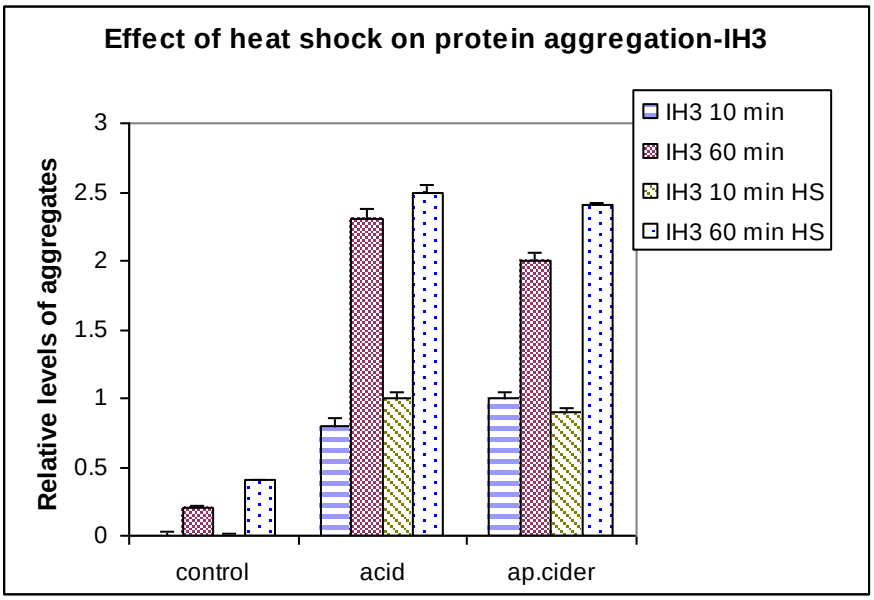


Fig. 2. Fluorescence micrographs of pre-heat shocked and non-heat shocked HfrH3000 and IH3 cells stained with SYTO9 and propidium iodide showing the effect of pH 3 and apple cider and the effect of heat shock on permeability of the cells at low pH. HS= pre-heat shocked cells.



A



B

Fig.3. Effect of acidic LB and apple cider on aggregation of cell proteins in non-heat shocked and pre-heat shocked HfrH3000 and IH3 cells. HS= pre-heat shocked cells.

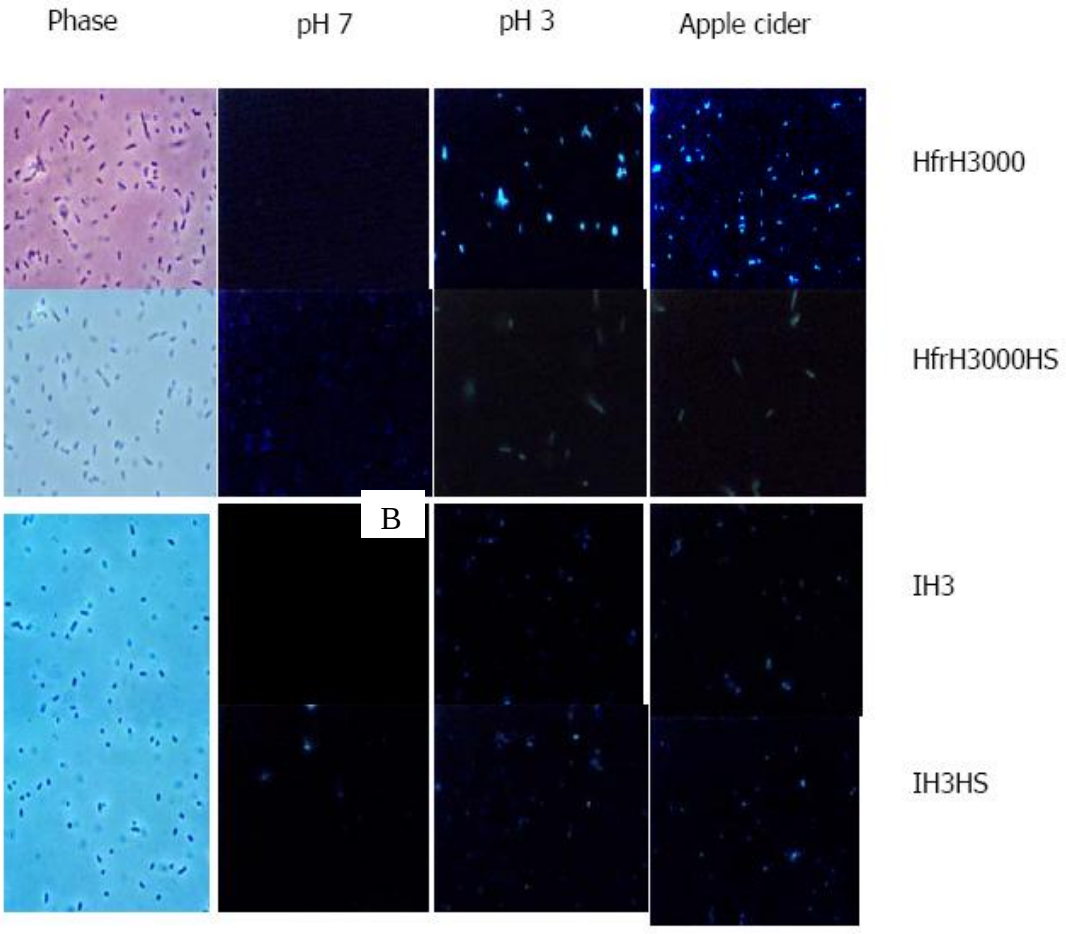


Fig.4. Fluorescence micrographs of non-heat shocked and pre-heat shocked HfrH3000 and IH3 cells stained with the apolar fluorescent dye 1-anilino-8-naphthalene sulfonate (ANS), showing the effect pH 3 and apple cider and the effect of heat shock on degree of aggregation of the membrane proteins at low pH. HS= heat shocked cells.