# Molecular Studies on EctC gene (Ectoine) in some halophilic Bacterial Isolates.

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**Abstract:** The ability of microorganism to adapt the changes in the osmolality of the external milieu is of fundamental importance for growth and survival, and thus prokaryotic cells have evolved a number of osmoadaptative mechanisms to cope with elevated osmolality. One hundred bacterial isolates were isolated from soil from Egypt and screened for salt tolerance. The best four isolates were identified by manual and API 20E system methods. Identification of bacterial isolates showed that strains were *Pseudomonas mallei, Halococcus salifodinae, Pseudomonas flourecens, Pseudomonas aeruginosa*. The primers were designed for screening the bacterial isolates about the EctC gene from octoine operon. The positive result was with bacterial isolate *Pseudomonas mallei*. The length of observed fragment was between 300-400 bp. SDS-PAGE analysis of the four isolates revealed that the protein profile of the six isolates were different from each other in their banding pattern at different concentrations of NaCl (0.0 ppm,40.000 ppm and 58.500 ppm) respectively.

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## 1. Introduction

Bacteria have evolved complex stress management strategies to sense and respond to change in their external environment (Bremer and Krämer, 2000 and Sleator and Hill, 2002).One such environmental parameter is the osmolality of the external growth medium. Bacteria, in principle, require an intracellular osmotic pressure greater than that of the surrounding growth medium to maintain cell turgor, which is generally considered to be the driving force for growth extension and cell division (Bremer and Krämer, 2000 and Sleator and Hill, 2002).

The ability to adapt to changes in the osmolality of the external milieu is therefore of fundamental importance for growth and survival, and thus prokaryotic cells have evolved a number of osmoadaptative mechanisms to cope with elevated osmolality (Bremer and Krämer, 2000 and Csonka and Hanson, 1991).

Microorganisms that adapt to moderate high salt environments use a variety of solutes, (organic and inorganic) to counter external osmotic pressure. The organic solutes can be zwitterionic, noncharged, or anionic (along with an inorganic cation such as $K^+$.

These organic solutes are accumulated by many microorganisms through synthesis or through uptake from the environment to counteract the outflow of water under hypertonic growth conditions. Accumulating organic solutes is the mechanism of Bacteria to avoid the salt stress. In this study will try to isolate one of salt tolerance genes and will characterize it.

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The genes involved in biosynthesis of the major compatible solute ectoine (1, 4, 5, 6-tetrahydro-2-methylpyrimidine carboxylic acid) in halo tolerant obligate methanotroph ‘‘Methylomicrobium alcaliphilum 20Z’’ were studied. The complete nucleotide sequences of the structural genes encoding L-aspartokinase (Ask), L-2,4-diaminobutyric acid transaminase (EctB), L-2,4-diaminobutyric acid acetyltransferase (EctA), and L-ectoine synthase (EctC) were defined and shown to be transcribed as a single operon EctABC ask (Alexander *et al* 2006).

The ectABC genes of the biosynthetic pathway of ectoine from the Gram-positive, moderately halophilic bacterium *Halobacillus dabanensis* was obtained by inverse polymerase chain reaction. Subsequently, the entire ectABC cluster was cloned and analyzed. It revealed that the intergenic regions of the ectABC genes from *H. dabanensis* are more tightly spaced than those of *Chromohalobacter salexigens*, *Halomonas elongata*, *Marinococcus halophilus*, and *Salibacillus pasteurii*. The amino-acid sequence deduced from ectABC was highly homologous that from *Virgibacillus pantethenticus* (EctA 52%, EctB 60%, EctC 67%, respectively).The ectABC genes were cloned in the expression plasmid pMXB10 resulting in

pMXB10ectABC.The ectoine was detected from cell extract in *Escherichia coli* ER2566 containing pMXB10 ectABC using $^{13}C$ nuclear magnetic resonance spectroscopy (Zhao, 2006).

The compatible solute 1,4,5,6-tetrahydro-2-methyl-4- pyrimidine carboxylic acid (ectoine) acts in microorganisms as an osmotic counterweight against halostress and has attracted commercial attention as a protecting agent. Its production and application are restricted by the drawbacks of the discontinuous harvesting procedure involving salt shocks, which reduces volumetric yield, increases reactor corrosion, and complicates downstream processing. In order to synthesize ectoine continuously in less-aggressive media, we introduced the ectoine genes *ectABC* of the halophilic bacterium *Chromohalobacter salexigens* into an *Escherichia coli* strain using the expression vector pASK-IBA7 (Schubert, 2007 and Schubert *et al.* 2007).

1, 4,5,6-Tetrahydro-2-methyl-4-pyrimidine carboxylic acid (ectoine) is an excellent osmoprotectant. The biosynthetic pathway of ectoine from aspartic β-semialdehyde (ASA), in *Halomonas elongata*, was elucidated by purification and characterization of each enzyme involved. 2, 4-Diaminobutyrate (DABA) aminotransferase catalyzed reversively the first step of the pathway, conversion of ASA to DABA by transamination with L-glutamate. This enzyme required pyridoxal 5-phosphate and potassium ions for its activity and stability. The gel filtration estimated an apparent molecular mass of 260 $_{kDa}$, whereas molecular mass measured by sodium dodecyl sulfate polyacrylmide gel electrophoresis (SDS-PAGE) was 44 $k_{Da}$. This enzyme exhibited an optimum pH of 8.6 and an optimum temperature of 25°C for L-glutamate and DL-ASA. DABA acetyltransferase catalyzed acetylation of DABA to g-*N*-acetyl-a, g-diaminobutyric acid (ADABA) with acetyl coenzyme A and exhibited an optimum pH of 8.2 and an optimum temperature of 20°C in the presence of 0.4 *M* NaCl. The molecular mass was 45 $_{kDa}$ by gel filtration. Ectoine synthase catalyzed circularization of ADABA to ectoine and exhibited an optimum pH of 8.5 to 9.0 and an optimum temperature of 15°C in the presence of 0.5 *M* NaCl. This enzyme had an apparent molecular mass of 19 $_{kDa}$ by SDS-PAGE in the presence of 0.77 *M* NaCl. DABA acetyltransferase and ectoine synthase were stabilized in the presence of NaCl (>2 *M*) and DABA (100 *mM*) at temperatures below 30°C Hisayo Ono *et al* (1999).

## 2. Material and Methods

### 1. Sample sites and microbial strains:

Soil and rhizospheric soil samples were collected from Wadi El Netroun, the cost north, Giza and El sharqia, for isolation of salt tolerance. Bacterial reference strains used in this study, *Pseudomonas aeruginosa* and *Bacillus subtilis* were obtained from Faculty of Agriculture – Botany department –Benha University, Qalubia, Egypt.

### 2. Isolation and identification of bacteria:

Suspensions were made by adding 5g of soil to 50ml sterile basic salt solution. Ten fold dilutions of these suspensions were plated on Modified medium (MM) agar. Only colonies from the highest dilution of the soil suspensions were selected for isolation of bacteria.

A number of representative soil samples were collected from these locations. Five grams of five sampling points were collected from each location. Then the samples were mixed by sterile spatula or glass rod, resulting in representative sample of 25 grams. The sample depth was 0-25 cm. If necessary, samples were stored at 2-4 ºC (Alef and Nannipieri, 1995).

One gram of each soil sample was added to 100 ml of sterile saline solution in sterile flask. Placed on magnetic stirrer for at least 10 min. (this is for dilution), fixed volume (100 µl) of appropriate dilution was speeded on SM agar plates which were incubated 37 ºC for 72 hr.

Different single colonies were then picked, according to their strong growth and different morphology. Picked colonies were streaked on SM medium for purification and then maintained on LB medium for further examination.

Isolates were stained according to Gram's procedure (Cruickshank et al., 1975). Motility was detected by semi- solid agar method or by microscopy of unstained wet film (Colle et al., 1996).

Nitrate reduction, and Voges- Proskauer test were done for bacterial isolates; according to (Collins et al. 1991).

### 3. DNA extraction from Bacterial Strains:

DNA was extracted from each strain, following a modified protocol reported previously by Rosso and Delecluse (1997). Fresh 30-ml Luria-Bertani broth cultures (optical density at 600 nm,1) were centrifuged at 3,000 x g for 5 min at 4°C, and the pellets were washed again in 10 ml of J buffer (1.0 M Tris-HCl, 0.1 M EDTA, 0.15 M NaCl [pH 8]). Pellets were resuspended in 4 ml of J buffer, and lysozyme was added to a final concentration of 4 mg/ml, followed by incubation at 37°C for 30 min. Then, 50 µl of RNase (10 mg/ml) was added, and suspensions were incubated for 15 min at 50°C. Next, 200 µl of 20% sodium dodecyl sulfate was added and incubated for 20 min at 70°C, followed by the addition of 120 *µl* of proteinase K (10 mg/ml) and

incubated overnight at 55°C. A total of 1.15 ml of 6 M NaCl was then added, gently mixed in ice for 15 min, and centrifuged at 3,900 x g for 20 min at 4°C. The supernatant was mixed with an equal volume of isopropanol and centrifuged at 17,000 x g for 20 min at 4°C. The pellets were washed with 70% ethanol, air dried, and dissolved in 200 µl of Tris-EDTA buffer (pH 8). DNA was quantified by spectrophotometry, and samples were stored at –20°C until further use.

**4. Polymerase Chain Reaction (PCR):**

The target DNA was prepared by using appropriate procedure for isolation genomic DNA.

**PCR for EctC gene:**

The primers were designed by wobble theory for conserved region for many different species; the primers sequence were ectC1 (5\CGTACGAAACTCATATTTGGTATCAA-3\) and ectC2 (5\CGCCATTTTCATCATGGACTTCGTTTCCTCC - 3\).

The extracted DNA was used for PCR, which was performed in 25µL reaction volumes containing: 20 ng genomic DNA, 100 µM dNTPs, 1 mM MgCl2, 2.5 µL 10X PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, pH 8.3), 0.2 µM of each primer pair and 1 U Taq DNA polymerase (Invitrogen®); distilled water was added to complete the final volume of the reaction. Cycling conditions were: initial denaturation step at 95°C for 3 min, followed by 30 cycles, each consisting of 95°C for 50 s, annealing temperature 52°C for 50 s, and 72°C for 1min, with a final extension at 72°C for 7 min.

Amplification products (25µl) were mixed with 3µl loading buffer and separated on 1.3%agarose gel and stained with 0.5 µg/ml ethidium bromide, and visualized under ultraviolet light and photographed. DNA fragment sizes were determined by comparisons with the 100bp DNA ladder marker.

**5. Cloning of PCR product:**

The PCR products were cloned in pGEM-T Easy vector plasmid (Promega, USA) and transformed into Escherichia coli DH5α; white colonies were picked and screened for the presence of the cloned gene of interest through digestion with EcoRI (Sambrook et al.1989).

**6. one-dimension protein electrophoresis analysis:**

Cell-free extracts of whole-cell proteins were prepared from cultures grown overnight on MM. 50 ml culture was span and the pellet was washed in 0.9% NaCl for removing any sugars. The bacterial pellet suspended in 0.1 ml of sodium dodecyl sulphate (SDS) 1 % w/v and boiled for 3 min. The samples were allowed to cool to room temperature and centrifuged at 12000 rpm for 10 min.

Protein analysis was done by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with 12 % gels (Laemmli, 1970). 100 µl of crude supernatants proteins were mixed with 100 µl of 2X sample buffer, then load. The gel were stained with 0.25% coomassie brilliant blue G-250 (Bio-Rad), destained with 10% acetic acid and 40 % methanol.

**3. Results and Discussion**

**1.Identification of Bacteria:**

The bacterial isolates were identified as aerobic, gram negative, some of them forming spores, cocci, different rods, and some of them motile as shown in table (1). The bacterial isolates were grown on different media for identification of biochemical tests like as T3, LB, and MM media. The gram negative cells differ from gram positive cells in the structure of the cell membrane. The cell membrane of gram negative releases the complex between crystal violet stain, RNA proteins and potassium iodide. The bacterial cells were observed with saffranin stain. The bacterial isolates had grown in both salt medium and free salt medium. One mechanism of bacterial isolate to defense itself from salt stress is released some polysaccharides in the environment. The polysaccharides were found in the medium was affected on protein extraction from bacteria. The size and the shape of bacterial isolates had a role in defense against salt stress, then the bacterial cells had to aggregate together to protect itself from salt stress. This result was obtained by examination of microscope for Halococcus salifodinae. The growth curve of bacteria was consumed more time with increased the level of salinity.

The characteristics of *Halococcus sp.* were gram negative, cocci and aerobic as mentioned in table (2). The salt tolerances of bacterial isolates growth were differed with increasing NaCl as shown in table (3). With increasing the salinity the growth of bacterial cells would decreased. The used concentrations in the test of salinity were 0.0, 40.000, 58.500 and 117.000 ppm as shown in the Table (3).

**Table (1): Morphological and biochemical characterization of halophilic bacteria isolated from of Pseudomonas sp.**

| Characteristics | Pseudomonas mallei | Pseudomonas flourecens | Pseudomonas aeruginosa |
|---|---|---|---|
| Motility | + | + | + |
| Morphology | Rods in chains | Short rods | Short rods |
| Colony shape | Mat | Mat | Mat |
| Colony pigment | chalk | chalk | chalk |
| Gram stain | - | - | - |
| Spore forming | + | + | + |
| Voges- proskauer V.P test | - | - | - |
| Oxidation formation test lactose | - | - | - |
| Nitrate reduction | - | + | + |
| Litmus milk | | Alkaline | peptonization |
| Growth in Media With NaCl | 0.0 -11.7% | 0.0 -11.7% | 0.0 -11.7% |
| Hydrolysis of Gelatin | - | - | + |
| Hydrolysis of Starch | - | - | - |
| Growth at $42^0$c | - | - | + |

**Key: (+): Positive, (-): Negative, ND: Not done/determine.**

**Table (2): Morphological and biochemical characterization of halophilic bacteria isolated from Halococcus Sp.**

| Characteristics | Halococcus morrhuae | Halococcus saccharolyticus | Halococcus salifodinae |
|---|---|---|---|
| Morphology | cocci | cocci | cocci |
| Oxidase | + | + | + |
| Catalase | + | + | + |
| Gram stain | - | - | - |
| Acid production from | | | |
| Glucose | - | + | + |
| Tween – 8o hydrolysis | + | - | ND |
| Utilization of compounds as carbon and energy sources : | | | |
| Lactose | - | + | ND |
| Arabinose | - | + | + |
| Glycerol | d | + | + |
| Indole production | ND | ND | - |
| Voges- proskauer V.P test | ND | ND | - |
| H2S production | ND | ND | - |
| Citrate utilization | ND | ND | - |
| Growth in NaCl | ND | ND | 0.0%- 23.4% |
| Growth in pH 9.5 | - | - | + |
| Sensitivity of antibiotics | | | |
| Chloramphenicol | - | - | + |
| Tetracycline | - | - | + |
| Novibiocin | + | - | + |
| Plasmid existing | ND | ND | + |

**Key: (+): Positive, (-): Negative, ND: Not done/determine.**

**Table (3): Salinity tolerance of the isolates.**

| Bacterial isolates | Concentrations of NaCl/ ppm | | | |
|---|---|---|---|---|
| | 0.0 ppm | 40.000 ppm | 58.500 ppm | 117.000 ppm |
| Pseudomonas mallei | G | V | G | L |
| Pseudomonas aeruginosa | G | V | G | L |
| Pseudomonas flourecens | G | V | G | L |
| Halococcus salifodinae | G | V | G | L |

**Key: G: good growth , V: very good growth and L: low growth**

**SDS-PAGE for Salt Stress Proteins:**

The protein was extracted from six isolates at different concretion of Sodium Chloride (0.0 ppm, 40.000 ppm).The electrophoresis of extracted protein on polyacrylmide gel 12%was obtained the different patterns for six isolates at three different concatenation . Which were mentioned above? Some bands were disappeared and the others were appeared. The mechanism of defense of bacterial cell against salt stress might back fore these bands. Some bands were increased in density and the others were decreased in density. There are some bands stable in three different conc. These bands were expressed from house keeping genes for each strain; the house keeping genes are necessary for living any microorganism.

Showed in table (4) and figure (1) the protein profile of the normal and induced *Pseudomonas mallei* bacterial cells showed that there are four newly induced protein(175.89, 116.6, 91.0 and 38.63 KDa), two over repressed (28.8 and 25.19 KDa) and four repressed proteins (97.73,88.29,81.25and 37.34KDa).

In addition, the protein profile of the normal and induced *Pseudomonas aeruginosa* bacterial cells showed that there are four newly induced protein(83.99, 65.07, 59.39, and 43.74 KDa), two over repressed ( 27.39 and 22.55 KDa) and seven repressed proteins (82.89, 64.45, 58.79, 55.96, 45.30, 26.15 and 24.84 KDa).

Also, found the protein profile of the normal and induced *Pseudomonas flourecens* bacterial cells showed that there are eight newly induced protein(102.62, 89.33, 79.62, 72.93, 59.39, 51.48, 47.60 and 43.74 KDa), only one over repressed (55.96KDa) and three repressed proteins (92.31, 64.45 and 49.86 KDa).

Showed in table (4) and figure (2) the protein profile of the normal and induced *Halococcus salifodinae* bacterial cells showed that there are two newly induced protein (46.31 and 19.44 KDa), and three repressed proteins (91.89, 72.44 and 20.75 KD).

Also, found the protein profile of the normal and induced *Bacillus subtilus* bacterial cells showed that there are eleven newly induced protein (134.78, 98.96, 72.44, 62.65, 51.54, 48.15, 43.14, 28.26, 26.96, 25.45 and 23.86 KDa).

There are various reports on the induced proteins in Gram- negative and Gram- positive bacteria upon salt stress (Duche *et al*., 2002). There has also been reported adown regulation of proteins in response to salt stress (Dartois *et al.*,1998) proteins induced by stress have been shown to play an essential role in bacterial physiology. For example, the ectABC genes of the biosynthetic pathway of ectoine from the Gram-positive, moderately halophilic bacterial are well for roles in protein folding as molecular chaperones (Alexander *et al.*, 2006 and Zhao, 2006). These induced and repressed proteins need to be characterized for understanding its role in salt adaptation.

The Salt Stress Proteins associated with cell membrane of halophilic bacterial, its location and components, the cytoplasmic membrane has been unconventional suggested to sense environmental changes through certain proteins that expand into the periplasm to interact with stress (Neidhardt, 2002). The primary response of bacteria to osmotic up shifts involves the activation of transporters in the cell membrane, which are mechanosenitive pathway proteins to effect the rapid accumulation of osmoprotectants, and sensor to increase the transport and biosynthetic capacity for these solutes (Poolman *et al.*, 2002).

**Table (4): Profile of total Salt stress proteins in bacterial isolates (*Pseudomonas aeruginosa, Pseudomonas flourecens, Pseudomonas mallei, Halococcus salifodinae and Bacillus subtilus).***

| *Bacterial isolates* | Character of salt stress protein | Molecular weight (KDa) |
|---|---|---|
| *Pseudomonas mallei* | I. Newly induced | 175.89, 116.61, 91 and 38.63. |
| | II. Repressed | 97.73, 88.29, 81.25 and 37.34. |
| | III. Over expressed | 28.80 and 25.19. |
| Pseudomonas aeruginosa | I. Newly induced | *83.99, 65.07, 59.39 and 43.74.* |
| | II. Repressed | *82.89, 64.45, 58.79, 55.96, 45.30, 26.15 and 24.84.* |
| | III. Over expressed | *27.39 and 22.55.* |
| *Pseudomonas flourecens* | I. Newly induced | 102.62, 89.33, 79.62, 72.93, 59.39, 51.48, 47.60 and 43.74. |
| | II. Repressed | 92.31, 64.45 and 49.86. |
| | III. Over expressed | 55.96. |
| Halococcus salifodinae | *I. Newly induced* | *46.31 and 19.44* |
| | ***II. Repressed*** | *91.89, 72.44 and 20.75* |
| Bacillus subtilus | *I. Newly induced* | *134.78, 98.96, 72.44, 62.65, 51.54, 48.15, 43.14, 28.26, 26.96, 25.45 and 23.86* |

**Fig (2): Whole-cell protein profiles of Salt tolerance bacterial isolates *Bacillus subtilus(Bs), Pseudomonas aeruginosa(Pa) and Halococcus salifodiniae* (C)**

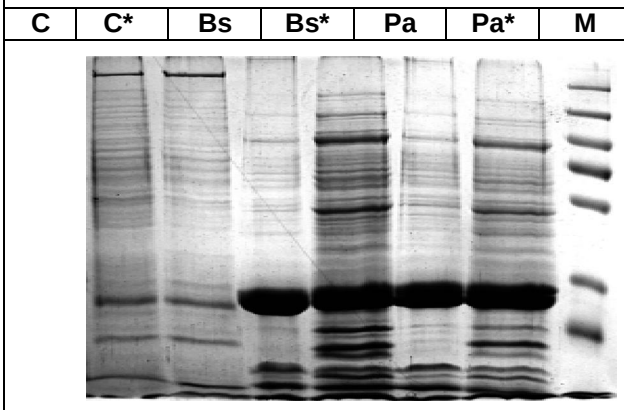
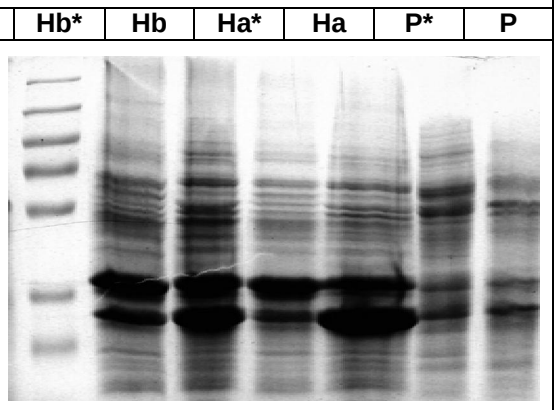



**Fig (1): Whole-cell protein profiles of Salt tolerance bacterial isolates *Pseudomonas aeruginosa (Hb), Pseudomonas flourecens (Ha) and Pseudomonas mallei* (P).**




**Specific PCR for EctC gene from ectoine operon:**

The primers were designed by wobble theory. The different sequences for ect C gene from different species were alignmented by N star program for choosing the conserved box. By using the designed primers with PCR for different six genomic DNA, the positive result was with *Pseudomonas mallei*. The length of the fragment between 300-400 bp as shown in fig (3). The fragment was purified from agarose gel was inserted into pGEM-T Easy cloning vector and sealed by ligation reaction. The ligation reaction was transformed to competent cells from *E.coli*. The transformed cells were grown in LB\Amp.\IPTG\X-Gal plates for screening the cells that carry the insert DNA.

The following pathway essentially similar to that described by Peters *et al*. (1990) aspartic β-semialdehyde (ASA) is converted to 2, 4-diaminobutyric acid (DABA) by transamination, and DABA is converted to ADABA by acetylation with acetyl coenzyme A (CoA), which in turn yields ectoine by circularization (Fig. 1). The three enzymes involved in this pathway are DABA aminotransferase, DABA acetyltransferase, and

ectoine synthase in order of the reactions to ectoine. Peters *et al*. (1990) detected the activity of the first and the second of the three steps by using crude extracts of *E. halochloris* and *H.elongata*. However, the characterization of these enzymes was limited; in particular, their responses to various salt concentrations remained unknown.

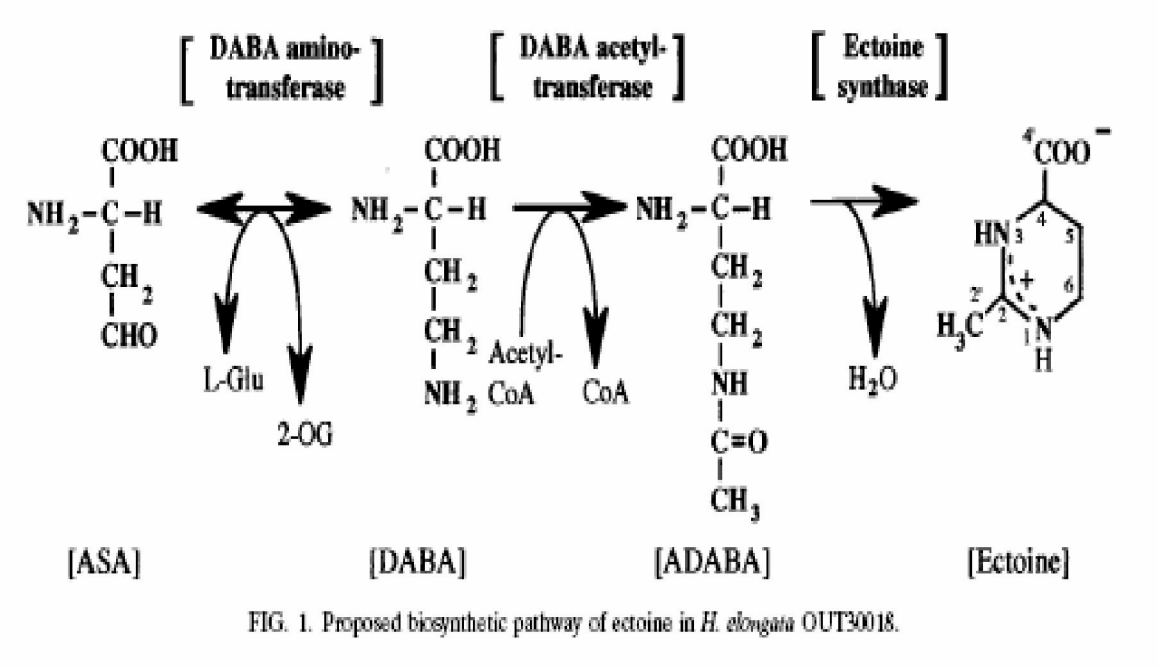



FIG. 1. Proposed biosynthetic pathway of ectoine in *H. elongata* OUT30018.

**Fig (3): PCR product for EctC gene: The best result was with salt tolerance isolate ( *Pseudomonas mallei* ).**

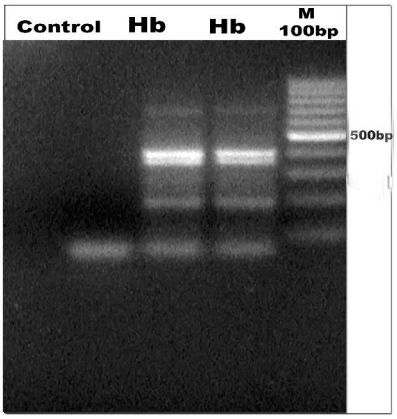



**Fig (4): PCR gradient product for EctC gene:**

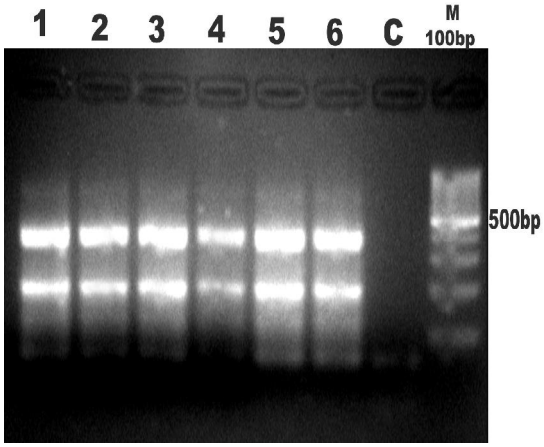


Fig (5): **Quick screening of cloning fragment of EctC gene.**

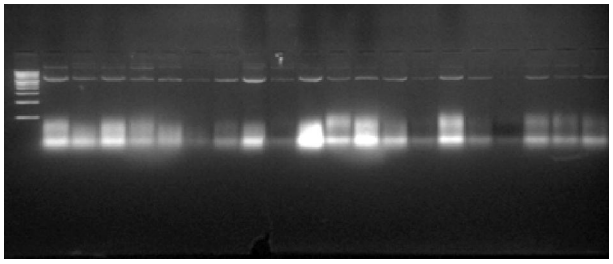
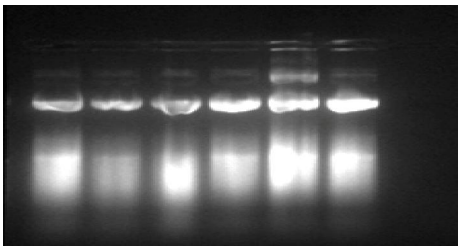


**Fig (6): Cloning for fragment of EctC gene.**



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