Women's Coll. Ann. Rev.
Vol 17 (1992).

EXISTANCE AND GROWTH OF HALOPHILIC BACTERIA FROM HYPERSALINE ENVIRONMENTS

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ABSTRACT

The studied localities in Qatife area of Kingdom of Saudia Arabia (KSA) are different in salt concentration. The effect of these environments on their bacterial population was studied. The increase in salt concentration was correlated with an increase in counts of some species of bacterial population. Many species of bacteria were recorded at 10% (w/v) total salt. Many of their individuals were normal flora of both salt water and fresh water. At over 10% salt concentration the bacterial isolates showed halophilic behaviour. Salt concentration induced the following criteria: Between 10 to 20% salt concentration the population of ***Pseudomonas; Flavobacterium*** and gram positive cocci were increased and represented as moderately halophilic bacteria. At 35% salt concentration some bacteria which were found at the lower concentration disappeared; and large individuals of halobacteria were developed. ***Halobacteria salinarum,*** 32 ***H. halobium*** 77 and ***Halococcus morrhuae*** 98 were identified and recorded as the most tolerant hyper salinity organisms. Growth of these organisms was followed by cell dry weight and total cell nitrogen in intensive studies. Potassium can increase the growth of the studied organisms. In all concentrations of potassium they were forced to produce the good growth. The selected amino acids; glutamic, glycine and proline have stimulatory effect on the growth of the studied organisms. These results may explain the role of these substances in haloadaptation of the organisms in salted locality. Finally these substances were considered as osmoregulator and/or osmoprotectant which help bacterial cell to grow in hyper saline environments.

INTRODUCTION

Hypersalsine environments have been defined as those in which a low species diversity is found and where whole taxonomic groups are missing (Brock, 1978). These environments are produed by the action of environmental factors that reach values far from the average in the biosphere. However, sometimes a given factor can reach very different values above those considered normal, creating different degrees of extreme conditions. A typical example of this kind of environmental factors is the concentration of salts present in seawater when it is concentrated by evaporation, giving rise to hypersaline environments (Brock 1979).

Many different groups of microorganisms which were isolated from hypersaline environments were classified as either of extreme halophilic behavior (*Halobacterium* and *Halococcus* - Woese et al, 1978) or other groups of moderately halophiiic behavior. The latter groups are numerous both gram-positive and gram negative bacteria of the aerobic and anaerobic type (Raymond and Sistrom, 1969; Yopp et al, 1978; Imhoff et al., 1981; Imhoff and Trüper, 1981; Ventosa et al, 1982 and Quesada et al. 1983).

The osmotic strength of the environment is the one of the physical parameters that determines the ability of the organisms to proliferate in a given habitat. There are remarkable similarities between bacteria and plants in their cellular responses to osmotic stress because organisms from both Kingdoms accumulate the same set of cytoplasmic solutes upon exposure to conditions of hyperosomolarity. Thus it is likely that these organisms employed to regulate responce to osmotic stress. Osmoregulation is the active processes carried out by organisms to adapt with osmotic stress. Haloadaptation in microorganisms occurred by: the molecular biology of the accumulated cytoplasmic osmolytes (Le Rudulier et al. 1984); or the interactions of biological macromolecules with solutes accumulated by organisms (Yancey et al, 1982); or effect of potassium which transported to the cell from the outer medium (Walderhaug et al, 1987).

The studied localities are salted environments with different concentrations which was mainteained almost constant through the time of investigation, therefore, this factor is considered for studying the effect of concentration of salts on the counts of microorganisms. In this work the counts, distribution and growth of microorganisms was studied in these salted localities.

MATERIALS AND METHODS

The studied salted localities (Qatif area) are located about 10 to 15 Km in the north of Dammam city (North eastern of S.A.K.). These localities are named Qatif, 1; Qatif, 2, and Qatif, 3 (Q_1, Q_2 and Q_3).

The physico-chemical analysis of water:

The total salt concentrations were determined by the ash content of 10 ml of water dried at 110°C during 48 hours. Cl^- content was determined by titration with $Ag\,N0_3$. Temperature and pH was measured in situ with calibrated mercury thermometer and a portable pH-meter. Oxygen content was measured by the modified Winkler method (water analysis. Merck, Darmstadt) Total Kjeldahl nitrogen was determined as indicated by standard methods for the examination of water and wastewater (APHA 1980).

Sample collection: (water sampling)

Water samples were collected in sterile sample bottles and transferred directly to the laboratory and plated as soon as possible on the chosen medium.

Bacterial isolation

i) The heterotrophic moderately halophilic bacteria were isolated according to ventosa et al. 1982 and Quesada et al. 1983 methods. The medium (basal medium) used for isolation had an adequate salt concentration for moderate halophilics from their enviroments and contained a lower concentration of magnesium salts than the corresponding proportion in the marine salt mixture (below) to impede the growth of halobacteria that have a high Mg^{++} requirement. The composition was as follows (% w/v): NaCl, 17.8; $MgS0_4.7H_20$,).1; KCl, 0.2; $CaCl_2, 2H_20$, 0.036; $NaHC0_3$, 0.006; NaBr, 0.023; $FeCl_3.6H_20$ trace; yeast extract (Difco), 1; protease-peptone no 3 (Difco), 0.5, glucose, 0.1; Bactoagar (Difco), 2. .The samples were surface-inoculated and incubated at 30°C for 7-10 days. Colonies were isolated at random and successively subcultured on the same medium to ensure purity. The strains were maintained on agar slants of medium containing marine salts at a final concentration of 10% (w/v), supplemented with 1% yeast extract (Difco); 0.5% protease-peptone no. 3 (Difco) and 0.1% glucose. For the characterization of the isolates the following tests were carried out, as previously described (by Bergey's 1974; Ventosa et al, 1982 and Quesada et al, 1983): gram stain; motility; growth at different salt concentrations; anaerobic growth; catalase test; oxidase test; acid production from glucose,

lactose, mannitol and sucrose; gelatine, Tween 80 and starch hydrolysis; and nitrate and nitrite reduction.

ii) Isolation of halobacteria:

For isolation of halobacteria the above medium (Basal medium, BM) was used. But the final concentration of NaCl was 25% (w/v) instead of 17.8% (w/v), and Yeast extract was 0.5% (w/v) instead of 1.0% (w/v). It was supplemented with 500 i.u/ml of penicillin G to avoid the growth of halophilic cubacteria (Rodiriguez-valera et al, 1981). The pH value of media was adjusted to 7.5 with 1 N KOH, and the plates were incubated at 38°C. Colonies were isolated at random and subcultured towice to ensure purity. Strains were maintained on the same medium without penicilline G. To classify the isolates, The following phenotypic characteristics were determined: colony appearance and pigmentation; growth at different salt concentrations; motility; catalase test; oxidase test; acid production from glucose, lactose, maltose, mannitol and sucrose; hydrolysis of gelatine, starch, casein and Tween 80; nitrate and nitrite reduction; H_2S and indole production. These methods are described previously.

Preparation of bacterial inoculum:

Growth of each (isolates) of ***Halobacterium salinarum, Halobacterium halobium*** and ***Halococcus morrhuae*** on basal medium was suspended under aseptic conditions in sterilized water to get cell counts of about $5x10^7$ per ml inoculum using a counting chamber (Haemocytometer made in DDR).

Method used for screening the most salt tolerant bacteria:

Growth of each isolate of ***Halobacterium salinarum, H. halobium*** and ***Halococcus morrhuae*** on the basal medium were determined as dry weight to detect the most salt tolerant bacteria.

Determination of dry weight & total cell nitrogen

Cells were collected by centrifugation whenever required; the clear supernatant was poured off, without disturbing the sedement cells. The bacterial cells were quantitatively transfered to the small bottles left to dry in an electric oven at 65°C till complete dryness. The dry weight of the

cells was estimated. Dry cells were used for the estimation of total cell nitrogen by micro-Kjeldahal apparatus. Total cell nitrogen content was calculated on oven dry bases:

1 ml of N/70 sulphuric acid = 0.2 mg N_2.

Supplementation of potassium:

Potassium (KCl) was supplied to the basal medium at the concentration of 100; 200; 300; and 400 mg/100 ml. Basal medium without KCl was used.

Supplementation of selected amino acids:

Amino acids "Prolabo" selected were *L*-glutamic acid, glycine and DL-proline. They were added singly to the basal medium in equemollecular weight of N_2 of protease-peptone.

RESULTS

Table (1): some physico-chemical analysis of water in the studied localities (Qatef, 1,2 and 3).

Localities	pH	Temp°C	0_2 mg/ml	T.S. mg/ml	N_2
Q_1	8.5	29	6.5	10.0	3.5
Q_2	8.0	34	2.5	20.0	5.0
Q_3	7.8	38	1.5	35.0	6.0

ρ'H Temperature (°C); Oxygen (mg/ml), total Kjelelahl nitrogen (mg/10 ml) and total salt concentration (mg/ml).

The studied localities showed concentrations of 10 mg/ml, 20 mg/ml and 35 mg/ml total salt for Q_1, Q_2 and Q_3 respectively. The environmental conditions varied in localities of different salt concentrations (Table 1). The locality with higher salt concentration had lower pH values and higher maximum temperature. The total Kjeldahl nitrogen increased in Q_3 which had a higher total salts concentrations. Oxygen concentration decreased with the increasing salt concentration (Table 1).

Table (2): Distribution of halophilic bacterial groups from salted localities in Qatif area (Q_1, Q_2, & Q_3)

Bacterial groups	Average No. of bacterial individuals from different sites at Qatif area		
	Q_1 10% salts concent.	Q_2 20% salts concent.	Q_3 35% salts concent.
1) Gram negative facultative anaerobic			
Chromobacterium	0.0 (0.0)	0.0 (0.0)	4 (1.6)
Enterobacteriaceae	5 (4.24)	0.0 (0.0)	0.0 (0.0)
flavobacterium	10 (8.48)	20 (15.0)	30 (12.00)
Vibrio	60 (50.87)	40 (31.0)	15 (6.00)
2) Gram negative aerobic			
Acinetobacter	6 (5.08)	10 (7.75)	27 (10.80)
Pseudomonas	20 (16.92)	35 (27.15)	75 (30.00)
3) Gram positive rods	4 (3.39)	9 (6.97)	19 (7.60)
4) Gram positive cocci	10 (8.48)	15 (11.63)	80 (32.00)
5) *Actinomycetes*	3 (2.54)	0.0 (0.0)	0.0 (0.0)

A total of 459 bacterial individuals were isolated from the 4 samples collected in February, April, June and October 1989 from each sites (Q_1, Q_2 and Q_3). The results are summarized in Table 2. The general observation was obsered as follows: A group of microorganisms increased in number with the increase of salt concentration. While the other group decreased. The first group was represented by *Pseudomonas* (20, 35 & 75 from Q_1, Q_2 and Q_3 respectively); gram positive cocci (10,15 & 80 from Q_1, Q_2 and Q_3 respectively); *Flavobacterium* (60 from all sites), *Acinetobacter* (43 from three areas) and gram positive rods (32 from all localities). The second group was represented only by *Vibrio* in a total

of 115 from all localities (60,40 & 15 from Q_1, Q_2 and Q_3 respectively. The following groups were recorded only in one site. *Chromobacterium* was isolated from Q_3 at a concentration 35% salts. While *Enterobacteriaceae* and Actinomycetes were recorded only at 10% salt concentration in Q_1.

The most common isolates in the first group were gram positive cocci (80) and*pseudomonas* (75) especially at 35% salt concentration in Q_3. The genus *Vibrio* was the next highest number of representative and was isolated frequently from the site with 10% salt concentration (Q_1).

No effect of seasonal variation on bacterial isolates was recorded.

Table 3: Distribution of 134 of individuals of *Halobacteriaceae* isolated from salted sites at Qatif area.

Bacterial isolates	Number of individual spp. of family Halobacteriaceae from different sites at Qatif area.		
	Q_1 10%	Q_2 10%	Q_3 10%
Halobacterium S.	4 (25.00%)	12 (37.5%)	28 (32.55%)
halobacterium H.	5 (31.25%)	8 (25.0%)	25 (29.06%)
Halococcus M.	7 (43.75%)	12 (37.5%)	33 (38.37%)

In this experiment, the halobacteria appeared in all localities. A total of 134 isolates were recorded from the same sampling described above. The isolates were classified according to their response to physiological tests to two genus (*Halobacterium* and *Halococcus*) of Family *Halobacteriaceae*.

The counts of *Halobacterium salinarum, H. halobium* and *Halococcus morrhuae* gradually increased with the increase of salt concentration to reach their highest numbers (86 isolates) at 35% salt concentration in Q_3 area (Table 3). The counts of all isolates of halobacteria from Q_1 (10% salts) and Q_2 (20% salts) were 16 and 32 respectively.

Table three also shows that the counts of *Halococcus M* was 52 from all localities, while the counts of *Halobacterium S.* and *H. halobium* were 44 and 38 isolates respectively.

Screening test for the most salt tolerant strains belonging to the species of *Halobacterium salinarum, H. halobium* and *Halococcus morrhue.*

This experiment show that, (data not tabulated) *Halobacterium salinarum,* 32; *Halobacterium halobium,* 77 and *Halococcus morrhuae* 98 produced the large amount of dry weight. These strains are considered the most salt tolerant bacteria.

Data in Table 4 represent the effect of different concentrations of potassium on the growth of *Halobacterium S.* 32; *H. halobium* 77 and *Halococcus morrhuae* 98. The three concentrations of potassium were definitely stimulating growth (dry weight and total cell nitrogen). Good growth was obtained when more potassium was added.

An expriment was also carried out on the effect of selected amino acids on the growth of the studied organisms as measured by dry weight and total cell nitrogen (Table 5). These amino acids (glutamic, Glycine and proline) activated the growth of the isolated Halobacteria. Proline had relatively activated effect on growth (Table 5).

Table (4): Effect of different concentrations of potassium KCl (B.M. without K; 100; 200; 300 and 400 mg/100 ml) on the growth of *Halobacterium*, 32; *H. hablobium*, 77, and *Halococcus morrhuae*, 98.

Organisms	Amount of KCl mg/100	Dry weight of cells mg/ culture of 10 ml)	Total cell nitrogen (mg/ culture of 10 ml)
Halobacterium	B.M. without K	12.3 ± 0.9	0.334±0.02
Salinarum (32)	B.M.(100 mg/100)	14.6±0.7	0.415±0.02
	200 mg/100	16.7±0.6	0.508±0.07
	300 mg/100	19.8±0.5	0.590±0.01
	400 mg/100	20.7±0.4	0.632±0.06
Halobacterium	B.M. without K	12.4±0.37	0.401±0.010
halobium (77)	B.M. (100 mg/100)	15.6±0.63	0.509±0.013
	200 mg/100	17.3±0.13	0.583±0.029
	300 mg/100	20.3±0.37	0.611±0.033
	400 mg/100	21.8±0.5	0.700±0.013
Halococcus	B.M.without K	12.4±0.38	0.410±0.013
morrhuae (98)	(B.M.100 mg/100)	16.7±0.50	0.529±0.017
	200 mg/100	18.6±0.70	0.597±0.007
	300 mg/100	20.5±0.30	0.630±0.006
	400 mg/100	22.1±0.50	0.710±0.07

Table (5): Effect of the selected amino acids (Glutamic, glycine and proline) on the growth of *Halobacterium salinarum*, (32)*H. halobium* (77) and *Halococcus morrhuae* (98).

Organisms	Treatments	Dry weight of cells mg/ culture of 10 ml)	Total cell nitrogen (mg/ culture of 10 ml)
Halobacterium	B.M.	13.3±0.90	0.334±0.02
Salinarum (32)	Glutamic	25.1±0.23	0.420±0.03
	Glycine	26.2±0.15	0.475±0.13
	Proline	28.2±0.73	0.589±0.03
Halobacterium	B.M.	12.4±0.37	0.401±0.010
halobium (77)	Glutamic	20.2±0.3	0.530±0.03
	Glycine	21.7±0.09	0.590±0.08
	Proline	23.4±0.79	0.638±0.02
Halococcus	B.M.	13.4±0.38	0.410±0.013
morrhuae(98)	Glutamic	23.3±0.13	0.500±0.043
	Glycine	24.8±0.18	0.560±0.011
	Proline	26.5±0.24	0.640±0.024

DISCUSSION

The studied localities are different in salt concentration, however this factor leads to differences in the characteristic and nature of these localities. For example the increase in salt concentration both resulted in the decrease of 0_2 and pH, while the total halobacteria & daily temperature increased. Those effect can partially be explained by physico-chemical characteristics of the concentrated salt solution; such as low oxygen solubility or the low specific heat (Strickland & Parsons, 1972). Also the biological factors could contribute to this phenomena. For example the absence of planktonic algae at high salt concentration may decrease the oxygen content of these environment.

A general conclusion that can be observed in these environments is that high salt concentration are related with the decrease of the oxygen content and increase of temperature.

At 10% salt concentration many groups of bacteria appeared; many of them are normal flora of fresh and sea water. At both 20% and 35% all organisms appeared are of halophilic behaviour and able to live in hypersaline environment (extreme environment Q_2 and Q_3).

Through this study we can observed that, the presence of two groups of bacteria. The first group which was represented in ***vibrio, Enterobacteriaceae*** and***Actinomycetes*** prefer the lower salt concentration while the second group was represented in Gram positive cocci and ***Pseudomonas*** prefer the high salt concentration. This observation reflects their different physiological salt response (halophilic behaviour) which is moderate for the 1st group and an extreme for the 2nd group. This observation is in agreement with Kushener 1978. The second group having the complex nutrient requirement therefore increased at 35% salt concentration.

The ***Halobacterium spp.*** appeared in low number at 10% salt concentration (Q_1) and increased at 20% salt concentration (Q_2). At 35% salts concentration (Q_3), the counts of ***Halobacterium salinarum*** ; ***H. halobium,*** , and ***Halococcus murrhuae*** increased 28, 25 and 33 respectively. These three species seems to have adapted to the high salt concentration (35%) like the second group of halophilic behaviour

bacteria (*Flavobacterium, Pseudomonas* and gram positive cocci). The increasing in counts of these organisms were restricted at 35% salt concentration. It is interesting that the 2 different group (Halobacteria and halophilic bacteria) having different physiological activity (behaviour) to regulate the osmotic stress.

Osmoregulation, osmoprotectant were the most observations which were reported by many investigators in some species of bacteria. The osmotic stress tolerance (osmoregulation, osmoprotectant) is not necessarily dependant on the interaction of a large array of gene products but can be dependant on simple phenomena, such as the accumulation of compatible solutes (Christian 1955). The existence of ***Halobacterium salinarum*** ; ***H. halobium*** and ***Halococcus morrhue*** at three different concentrated salted area (Q_1, Q_2 and Q_3) were not correlated with gene activity. This observation was ingreement with christian (1955) postulation. ***Halobacterium salinarum*** (32); ***H. halobium,*** (77); and ***Halococcus morrhue*** (98); were the most salt tolerant halobacteria.

Compatible solutes can be accumulated by bacteria by de novo synthesis or by transport from culture medium. This solutes are called osmoregulator or osmoprotectant.

Basal medium with potassium (BMK) was petter for growth of halobacteria than the BM without K^+. The result here in shows that, when more potassium was added the growth of the studied organisms was enhanced. Potassium was essential for halophilic enzyme, ribosome, and cell wall structure (Kushner, 1968). Brown & Gibbons (1955) found that potassium was essential for growth of some halobacteria and *Sarcina*. The results here in were in agreement with the previous results. In addition Sen et al, 1988 found that potassium is the most prevalent cations in bacterial cytoplasm. The intercellular content of potassium play an important role in serving the turgor condition of bacterial cell (Road & Stwart, 1985).

Meury and Kepes (1982) found that the rapid exchange between extracellular and intracellular concentration of potassium was responsible for intracellular content.

Generally this results and others of many workers confirmed the pressence of positive correlation between the potassium content and both good growth (dry weight and total cell nitrogen in these results); and the ability of bacteria to tolerant the conditions of hypersalinity (Christian & Waltho, 1961).

Many investigator reported that the cytoplasmic levels of glutamine and glutamate. Tempest et al. 1970) increase in response to osmotic stress in gram negative bacteria (Botsford, 1984; Richey et al, 1987; and Csonka, 1988). The increasing of concentration of glutamine and glutamate stimulated the growth of E. coli (Richey et al, 1987) and Gram negative bacteria (Brown & Stanly 1972). Tempest (1970) found that the activity of glutamate dehydrogenase enzyme of *Enterobacter aerogenes* in case of exposure of their cells to osmotic stress. These results were in agreement with the results here in (in this work).

The presence of glycine in basal medium increased the cell dry weight and total cell nitrogen of the test organisms. This results were in agreement with the results of many workers. Sakaguchi 1960 found that the glycine & its derivatives could activate the growth of *pediococcus* in salted medium. Rafaeli-Eshkol and Avi-Dor (1968) found that glycinebetaine stimulated the respiratory rate of halophilic bacteria. Le Rudulier and Bouillard (1983) also observed that this compound is osmoprotectant for a number of *Enterobacteriaceae*. The result here in and the results of other worker as seen above confirmed that the osmoprotectant effect of glycine and glycine derivatives leads to good growth of the studied organisms.

High intercellular concentration of proline in gram negative bacteria during osmotic stress was dependant on the enhanced transport (Brady & Csonka 1988). Proline was able to function as osmoprotectant of some species of bacteria (Csonka 1983). This function was depended on its activation on metabolism by the bacterial cell in the medium of high salt concentration. The effect of proline on the studied organisms was increasing in both cell dry weight and total cell nitrogen. These increases were due to the activation of metabolism of the studied organisms. The results here in were in agreement with the results previous workers.

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