Cloning, Sequencing and Expression of Aldehyde Dehydrogenase Gene from *Halomonas salina* AS11

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Thesis Title Cloning, Sequencing and Expression of Aldehyde Dehydrogenase Gene from *Halomonas salina* AS11

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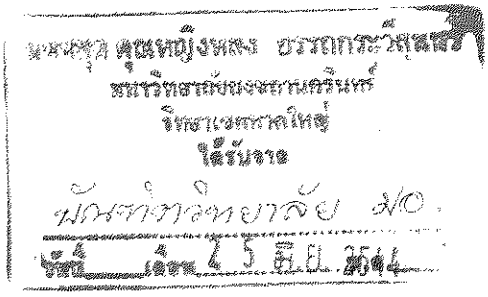

Abstract

The moderatedly halophilic bacteria from prawn pond's sediment in Songkhla region (Thailand) were screened on medium containing sodium chloride. Five of thirty-six isolated halophilic bacteria show aldehyde dehydrogenase activity on ATCC culture medium 1270 for halobacterium. One isolate designated as AS11 was selected based on its activity. The organism can grow at sodium chloride concentrations ranging from 2.5 to 25% with optimum growth at 5%. Phenotypic and phylogenetic studies indicated that AS11 referred to *Halomonas salina*. The *aldh* gene coding an aldehyde dehydrogenase has been cloned from *Halomonas salina* AS11. The genomic sequence of the *aldh* gene consisted of 1521 bp encoding 506 amino acid residues with a calculated molecular mass of 54.9 kDa. The gene encoding for *aldh* was confirmed by expression in *E. coli* system. The recombinant cells produced ALDH protein and corresponding to 55 kDa. Comparison of the deduced amino acid sequence of the cloned aldehyde dehydrogenase gene product (*aldh*) with other dehydrogenases revealed a high homology (76% identify) with NAD-dependent acetaldehyde dehydrogenase gene from *Pseudomonas aeruginosa*.

ชื่อวิทยานิพนธ์ การโคลน, การหาลำดับเบส และการศึกษาการแสดงออกของยีน aldehyde dehydrogenase จากเชื้อ *Halomonas salina* AS11

ผู้เขียน นาง ธัญญา ศรีโพธิ์

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บทคัดย่อ

แบคทีเรียกลุ่มทนเค็มที่แยกจากตะกอนดินของบ่อเลี้ยงกุ้งในจังหวัดสงขลาประเทศไทยจำนวน 36 สายพันธุ์สามารถเจริญบนอาหารที่มีเกลือโซเดียมคลอไรด์ 2.5-25% ในจำนวนนี้มี 5 สายพันธุ์ที่มีกิจกรรมของเอนไซม์ aldehyde dehydrogenase ได้เลือกสายพันธุ์ AS11 ซึ่งสามารถผลิตเอนไซม์ในกลุ่ม aldehyde dehydrogenase ในระดับที่น่าสนใจและเจริญได้ดีที่สุดในอาหารที่มีเกลือโซเดียมคลอไรด์ 5% มาทำการศึกษา พบว่าเมื่อนำมาจำแนกด้วยวิธีทางชีวเคมี, การใช้คาร์โบไฮเดรต และ เปรียบเทียบลำดับเบสของ 16S ribosomal DNA ระบุสายพันธุ์ได้ว่าเป็น *Halomonas salina* นำโครโมโซมดีเอ็นเอจากเชื้อ AS11 ไปโคลนและหาลำดับเบส ในที่สุดได้ยีน aldehyde dehydrogenase มีขนาด 1,521 เบสประกอบด้วย 506 กรดอะมิโนและมีค่าน้ำหนักโมเลกุลจากการคำนวณเท่ากับ 54.9 กิโลดาลตัน การแสดงออกของยีน aldehyde dehydrogenase ในเชื้อ *E. coli* พบว่า เชื้อลูกผสมที่มียีนนี้ผลิตโปรตีนขนาด 55 กิโลดาลตันเช่นกัน การเปรียบเทียบลำดับกรดอะมิโนของยีนกับธนาคารยีน พบว่ามีความเหมือนกับลำดับของยีน acetaldehyde dehydrogenase ของเชื้อ *Pseudomonas aeruginosa* 76%

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Thanya Sripo, Ph. D

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List of Abbreviations

ABA	=	Abscisic acid
ADH	=	alcohol dehydrogenase
ALDH	=	aldehyde dehydrogenase
aldh	=	aldehyde dehydrogenase gene
APS	=	ammonium persulfate
BAP	=	6-benzylamino-purine
BSA	=	Bovine serum albumin
bp	=	base pair (s)
°C	=	degree Celsius
CIM	=	callus inducing medium
cm	=	centimeter
CTAB	=	cetyltrimethylammonium bromide
dATP	=	deoxyadenosine-5'-triphosphate
dCTP	=	deoxycytidine-5'-triphosphate
dGTP	=	deoxyguanosine-5'-triphosphate
dTTP	=	deoxythymidine-5'-triphosphate
dNTP	=	dATP, dCTP, dGTP, and dTTP
DNA	=	deoxyribonucleic acid
E. coli	=	*Escherichia coli*
EDTA	=	ethylenediamine tetraacetic acid
et al.	=	Et. Ali (Latin), and others

List of Abbreviations (continue)

etc.	=	Et. cetera (Latin), other things
g	=	gram
H.	=	*Halomonas*
i.e.	=	id. est, for example
IPTG	=	isopropyl-β-D-thiogalactopyranoside
kbp	=	kilobasepair (s)
kDa	=	kilodalton (s)
LB	=	Luria-Bertani (medium)
M	=	molar
mA	=	milli ampare
MCS	=	multiple cloning site
mg	=	milligram
ml	=	milliliter
mM	=	milli molar
mm	=	millimeter
MT	=	metricton
µg	=	microgram
µl	=	microliter
NAA	=	1-naphthaleneacetic acid
nm	=	nanometer
O.D.	=	optical density

List of Abbreviations (continue)

PAGE	=	polyacrylamide gel electrophoresis
PCR	=	polymerase chain reaction
PEG	=	polyethyleneglycol
pfu	=	plaque-forming unit
RNA	=	ribonucleic acid
rDNA	=	ribosomal deoxyribonucleic acid
rRNA	=	ribosomal ribonucleic acid
Rnase	=	ribonuclease
rpm	=	revolution per minute
SDS	=	sodium dodecyl sulfate
TEMED	=	N,N,N'N'-tetramethyl-ethylenediamine
Tris-HCl	=	Tris-(hydroxymethyl)-aminoethane hydrochloric acid
U	=	unit (s)
V	=	volt
v/v	=	volume by volume
w/v	=	weight by volume

1. INTRODUCTION

Introduction

The growth of the shrimp aquaculture industry in Thailand has benefited the social and economic well being of numerous individuals but also had a significant environmental impact on many parts of the country. This industry has led to the destruction of wetlands and a deterioration of water-quality as a result of silitation, eutrophication, oxygen depletion, and the release of toxic sulfides, ammonia, and xenobiotics compounds from therapeutic and wastewater treatment chemicals (Dierberg and Kiattisimkul, 1996). The biotransformation of a large number of drugs and other xenobiotics generates aldehyde as intermediates or as products resulting from oxidative deaminations and dealkylations, as well as the oxidation of primary alcohol (Lindahl, 1992). Aldehydes frequently are highly reactive, and many have significant biological effects, including cytotoxicity, mutageneticity, genotoxicity and carcinogeneticity, on organisms exposed to them. However, the majority of aldehydes are encountered as physiologically derived intermediates in the metabolism of other compounds. Endogenous sources include aldehydes arising from the metabolism of amino acids, biogenic amines, carbohydrates, vitamins, steroids and lipids. A variety of enzymes have evolved to metabolize aldehydes to less reactive forms. The most effective pathway for aldehyde metabolism is their oxidation to carboxylic acids by ALDHs. Regardless of their specificity, these NAD/NADP-dependent enzymes share

common structural and functional features that catalyze the oxidation of a broad spectrum of aliphatic and aromatic aldehydes (Hempel *et al.*, 1993).

The use of bacterial remediation treatments is becoming more commonplace in the aquaculture industry in the belief that the growth of selected microbial species will help maintain low ammonia concentrations, reduce organic matter concentrations and improve the quality of sediment accumulated in the pond (Funge-Smith and Briggs, 1998). Several research groups have isolated novel microorganisms from soil, wastewater, and culture collections that have proven capable of catabolizing some of the more exotic compounds introduced into the environment by today's agricultural practices. The ponds and run-offs of shrimp-farming enterprises present a particularly difficult challenge to bioremediative programs. Most of the organisms that have proven effective in soil cannot grow well in the saline environments of these ponds. On the other hand, these ponds are the natural home of halophilic bacteria that are very well adapted to live in environments with high salinities. They are easy to grow and have simple nutritional requirements (Larsen, 1962; Vevtosa, 1994). Moreover, many of them produce compounds of industrial interest including enzymes, polymers and osmoprotectants.

This thesis describes the isolation of a halophilic microorganism from prawn pond sediment. Isolation and characterization of this bacterial strain is discussed. A gene cluster encoding three proteins, aldehyde dehydrogenase, alcohol dehydrogenase and a possible regulatory protein are cloned and sequenced. This bacterium may prove to be useful in future programs to accelerate the breakdown of toxic metabolites produced by the prawn industry.

Review of Literatures

1.1 Shrimp culture affects environment

Over the last 10 years, intensive shrimp culture has become one of the most important worldwide agricultural industries. Thailand has managed to win and maintain its title as "the world's largest cultured shrimp producer" for the tenth consecutive year. Although Thailand started off the year 1998 with a poor first quarter, production during the third and fourth quarters more than make up for it so that the year finished with a record high yield of 220,000 MT (Table1). One of the greatest sources of concern in the shrimp farming industry, yellow-head disease which is caused by yellow-head virus (Flegel *et al.*, 1996), appeared to have been very well controlled and contained. Despite disease problems plaquing shrimp farming in the first quarter of the year 2000, Thailand retained the world's title with the record production of 245,000 MT, an impressive 5,000 MT or a 7% increase over the previous year 1999.

The rapid development of intensive marine shrimp culture in Thailand and other Southeast Asian countries has imposed a serious strain on the coastal aquatic environment as a result of wastewater effluent discharges from intensive aquaculture. Intensive marine shrimp farming requires inputs of high protein and phosphorus diets and high rates of water exchange. Much of organic material provided to the shrimp is never fully utilized. As a result, a large proportion of the nutrients in shrimp feed becomes waste that is discharged directly into the coastal waters causing rapid eutrophication. As a result, the nitrogen and phosphorus concentrations in shrimp pond effluent increase drastically compared with those in the inflow source water.

Table 1 Thailand's quarterly shrimp harvest during 1991-2000 (MT)

year	Quarter				Total
	1st	2nd	3rd	4th	
1991	30,072	36,911	46,808	39,586	153,317
1992	37,008	35,534	46,100	44,050	162,692
1993	32,500	36,800	69,900	69,800	209,000
1994	51,000	58,000	75,000	66,000	250,000
1995	46,500	60,000	70,000	48,500	225,000
1996	30,000	50,000	80,000	45,000	205,000
1997	30,000	45,000	60,000	35,000	170,000
1998	30,000	40,000	60,000	90,000	220,000
1999	40,000	45,000	75,000	70,000	230,000
2000	40,000	55,000	80,000	70,000	245,000

Source: Aquaculture news from Charoen Pokphand Group (C.P.G.), December 2000

The amounts of nitrogen and phosphorus contained in wastewater from producing one ton of shrimp range from 26-117 kg and 13-38 kg, respectively depending on the Feed Consumption ratio (FCR) (Table 2). The FCR is calculated by the following formula:

$$\text{FCR} = \frac{\text{dry weight of feed consumed}}{\text{wet weight of shrimp gained}}$$

The use of feeds will improve shrimp production and increase profits. However, feeds are expensive and can range from 50% to 70% of the total variable cost of production. As feed quality and cost are directly related, an improvement in feed

quality will inevitably increase feed cost. Therefore, feed quality and cost are critical factors in determining the profitability of a shrimp farm. By the way, feeds with high levels of fiber will increase fecal production and consequently, pollute the water environment.

Table 2 Feed conversion ratio and waste production per MT of cultured shrimp

FCR	Wastes/ MT shrimp		
	Organic matter	nitrogen	Phosphorus
1.0	500	26	13
1.5	875	56	21
2.0	1250	87	28
2.5	1625	117	38

Source: Lin and Nash (1996)

The use of good quality feeds will definitely increase shrimp production and profits. However, there is a growing concern about environmental pollution from shrimp farming which directly related to excessive feed use. For each metric ton of shrimp produced with an FCR of 2:1, approximately 1250 kg of organic material, 87 kg of nitrogen, and 28 kg of phosphorus are generated as wastes (Table 2). Some of these wastes are utilized by phytoplankton and other organisms in the ponds. However, the majority of wastes are flushed out of the pond and into the water sources. The dissolved nutrients and organic solids stimulate rapid growth of microorganisms, including bacteria, phyto- and zoo- plankton and benthic fauna.

Bacterial growth in intensive shrimp rearing ponds takes place mostly in the water column, where the microbes can have a doubling time of only a few hours and increases linearly with increasing feed input. Rapid microbial growth can then deplete the water of oxygen leading to significant changes in the natural ecosystem. In addition to what might be thought of as "natural" wastes, there is an increasing association of these farms with other kinds of chemicals. For example, crowding animals in small spaces promotes the growth and spread of various pathogens. Because of this, farmers may add drugs and other chemicals to the growth ponds to keep bacterial, fungal, and algal populations under control. Although antibiotics and herbicides may be effective initially, they can promote the growth of antibiotic-or multiple-drug-resistant microbes that are hard to control.

All of these factors contribute to increasing concerns about the negative effects that intensive shrimp culture may have on the local economy. The benefits derived from shrimp culture operations in terms of economic and social well-being are now being outpaced by the loses incurred from environmental degradation. As is usually the case in many countries, large-scale shrimp farms are preferentially located in certain suitable areas. Crowding of farms in these areas is usually due to lack of appropriate governmental planing and regulations. Shrimp farms are usually constructed without consideration of the neighboring farms. No comprehensive plan is followed which takes into account the relationship between the total area of shrimp ponds that can be constructed in such a site and the carrying capacity of the supply/receiving water body. Thus, pollution has become a serious problem in such cases. The water discharged by one farm is pumped in by a neighboring farm making disease outbreaks more likely and the rates of production lower.

The water discharged from intensive farms is generally of low quality. Because of high stocking densities there is a build-up of dissolved organic matter from metabolites and decomposition of uneaten feeds in the ponds. An understanding of the nature of wastes from shrimp pond effluents is important. The physical, chemical and biological characteristics of intensive shrimp pond effluents and their sources are listed in Table 3. The particular contaminants of concern are listed in Table 4.

Table 3 Physical, chemical and biological characteristics of intensive shrimp pond effluents

Characteristic of effluents	Sources/causes
Physical properties	
Color	Decomposing organic matter; algal populations; leaching of inorganic substances from soil.
Odor	Decomposing organic matter from uneaten feeds and metabolites.
Solids	Uneaten feed particles; detritus; coagulated flocculated products of dead organisms and/or inorganic particles; scoured soil particles.
Chemical constituents	**Organic**
Oxygen-demanding waste (BOD)	Biologically produced breakdown products of proteins, carbohydrates, and fats from uneaten feeds and/or metabolites.
Pesticides	Applied in ponds for ponds for predator control.
Inorganic	
pH	Interaction among biological and chemical constituents of water and soil.
Total Nitrogen	Decomposition of organic matter from uneaten feeds, metabolites and dead organisms.
Phosphorus	Decomposition of organic matter, dead plants/animals.
Gases	
Other drugs and chemicals	Antibiotics applied in ponds for disease control or incorporated in feeds; disinfectants, other chemotherapeutants.
Biological constituents	
Planktonic organisms	Phyto-/zooplankton flourishing in ponds.
Pathogens	Bacteria, viruses from diseased shrimp in ponds.

Cited by Lin and Nash (1996)

Table 4 Contaminants of concern in intensive shrimp pond effluents

Contaminants	Significance
Biodegradable organics	If discharged at relatively high concentration, biological stabilization leads to depletion of oxygen resources of the receiving water boby causing septic conditions.
Suspended solids	Can lead to the development of sludge deposits and aerobic conditions.
Pathogens	Diseases can be transmitted by pathogenic organisms.
Nutrients	When discharged into the aquatic environment, these lead to the growth of undesirable aquatic life.
Other drugs/ chemicals	Presence of antibiotics in the environment can lead to resistant strains of bacteria; most pesticides cannot be naturally decomposed in the environment.

Cited by Lin and Nash (1996)

Shrimp feed and waste management need to be improved for the longer-term viability of shrimp farming. With better farm management and lower feed conversion ratios, pollution of shrimp farming will be reduced and the profitability of the shrimp farm will increase. The maximum feeding rate should be determined by existing water quality parameters and not by expected production levels.

1.2 Bioremediation technology for shrimp culture

It's a golden rule that successful intensive shrimp culture requires intensive management to maintain good pond water quality. The pond water quality changes quickly because of the input of large quantities of high quality feeds. Most of those feeds eaten by shrimps are eventually excreted as metabolic wastes that add inorganic

nutrients and organic matter to the water and bottom of the ponds. The ponds thus become eutrophic, with active decay and assimilation of leftover feed and metabolic wastes carried out by microorganisms. The basic groups of microorganisms in the aquatic environment consist mostly of bacteria, fungi, protozoa and phytoplankton, which may exist ubiquitously in large variety in shrimp ponds. The microbial decomposition of organic compounds requires dissolved oxygen from water and the amount oxygen required is called biological oxygen demand (BOD). As a result of microbial activity under aerobic conditions the organic matter is converted to inorganic compounds, such as phosphate (PO_4), ammonia (NH_3), carbon dioxide (CO_2). The microbial process of converting organic matter to inorganic compounds is called mineralization. Some of those organic compounds serve as nutrients to stimulate algal growth. That's why the phytoplankton bloom intensively in shrimp ponds. In a way, the bacteria and phytoplankton exist as symbiotic partners in the onds. The microbial functions and bacterial-algae relationships are illustrated in Figure 1. These microorganisms are part of a natural process of self-purification that disperses "dirty organic matter" through other trophic levels.

As shrimp culture intensifies by using a higher stocking density and increased feed inputs to enhance productivity, a greater effort is required to deal with the increasing amount of wastes produced by the shrimp. The traditional method for removing wastes from ponds has been by frequent water exchange, which pollutes nature waters and is considered to be environmentally unfriendly. To reduce the waste contaminants discharged from shrimp ponds to the external environment, a closed recycling system has been recently developed. This apparatus can not only reduce organic wastes, but also prevent the importation toxic contaminants and diseases-

causing organisms that may exist in the external water source. The carrying capacity of the recycling system is based on the balance between the quantity of waste products being generated by shrimp ponds and the capacity to assimilate those wastes by microorganisms.

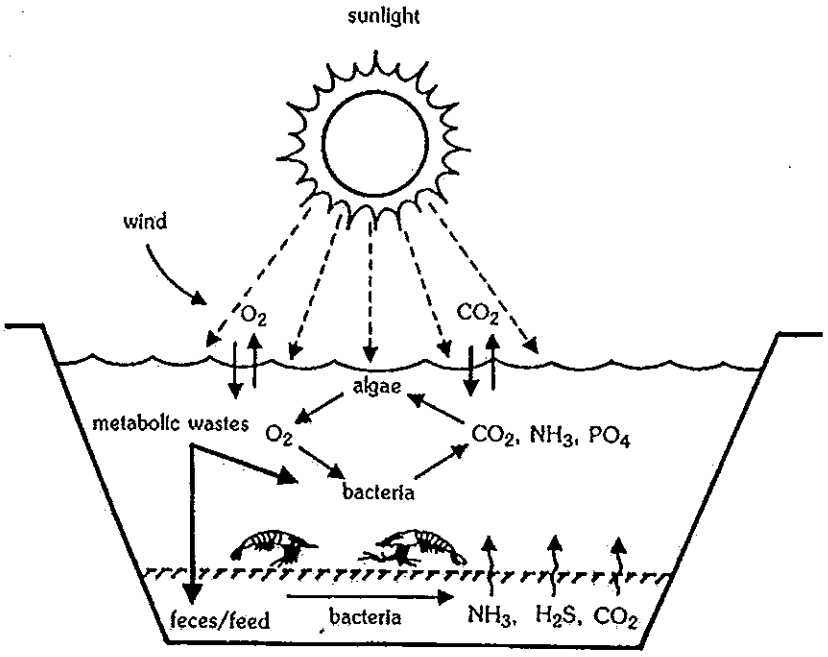


Figure 1.Role of microorganisms in the metabolic waste cycle in shrimp ponds.

The newest approach towards the improvement of water quality in intensive shrimp culture is the application of bacteria or enzymes to the ponds. This type of biotechnology is known as "bioremediation", which involves manipulation of microorganisms in ponds to enhance mineralization of organic matter and eliminate

undesirable waste compounds. This approach is not new. It is merely an extension of older practices that employed microbes to condition soil, prevent disease, and add nutrients needed to improve crop production. Those microbes are called "effective microorganisms" or EMs for short. EMs isolated from the natural environment have also been used to enhance the degradation of organic compounds in industrial and domestic wastewater treatment.

As the problems affecting water quality and disease have become more visible, farmers have resorted to the use of numerous chemical products to rescue the endangered shrimp from ill health or to improve the water quality in ponds. In addition to commonly known antibiotics and other therapeutic agents, cases of outrageous abuses of chemicals, such as the application of household detergents, have been reported. Farmers are often persuaded by sales agents to "clean up" the ponds with those products, as if they were washing dirty clothes. They have also been led to believe that all microorganisms are harmful to shrimp, therefore ponds should be sterilized.

A very different, and potentially useful, alternative to the previously kinds of products and sales pitches would be one that uses bacteria and bacterial products for the remediation of shrimp ponds. At least in theory, bioremediation technology could be used to improve shrimp pond water quality or even combat diseases. In order to ensure that the organism will be able to adjust to the extreme conditions of shrimp ponds, these microorganisms should be isolated from those habitats. One such group of organisms known to exist there is halophilic bacteria.

1.3 Physiology of Halophilic Bacteria

Halobacteria are a group of prokaryotes very well adapted to live in environments with high salinity. Some grow optimally in media with 15 to 25% sodium chloride while others are able to reproduce over a range of salt concentrations, from the salinity of seawater to the salinity found in concentrated brines with 25 to 30% salt (Rodriguez-Valera, 1988).

Moderately halophilic bacteria and halobacteria are microorganisms that inhabit hypersaline environments, in which a succession of different microbial populations can be observed at increasing salinities and in some instances both groups compete. The extensive modifications of halophiles involved in their adaptation to extreme conditions permitted researchers to speculate about the origin and evolution of these prokaryotes. Traditionally, halobacteria were related to *Pseudomonas* (rod-shaped cells) or to *Micrococcus-sarcina* (cocci) and several opinions were expressed with respect to the possible relationships between halobacteria and other groups of prokaryotes as well as about the ways in which they might be derived from other bacterial groups (Larsen, 1962). Kushner reported that it is a mistake to consider the red halophiles as very closely related to other known bacterial genus. In further their taxonomic status may classify by comparisons of specific ribosomal proteins, of RNA's and of other macromolecules of known function (Kushner, 1978).

During the last decade, the extensive studies on hypersaline environments that have been carried out in many geographical areas have permitted the isolation and taxonomic characterization of a large number of moderately halophilic species. The genera *Halomonas* and *Deleya*, which constitute the family *Halomonadaceae*, are difficult to differentiate on the basis of phenotypic and chemotaxonomic attributes. A

phylogenetic analysis of the 16S ribosomal RNA sequences of seven members of the *Halomonadaceae* indicated that the members of the genera *Halomonas* and *Deleya* do not form separate monophyletic subgroups, confirming the lack of any phylogenetic support for retention of these taxa as separate genera (Franzmann and Tindall, 1990; James *et al.*, 1990). The members of the genera *Halomonas*, *Deleya* and *Halovibrio* cannot be resolved on the basis of phylogenetic, chemotaxonomic, or phylotypic data. Dobson and colleagues (1993) concluded that the members of the genera *Halomonas*, *Deleya* and *Halovibrio* should be combined in a single genus. Later studies of the 16S ribosomal RNA sequences as well as phenotypic or chemotaxonomic features of *Halomonas*, *Deleya*, *Halovibrio* and *Volcaniella*, as well as *Paracoccus halodenitrificans*, indicated that they could form a monophyletic group within the gamma subclass of the *Proteobacteria* (Miller *et al.*, 1994; Mellado *et al.*, 1995). The levels of 16S ribosomal RNA sequence similarity among these species ranged from 91.5 to 100%; although several sub-groups, which might represent separate genera, were resolved, they could not be differentiated on the basis of phenotypic or chemotaxonomic features. For these reasons, Dobson and Franzmann (1996) proposed placing all members of the above four genera and *P. halodenitrificans* in a single genus, the genus *Halomonas*, and amended the description of the family *Halomonadaceae*. This family now comprises the species of *Halomonas* and *Zymobacter* and the moderate halophile, originally isolated from the Dead Sea.

Halophilic bacteria are a group of prokaryotes very well adapted to live in environment with high salinities, minimizing the risk of contamination. They are easy to grow and their nutritional requirements are simples. Therefore haloplilic bacteria have the potential for exciting and promising applications. Many of them produce

compounds of industrial interest (enzyme, polymers and osmopotectant). They also possess useful physiological properties which can facilitate their exploitation for commercial purposes.

1.4 Aldehyde

Aldehydes are ubiquitous in the environment. For example, certain aldehydes such as formaldehyde, acetaldehyde, and acrolein are products of combustion and are present in smog and cigarette smoke. In addition, many foods, especially fruits and vegetables, are sources of aldehydes, including a range of aliphatic and aromatic species (Table 5). Aldehydes are responsible for the flavors and odors of foods and beverages. One particular aldehyde, malondialdehyde, is present in many foodstuffs, increasing in concentration in spoiled foods and in microwave-cooked red meats.

Table 5 Examples of aldehydes found in foods

Formaldehyde	Benzaldehyde	Malondialdehyde
Acetaldehyde	Phenylacetaldehyde	Acrolein
Propanol	4-Hydroxynonenal	Glyoxal
Hexanal	Crotonaldehyde	Methylglyoxal
Octanal	Citral	
Decanal	C24-30 aldehydes	

Compiled from Schauentein *et al.* (1977)

Aldehydes also function as communication molecules (Table 6). They may act as information-transmitting molecules, either between species or within a species. Communication within a species is usually mediated by pheromones, which act to identify individuals or to attract conspecifics for mating (Schauentein *et al.*, 1977). Interspecies communication may be in the form of territory marking or defense. Plant-animal information exchange may also occur through aldehydes. The roles may be attractive, to assist in reproduction, or defensive, acting as natural pesticides.

Table 6 Some aldehydes that act as communication molecules

Propanal	Salicylaldehyde
Hexanal	Benzaldehyde
Hexenal	Crotonaldehyde
Octenal	Acrolein
Decanal	Citral

Compiled from Schauentein *et al.* (1977)

The majority of aldehydes, however, are encountered as physiologically derived intermediates in the metabolism of other compounds. In this context both endogenous and exogenous sources are important. Endogenous sources include aldehydes arising from the metabolism of amino acids, biogenic amines, carbohydrates, vitamins, steroids, and lipids (Table 7). Of considerable recent interest in the context of the present review is the generation of aldehydes as the result of membrane lipid peroxidation.

Table 7 Examples of endogenously produced aldehydes

Aldehyde	Source
Acetaldehyde	Threonine catabolism
Betaine aldehyde	Choline catabolism
21-Dehyrocorticosteroids	Corticosteroids catabolism
3,4- Dihydroxyphenylacetaldehyde	Dopamine catabolism
Glutamic-γ-semialdehyde	Proline biosynthesis
Hexanal	Lipid peroxidation
5-Hydroxyindoleacetaldehyde	Serotonin catabolism
4-Hydroynonenal	Lipid peroxidation
Malondialdehyde	Lipid peroxidation
Retinal	Vitamin A metabolism
Succinic semialdehyde	GABA shunt

Compiled from Schauentein *et al.* (1977)

Xenobiotics are the major exogenous source of aldehydes. The biotransformation of a large number of drugs and other xenobiotics generates aldehydes either as intermediates or as products (Table 8). Some of the reactions that can produce aldehydes include oxidative deaminations and dealkylations, as well as the oxidation of primary alcohols. Another mechanism of particular relevance to the present discussion is ring hydroxylation adjacent to a heteroatom, followed by tautomerization (Sladek *et al.*, 1988). This process occurs during metabolism of the

antitumor agent cyclophosphamide (CP). Metabolic intermediates can produce aldehydes by hydroxylation or ester linkage hydrolysis.

Table 8 Examples of exogenous sources of aldehydes

Source	Aldehyde	References
Combustion	Formaldehyde, acetaldehyde, acrolein	Schauenstein *et al.* (1977)
Ethanol	Acetaldehyde	Williams (1959)
2-Butoxyethanol	Butoxyaldehyde	Ghanayem *et al.* (1987)
Diethylnitrosamine	Acetaldehyde	Magee *et al.* (1973)
Succinylcholine	Betaine aldehyde	Taylor (1990)
Toluene	Benzaldehyde	Gillette (1959)
Xylene	Tolualdehyde	Patel *et al.* (1978)
Laetrile	Benzaldehyde	Haisman and Knight (1967)
Nicotine	γ-3-Pyridyl-γ-methylamino-butyraldehyde	Mckennis *et al.* (1957)

Aldehydes are generally relatively long-lived and highly reactive. Because aldehydes are long-lived, they can diffuse or be transported from their site of generation to distant sites, for example, to another cell compartment, another cell, or even another tissue. The electrophilic nature of their carbonyl group is responsible for the high reactivity of aldehydes.

Aldehydes are highly reactive molecules that may have a variety of effects on biological systems. They can be generated from a virtually limitless number of endogenous and exogenous sources. Although some aldehyde-mediated effects such as the oxidation product of retinal into retinoic acid is involved in embryonic differentiation, and retinal itself is required for vision. Many effects are deleterious, including cytotoxicity, mutagenicity and carcinogenicity. Aldehydes cause their effects by reacting with cellular nucleophiles, including proteins and nucleic acids. A variety of aldehydeprotein and aldehyde-nucleic acid adducts have been identified. Most physiologically relevant protein adducts are formed through sulfhydryl group. The relevant nucleic acid adducts are those with the nitrogen atoms of both purines and pyrimidines. With respect to carcinogenesis, aldehydes can act either as carcinogens or as carcinostatic agents (Schauentein *et al.*, 1977). Formaldehyde and acetaldehyde are respiratory tract carcinogens in rats and hamsters. In addition, reactive aldehydes can be generated during the metabolism of other nonaldehyde carcinogens. For example, the metabolism of many nitrosamines produces aldehydes as intermediates. In many cases it is not known whether these aldehydes can function as carcinogens. However, the fact that they can form adducts with cellular macromolecules and are mutagenic and/or genotoxic suggests that they may play some role in carcinogenesis.

In living systems, aldehydes are produced by many of the enzymes of intermediary metabolism, by monoamine and diamine oxidases, by a variety of esterases, and many of the phaseI and phaseII enzymes involved in xenobiotic metabolism. Because the number and substrate specificities of these enzymes are very large, the number of aldehydes that can be generated is virtually unlimited. Many of

these enzymes systems are operative in the metabolism of normal cells. It is also apparent that a number of pathophysiological conditions, including cancer, alter the metabolic behavior of cells and tissues. Many of these alterations are reflected in the composition or activity of the enzymes cited above.

1.5 Aldehyde-metabolizing enzymes

Aldehydes can be metabolized by three different enzyme systems: aldehyde oxidase, aldo-keto reductases, and aldehyde dehydrogenase. Isoforms of each enzyme family can be found in virtually all tissues. The three systems have broad and sometimes overlapping substrate preferences.

A. Aldehyde oxidase (EC 1.2.3.2, ALOX) is a cytosolic, molybdenum-containing flavoprotein. It is a large (mol wt 300,000) molecule composed of two apparently identical subunits. It can use molecular oxygen as the electron acceptor, generating the oxidized product together with H_2O_2 and $O_2^{\cdot}$.

B. Aldo-keto reductases (EC 1.1.1.2, ALRD) are a family of enzymes that reduce a variety of aldehydes and ketones to their corresponding alcohols. They are small (mol wt 30,000-40,000), monomeric, cytosolic enzymes that use NADPH as coenzyme.

C. Aldehyde dehydrogenases (EC 1.2.1.3, ALDH) are also a family of related enzymes. They catalyze the irreversible oxidation of a wide variety of aldehydes to their corresponding acids. Some forms display broad substrate specificity, oxidizing a variety of aliphatic and aromatic aldehydes, whereas other forms possess narrower substrate preferences.

One of the important steps in aldehyde metabolism is their oxidation to carboxylic acids by aldehyde dehydrogenase. Different types of ALDH have been distinguished based on their physicochemical and enzymological properties, subcellular localization and tissue distribution. ALDH isozymes play roles in the detoxification of acetaldehyde and in the metabolism of corticosteroids, biogenic amines and lipid peroxidation products (Yoshida *et al.*, 1998). At present, the best characterized ALDH genes are those isolated from bacteria, humans and *Saccharomyces cerevisiae*. In humans, there are at least two isozymes of ALDH involved in ethanol metabolism (Hsu *et al.*, 1988). Orientals are known to have a higher sensitivity to alcohol than other races, and this has been associated with a genetic deficiency of ALDH2 (Yoshida *et al.*, 1998). In *S. cerevisiae*, two ALDH isozymes have been described. One of them is located in the mitochondria and is activated by potassium (K^+) ions, and functions under aerobic conditions in the oxidation of ethanol (Wang *et al.*, 1998). The other is located in the cytosol and is activated by magnesium (Mg^{2+}) ions, and plays a role in fermentative metabolism in the pathway generating acetyl-CoA from pyruvate (Dickinson, 1996).

In 1996, the first gene encoding a plant mitochondrial ALDH was identified in maize (Cui *et al.*, 1996). This was found to be a nuclear-encoded restorer gene of Texas-type cytoplasmic male sterility (cms-T) (Schnable and Wise, 1998). Subsequently, in tobacco, two *aldh* gene (*aldh*2a and *aldh*2b) were identified, and the tobacco ALDH2a transcript and ALDH2a protein were found to be present at high levels in floral organs such as stamens, pistils and pollen (op den Camp and Kuhlemeier, 1997). Alcoholic fermentation and expressions of genes involved in alcoholic fermentation [pyruvate decarboxylase (PDC) and alcohol dehydrogenase

(ADH)] also increase during pollen development in tobacco, even under aerobic conditions. Therefore, it has been suggested that alcoholic fermentation and the oxidation of acetaldehyde by ALDH has some as yet undetermined function in biosynthesis and energy production during pollen development (Tadege *et al.*, 1999).

1.6 Molecular genetic of aldehyde dehydrogenase

The aldehyde dehydrogenase family has been widely studied at the biochemical, physiological, molecular and genetic level. Nagy and colleagues (1995) reported the identification and characterization of a unique gene cluster from *Rhodococcus* sp. strain NI86/21, containing a cytochrome P-450 system that confers both EPTC (S-ethyl dipropylcarbamothioate)-degrading and biosafening abilities to *Rhodococcus erythropolis* SQ1.

Priefert and his colleagues (1992) studied the structural gene for an AcDH-II (*aco*D) of *Alcaligenes eutrophus* that is involved in the catabolism of acetoin as well as of ethanol. Sequence analysis of a 2.8 kbp *Pst*I subfragment of D showed that it encoded a protein with a relative molecular weight of 54,819. Alignment of the amino acid sequence deduced from *aco*D with the primary structures of aldehyde dehydrogenases from other sources revealed a high degree of homology, amounting to 46.5% identical amino acids.

Xu and Johnson (1995) proposed that *ald*B encodes an aldehyde dehydrogenase in *Escherichia coli*. The complete DNA sequence encodes a 56.3 kDa protein which shares a high degree of homology with an acetaldehyde dehydrogenase encoded by *aco*D of *Alcaligenes eutrophus* and an aldehyde dehydrogenase encoded by *ald*A of *Vibrio cholerae*. It also had significant homology with a group of other aldehyde

dehydrogenases from diverse prokaryotes and eukaryotes. *ald*B reaches its highest level of expression during the transition from exponential growth phase to stationary phase, but can also be induced by ethanol.

Schobert and Gorisch (1999) studied the organization of a cluster with five genes encoding components of the quinoprotein/ethanol oxidation system in *Pseudomonas aeruginosa* ATCC17933. *P. aeruginosa* grown aerobically on ethanol produces a soluble cytochrome C550 together with a quinoprotein, ethanol dehydrogenase. A 3.2 kb genomic DNA fragment containing the gene encoding cytochrome C550 was cloned and sequenced. Two other complete and two truncated ORFs were also identified. A truncated ORF encoding the quinoprotein ethanol dehydrogenase was found upstream of the cytochrome C550 gene and in the reverse orientation. An ORF encoding an NAD^+-dependent acetaldehyde dehydrogenase was located downstream of the cytochrome C550 gene and in the same orientation. Another ORF showed similarity to the *pqq*A gene and a truncated ORF similarity to the *pqq*B gene both involved in the biosynthesis of the prosthetic group pyrroloquinoline (PQQ). The organization of these genes was found to be different from the well-studied methanol oxidation system in methylotrophic bacteria.

While studying a related bacterium, *Rhodococcus* sp. strain Q15 isolated from Lake Ontario, Canada, Whyte and colleagues (1998) characterized an aliphatic aldehyde dehydrogenase gene that was highly homologous to the *Rhodococcus erythopolis thcA* gene. The *thcA* gene was originally found to be induced in *R. erythropolis* NI86/21 during exposure to the herbicide thiocarbamate, where its gene product was most likely responsible for transforming aliphatic aldehydes to the corresponding carboxylic acids (Nagy *et al.*, 1995). The derived amino acid sequence

of Q15 ThcA contained glutamic acid-catalytic and cysteine-catalytic consensus sequences and the glycine NAD^+ coenzyme binding motif characteristic of aldehyde dehydrogenase. *Rhodococcus* sp. strain Q15 possessed the chromosomal DNA and a large plasmid of approximately 90 kbp which enhanced mineralization of some alkanes and growth on diesel oil at both 5°C and 25°C.

Bergeron and colleagues (1998) examined the molecular basis and properties of two chloroacetaldehyde (CAA) dehydrogenase encoding genes from *Xanthobacter autotrophicus* GJ10. The *ald*A and *ald*B, encoded on the pXAU1 plasmid and the chromosome of GJ10, respectively. The predicted amino acid (aa) sequences of the two proteins (505 aa in AldA and 506 aa in AldB) are 84% identical. Both AldA and AldB proteins of GJ10 share the highest degree of sequence identity with an acetaldehyde dehydrogenase encoded by *aco*D of *Alcaligenes eutrophus* (77.3-78% identity). The enzymology of the two genes was verified by expression them in the *E. coli* under the control of the T7 polymerase/promoter and the *lac* promoter systems. While both catabolized CAA, the enzymatic activity of *aldA* was higher than that of *aldB*.

Not all Aldh genes that have been studied came from microorganisms. Sebela and colleagues (2000) have recently isolated the enzyme aminoaldehyde dehydrogenase (AMADH) from etiolated pea seedlings. After an initial purification step involving three low pressure chromatographic steps, the partially purified enzyme was further subjected to chromatography on a Mono Q column and to affinity-interaction chromatography on 5'-AMP Sepharose. The purity of the final enzyme preparation was checked by SDS-PAGE and chromatofocusing. Studies of the enzyme revealed that pea AMADH exists as a tetramer of 230 kDa in the native state. Each subunit, in

turn was estimated to have a mass of 57 kDa. The enzyme was found to be an acidic protein with a p*I* 5.4. AMADH catalysed the oxidation of the monoaldehyde to N-(3-aminopropyl)-1,4-butanediamine-N'-propionic acid. An aldehyde dehydrogenase (NAD-dependent)in extracts from *Phaseolus aureus* catalyses the oxidation of indole-3-acetaldehyde to indole-3-acetic acid (Awal *et al.*, 1997). AMADH showed a broad substrate specificity utilizing various amino aldehyde (C_3-C_6) as substrates.

Although AMADH did not oxidize betaine aldehyde at all, the N-terminal amino acid sequence of the enzyme showed a high degree of homology with those of plant betaine aldehyde dehydrogenases of spinach (Weretilnyk and Hanson, 1989), sugar beet (McCue and Hanson, 1992) and amaranth (Legaria *et al.*, 1998).

1.7 Application of aldehyde dehydrogenase

Several research groups have isolated novel microorganisms from soil, wastewater, and culture collections that have proven capable of catabolizing some of the more exotic compounds introduced into the environment by today's agricultural practices. The newly isolated aerobic bacterium V2, for example, can grow on vinyl acetate using an enzymatic pathway that involves vinyl acetate esterase, aldehyde dehydrogenase and alcohol dehydrogenase (Neider *et al.*, 1990). Vinyl acetate was degraded to acetate as follows:

$$\text{vinyl acetate} + \text{NAD(P)}^+ \rightarrow 2\ \text{acetate} + \text{NAD(P)H} + \text{H}^+$$

The acetate was then converted to acetyl coenzyme A and oxidized through the tricarboxylic acid cycle and the glyoxylate by-pass.

In a similar way, *Pseudomonas fluorescens* transforms environmentally persistent compounds including volatile chlorinated aliphatic hydrocarbons into non-toxic, non-

chlorinated component (Vandenbergh and Kunka, 1988). A *P. fluorescens* PFL12 was isolated from soil and water that was contaminated with various chloroaliphatic hydrocarbons. The isolated microbe was able to metabolize 1,2-dichloroethane, 1,1,2-trichloroethane, 1,2-dichloropropane, 2,2-dichloropropane, and trichloroethylene.

The psychrotroph *Rhodococcus* sp. strain Q15 was examined for its ability to degrade individual n-alkanes and diesel fuel at low temperatures. Its alkane catabolic pathway was investigated by biochemical and genetic techniques. Q15 utilized a broad range of aliphatics (C_{10} to C_{21} alkanes, branched alkanes, and a substituted cyclohexane) present in diesel fuel at 5°C. (Whyte *et al.*, 1998)

A bacterium that is able to utilize a number of halogenated short-chain hydrocarbons and halogenated carboxylic acids as a sole carbon source for growth was identified as a strain of *Xanthobacter autotrophicus* GJ10. The organism constitutively produces two different dehalogenases. One enzyme is specific for halogenate alkanes, whereas the other, which is more heat stable and has a higher pH optimum, is specific for halogenated carboxylic acids. Haloalkanes were hydrolyzed in cell extracts to produce alcohols and halide ions, suggesting a pathway for the metabolism of 1,2-dichloroethane (Janssen *et al.*, 1985). Both dehalogenases show a broad substrate specificity, allowing the degradation of bromine- and chlorine-substituted organic compounds. It has been suggested that hydrolytic dehalogenase may be involved in the microbial metabolism of short-chain halogenated hydrocarbons in microorganisms living in contaminated environments. (Janssen *et al.*, 1985)

Cultures of the newly isolated bacterial strains AD20, AD25 and AD27, identified as strains of *Ancylobacter aquaticus*, were capable of growth on 1,2-

dichloroethane (DCE) as the sole carbon and energy source. These strains, as well as two other new DCE utilizers, were facultative methylotrophs and were also able to grow on 2-chloroethanol, chloroacetate, and 2-chloropropionate. In all strains tested, DCE was degraded by initial hydrolytic dehydrogenation to 2-chloroethanol, followed by oxidation by a phenazine methosulfate-dependent alcohol dehydrogenase and an NAD-dependent chloroacetaldehyde dehydrogenase. The resulting chloroacetic acid was converted to glycolate by chloroacetate dehalogenase (van den Wijngaard *et al.*, 1992). In the following year, van den Wijngaard *et al.* (1993) reported the biodegradation of several chloroethers and the growth of two different *Ancylobacter aquaticus* strains (AD25 and AD27) on 2-CVE (2-chloroethylvinylether) under aerobic conditions. They found that the organisms synthesize enzymes for the degradation of 2-CVE and also can grow on its spontaneously formed degradation products. Both cultures were also able to grow on 1,2-dichloroethane. The cells contained a haloalkane dehalogenase that dehalogenated 2-CVE, 2-chloroethylmethylether, 2-bromoethylethylether, and epichlorohydrin. Experiments with cell extracts indicated that an alcohol dehydrogenase and an aldehyde dehydrogenase were also involved in the degradation of 2-CVE. This suggests that 2-CVE is metabolized via 2-hydroxyethylvinylether and vinyloxyacetaldehyde to vinyloxyacetic acid. They proposed that *A. aquaticus* strains may be important for the detoxification and degradation of halogenated aliphatic compounds in the environment.

The degradation of 1,2-dichloroethane and 2-chloroethanol by *Xanthobacter autotrophicus* GJ10 proceeds via chloroacetaldehyde that encode plasmid and chromosomal DNA, a reactive and potentially toxic intermediate. The organism

produced at least three different aldehyde dehydrogenases, of which one is plasmid encoded. Two mutants of strain GJ10 could no longer grow on 2-chloroethanol and were found to lack the NAD-dependent aldehyde dehydrogenase that is the predominant protein in wild-type cells growing on 2-chloroethanol. Mutant selected on the basis of their resistance to 1,2-dibromoethane, proved to have lost the plasmid encoding the essential haloalkane dehalogenase activity. In both transconjugants, the aldehyde dehydrogenase that was absent in the mutants was overexpressed. The substrate range was rather broad, with the highest activity measured for acetaldehyde (van der Ploeg *et al.*, 1994).

Objective

1. To isolate and identify halophilic bacteria from shrimp farm's sediment and wastewater.
2. To study the enzyme activity of aldehyde dehydrogenase from the isolated strains.
3. To clone the chromosomal aldehyde dehydrogenase gene from isolated strains.
4. To sequence and analyze the aldehyde dehydrogenase gene.
5. To express the aldehyde dehydrogenase protein in *Escherichia coli*.
6. To express the aldehyde dehydrogenase protein in *Oryza sativa*.

2. Materials and Methods

Materials

Sample

Samples from sediment and wastewater were collected from a prawn pond in the Songkhla region of Thailand. Each of the samples was kept at room temperature and immediately transferred to the laboratory for isolation. Serial dilutions were then made using 0.85% sodium chloride solution and plated out for colony isolation on nutrient medium containing 5% sodium chloride.

Bacteria strain

E. coli LE392 [F^-, *hsd*R514 (r_k^-,m_k^-), *sup*E44, *sup*F58, *lac*Y1 or Δ(*lac*IZY), *gal*K2, *gal*T22,*met*B1, *trp*R55, λ^-] is a permissive host strain that can be utilized for most cloning experiments.

E. coli XL1-blue [F', ::Tn10, *proAB*$^+$, *lacI*^{q}, Δ(*lacZ*)M15, *rec*A1, *end*A1, *gyr*A96,(Nal^r), *thi*, *hsd*R17(r_k^-, m_k^+), *gln*V44, *rel*A1, *lac*] is used as a host for plasmid propagation.

E. coli M15 (Nal^S, Str^S, Rif^S, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^+$, Uvr^+, Lon^+) is useful for high level expression and easy to handle.

Plasmid vectors

Vectors and recombinant plasmids that were used in this study are described in Table 9 and Figure 2-5.

Table 9 Vectors and recombinant plasmids used in this study

Plasmids (MW.)	Description	Marker in *E. coli*	Marker in rice	Source
pGEM®-T (3.00 kb)	Recovery of PCR products after ligation. It was used for sequencing analysis.	*bla*	-	Promega
pGAS1101 (3.43 kb)	pGEM®-T containing a cloned copy of the PCR fragment of the *aldh* gene.	*bla*	-	This study
Lambda GEM®-11 (43 kb)	Bacteriophage-based cloning vector that will accept DNA fragments ranging from 9 kb to 23 kb.	-	-	Promega
LambdaA21 (39 kb)	Lambda GEM®-11 containing a *Bam*HI fragment of AS11 genomic DNA that encodes the *aldh* gene cluster.	-	-	This study
pSPORT1 (4.11 kb)	High copy number plasmid. It was used for sequencing analysis.	*bla*	-	Gibco BRL
pPP75 (11.61 kb)	A 7.5 kbp *Pst*I fragment containing the *aldh* cluster gene from LambdaA21 was subcloned into the same restriction site of pSPORT1.	*bla*	-	This study
2'-CAMBIA (9.5 kb)	Suitable for tobacco and rice transformation. It can transform to *E. coli* and *Agrobacterium*.	*npt*	*hyg*	Johnsen, S. (unpublished results)
2'-CALDH (11.3 kb)	A 1.8 kbp *Pvu*II-*Sma*I fragment containing the *aldh* gene from pPP75 was subcloned into the *Sma*I sites of 2'-CAMBIA.	*npt*	*hyg*	This study
pQE40 (4.0 kb)	*E. coli* expression vector containing T5 promoter and 6xHis tag	*bla*	-	QIAGEN
pQA802 (5.7 kb)	*Kpn*I-*Hin*dIII fragment containing *aldh* gene from pPP75 was subcloned into the same restriction sites of pQE40	*bla*	-	This study

bla = β-lactamase gene from *E. coli* (ampicillin resistant gene).

npt = neomycin phosphotransferase gene (kanamycin resistant gene).

hyg = hygromycin resistant gene for tobacco or rice transformation.

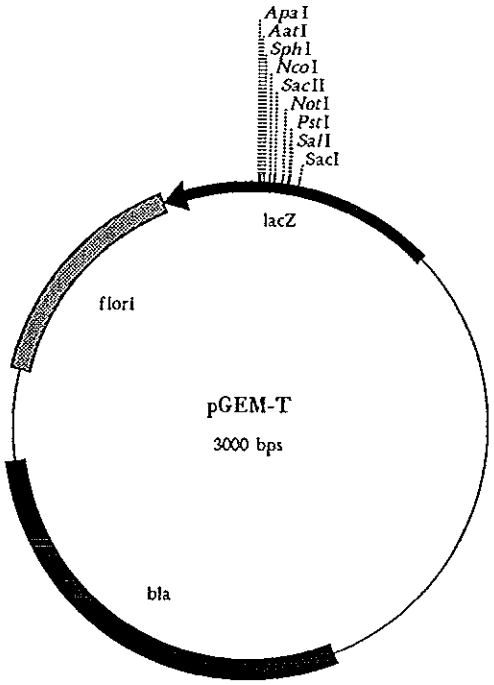


Figure 2 Physical map of plasmid pGEM-T

The figure illustrates plasmid pGEM-T containing lacZ gene, multiple cloning sites, origin of replication of *E. coli*, ampicillin resistant marker gene and 3'-T overhangs for ligation of PCR products.

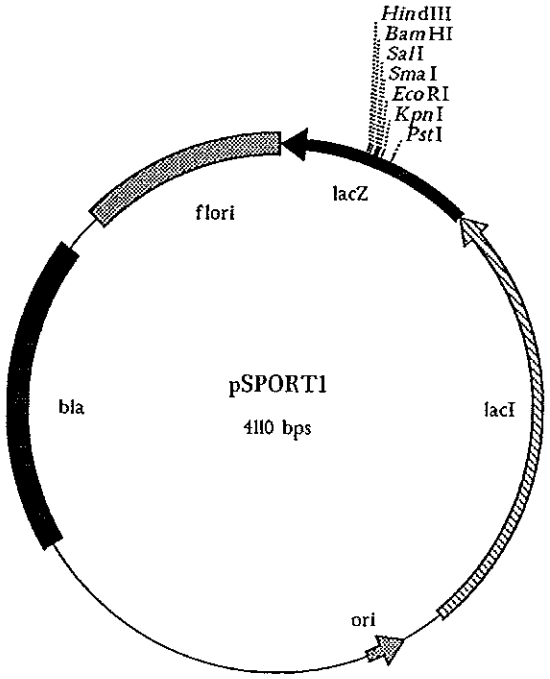


Figure 3 Physical map of plasmid pSPORT1

The figure illustrates plasmid pSPORT1 containing lacZ gene, multiple cloning sites, origin of replication of *E. coli* and ampicillin resistant marker gene.

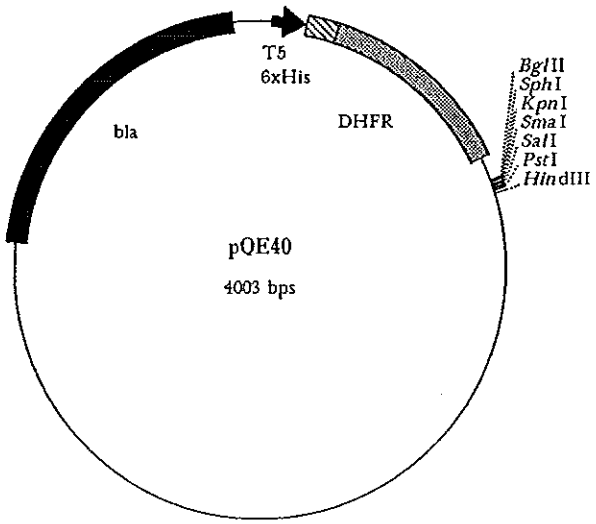


Figure 4 Physical map of plasmid pQE40

The figure illustrates plasmid pQE40 containing 6x His tag, DHFR, lacZ gene, multiple cloning sites, origin of replication of *E. coli* and ampicillin resistant marker gene.

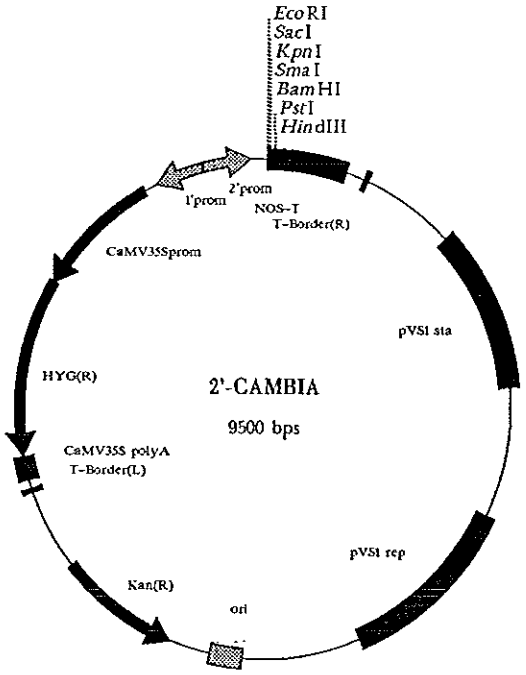


Figure 5 Physical map of plasmid 2'-CAMBIA

The figure illustrates plasmid 2'-CAMBIA containing hygromycin resistance for plant selection and kanamycin resistance for bacterial selection, multiple cloning sites, origin of replication of *E. coli*, NOS terminator and the mannopine synthase promoter.

Primers for PCR amplification

The nucleotide primers were synthesized by Gibco-BRL, USA and are shown in Table 10. The first pair of PCR primers was used for amplification of the 16S rRNA from the AS11 genomic DNA. The second set of PCR primers was used for sequencing of the *aldh* cluster gene. The third set of PCR primers was used for screening of the *aldh* gene from *Halobacteria*. The last set of PCR primers was used for amplification of the *aldh* fragment and contained *Kpn*I and *Hin*dIII sites in order to facilitate subcloning in an expression vector.

Table 10 Primers and sequence of primers for PCR amplification

Set	Name of primer	Size (bases)	Sequence
1	16S rRNA Forward	18	5'-CGGATTAGCTAGTTGGTG-3'
1	16S rRNA Reverse	18	5'-GCGATTACTAGCGATTCC-3'
2	SP6	19	5'-TATTTAGGTGACACTATAG-3'
2	T7	20	5'-TAATACGACTCACTATAGGG-3'
2	T3	20	5'-ATTAACCCTCACTAAAGGGA-3'
3	mix *aldh* Forward	16	5'-TCGAACTGGGYGGHAA-3'
3	mix *aldh* Reverse	17	5'- GGCCCGAAGATYTCYTC -3'
3	ADH11 *aldh* Forward	21	5'- GAACTGGGTGGCAAGTCGCCG -3'
3	ADH12 *aldh* Reverse	21	5'- CCCGAAGATCTCTTCCTGGAA -3'
4	ex *aldh* Forward*	24	5'-GAGAGGTACCATGATCTACGCCAA-3'
4	ex *aldh* Reverse*	24	5'- CCATAAGCTTTCTCCTCAACAACG -3'

* The underlined sequences of ex *aldh* Forward and ex *aldh* Reverse primers are recognition site of *Kpn*I and *Hin*dIII site (respectively).

Chemicals

All chemicals used were analytical grade. The major chemicals including aldehyde, NAD^+ and IPTG were purchased from Sigma, USA. The other chemicals and solvents (analytic grade) were purchased from Gibco BRL, USA; Fluka, Switzerland; Sigma, USA and Merck, Germany.

Enzymes

Enzymes and other modifying enzymes were purchased from Biolabs, USA; Boehringer Mannheim, Germany; Promega, USA; Gibco BRL, USA; QIAGEN, Germany and Stratagene, USA.

Instruments

Instruments	Model	Company
Analytical balance	Junior 2000C	Precisa
Autoclave	HA-300 MII	Hireyama
Hot plate stirrer	NuovaII	Sybron
Incubator	Im 550 R	K.S.L. Engineering Co., Ltd.
Incubator-shaker	3525-1C	Lab-Line Instrument, Inc.
Light microscope	CHS	Olympus Optical Co., Ltd.
Microcentrifuge	Centrifuge 5415C	Eppendorf
Refrigerated centrifuge	RC-5B plus	Sorvall
UV-spectrophotometer	Ultro-specIII	Phamarcia
Ultrasonicator		MSE

Methods

2.1 Isolation of halophilic bacteria from prawn pond

One gram of prawn pond sediment was suspended in 9 ml of sterile 0.85% sodium chloride solution. Dilutions of 1:10 and 1:100 were prepared and 0.1 ml of each dilution was spread onto the surface of nutrient agar media containing 5 and 10% sodium chloride. After incubation at 37°C overnight, several colonies were selected and streaked onto new agar plates. The procedure was repeated until pure cultures were obtained. The isolates were kept on nutrient agar slants and incubated at 37°C overnight. The cultures were stored at 4°C until used. Stock of each individual bacteria were stored at –70°C. To prevent bursting of the cells during freezing, glycerol was added into the cultured cell to give a final concentration of 15% (w/v).

2.2 Selection of halophilic bacteria with aldehyde dehydrogenase activity

2.2.1 Preparation of cell extracts

A full loop of each isolated strain was inoculated into 50 ml of modified ATCC culture medium 1270 and nutrient medium supplemented with 5% sodium chloride. Cell cultivation was carried out overnight on a rotary shaker at 200 rpm at room temperature. At the end of the period of cultivation, bacterial growth was monitored by measuring absorbance at 600 nm. The bacterial cells were harvested by centrifugation at 5,000x g for 10 minutes. The pellet was washed twice with 50 mM phosphate buffer, pH 7.0, suspended in 1 ml of the same buffer, and then disrupted by sonication at 24 kHz for 25 minutes. The resulting crude bacterial extract was

centrifuged at 12,000x g for 20 minutes, to remove unbroken cells and cell debris. This supernatant was used for enzyme assays.

2.2.2 Enzyme assays

Enzyme activity was determined spectrophotometrically (model Ultrospec III, Pharmacia) at 30°C by monitoring the reduction of NAD^+ at 340 nm. The reaction mixture consisted of 50 mM HEPES-KOH pH 8.0, 5 mM DTT, 1 mM EDTA, 10 mM aldehyde, 1 mM NAD^+ and 100 µL of enzyme solution. A blank was set using the reaction mixture without NAD^+. The reaction was started by adding NAD^+ to the mixture. The enzyme activity was determined by incubation of the cellular extract with 10 mM of each substrate [propionalaldehyde, acetaldehyde, betaine aldehyde, and ethanol (Sigma)] at 30°C according to the method of von Tigerstrom and Razzell (1968). One unit is defined as the activity that catalyzes the formation of 1 µmol of product (NADH) per minute.

2.3 Protein determination

Protein was measured with the Folin phenol reagent described by Lowry *et al.* (1951). 0.5 ml of the cell extract (see section 2.2.1) was added to 2.5 ml alkaline copper and incubated at 25°C for 10 minutes. After that, 0.5 ml Folin phenol reagent was added and it was incubated for a further 30 minutes. Protein content was determined with a spectrophotometer at 750 nm. Bovine serum albumin (Sigma) was used to prepare a calibration curve.

2.4 Identification of microorganism

The morphological and biochemical characteristics from each isolate was determined using cultures grown in nutrient media incubated overnight at 30°C. Gram straining was performed for non-halobacteria by the Hucker protocol (Norris and Swain, 1971). The smear was stained with crystal violet for 15 seconds, fixed with iodine for 15 seconds and decolorized with 95% ethanol. The smear was then washed with distilled water and counter stained with safranin for 15 seconds. Gram staining for halophilic bacteria was done by using the method of Dussault (1995) with the slight modification that a colony of microorganisms was suspended in sterile 20% sodium chloride and spread on a clean slide. After being air dried, the slide was fixed and desalted simultaneously by immersing in 2% acetic acid for 5 minutes. Then it was stained by the Hucker procedure as described above.

The optimal salt range for an isolated strain was determined by measuring growth in YEB medium containing 0.5, 2.5, 5, 10, 15, 20, 22.5, 25% sodium chloride. Precultures were prepared by growing the bacteria in each of the above media at 30°C overnight while shaking at 200 rpm. 100 µl sample of these overnight cultures were inoculated into the same medium and sampled every two hours.

Taxonomic characterization of the isolated strain was conducted according to Bergey's Manual of Systematic Bacteriology (Brinley-Morgan *et al.*, 1984) and on the basis of substrate utilization (Biolog© GN microplate identification system, Biolog, Hayward, Calif.). For the phylogenetic analysis of the 16S rRNA sequences, total DNA was extracted from the isolated strains according to the method of Ausubel *et al.* (1987). DNA fragments encoding 16S rRNA were amplified from the extracted total DNA by PCR and sequenced according to the method described by Edwards *et*

al. (1989). Phylogenetic analysis of the 16S rRNA sequences was performed as described by Maidak *et al.* (1994).

2.5 DNA amplification by polymerase chain reaction (PCR)

2.5.1 Primer design

For effective amplification, the appropriate size of the primer was judged to be 20-30 bases. An additional sequence can be added to the 5' end of the primer to facilitate cloning. In this study, two different primers were designed for amplifying a 16S rRNA PCR product.

A pair of degenerate oligonucleotide primers was synthesized based on conserved amino acid sequences ($\underline{NH_2\text{-ELGGKS-COOH;}}$ 5'- TCGAACTGGGYGG HAA -3' and $\underline{NH_2\text{-EEIFGP-COOH;}}$ 5'- GGCCCGAAGATYTCYTC -3') common to *aldh* genes of several bacteria. Two primers were designed for amplifying the *aldh* gene. Both primers contained recognition sites for a restriction enzyme at the 5' end, i.e ex *aldh* forward contained a *Kpn*I site while ex *aldh* reverse contained a *Hin*dIII site. The ex *aldh* forward also contained a translational start codon (ATG) to create an open reading frame beginning of the *aldh* gene which is suitable for subcloning into the expression vector.

2.5.2 Amplification of *aldh* gene by PCR

The standard procedure for a PCR reaction was performed in a 50 µl mixture containing 200 µM dNTPs; 1X PCR buffer (1.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl pH 8.4); 500 nM each of the PCR primers; 20-50 ng of AS11 genomic DNA and 1 U Taq DNA polymerase (Promega, USA). The reaction was

performed in an automated DNA thermal cycler model *Touch-Down* (Hybaid, UK) according to conditions shown in Table 11.

Table 11 PCR parameters for *aldh* DNA amplification

Segment	Step	Temperature (°C)	Time	Number of cycles
1	Denaturing	95	5 minutes	1
2	Denaturing	92	90 seconds	35
	Annealing	60	90 seconds	
	Extension	72	2 minutes	
3	Extension	72	7 minutes	1
	Holding	25	∞	

2.5.3 Agarose gel electrophoresis

In this study, gel electrophoresis was used for determining the size of the DNA of insert. A 1.0% (w/v) agarose solution was prepared using 1X TAE buffer (see Appendix A), melted, and poured on a plastic tray. A plastic comb was then placed in the gel. After the agarose gel was completely set (30-45 minutes at room temperature), the comb was carefully removed and the gel was installed on the platform in the electrophoresis tank containing 1X TAE buffer. The DNA samples were mixed with 30% (v/v) gel loading buffer (see Appendix A) and slowly loaded into the slots of the submerged gel using an automatic micropipette. Electrophoresis was carried out at a constant 80-120 V for 20-120 minutes, or until the running dyes had migrated the necessary distance. Next, the gel was stained with 2.5 μg/ml of ethidium bromide (EtBr) solution for 5 minutes and destained with water for 15 minutes. After that the DNA pattern was observed using a UV light box (Gel Doc

model 1000, BIO-RAD, USA) and the photograph was taken and printed out on the paper.

2.5.4 Purification of DNA using QIAGEN kit

The PCR-amplified fragment was purified by agarose gel electrophoresis. The expected DNA fragment was excised from the agarose gel and transferred to a microcentrifuge tube. Three volumes of Buffer QX1 were added to 1 volume of the gel (100 mg of gel ~ 100 µl), then the mixture was incubated at 50°C for 10 minutes or until the gel slice had completely dissolved. After that, 10 µl of 3 M sodium acetate, pH 5.0 and 1 gel volume of isopropanol was added to the sample. The sample was applied to the QIAquick column and allowed to stand at room temperature for 1 minute. The flow-through solution was discarded. Then 0.5 ml of Buffer QX1 was added and the mixture centrifuged for 1 minute into 1.5 ml collection tubes. The column was washed by adding 0.75 ml of Buffer PE to the column, left at room temperature for 5 minutes, and then centrifuged again at 12,000x g for 1 minute. The flow-through was discarded and the QIAquick column was centrifuged for 1 additional minute at 12,000x g. After that, 20 µl of 10 mM Tris-HCl, pH 8.5 was added into this column. The column was then placed in a new microcentrifuge tube, left to stand at room temperature for 10 minutes and centrifuged for 1 minute at the maximum speed to elute DNA. Finally, the concentration of eluted DNA was determined by running a small portion of it on an agarose gel.

2.5.5 DNA ligation

The purified DNA fragment of PCR product was ligated by T4 DNA ligase into plasmid pGEM-T (Promega). The ligation mixture was performed in a final volume of 20 µl. The reaction mixture contained the plasmid vector and *aldh*

fragment with a molar ratio of 1:3, 1 unit of T4 DNA ligase and 1X ligase reaction buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT). The reaction mixture was incubated at 14°C for 16-24 h.

2.5.6 Preparation of *E. coli* XL1-blue competent cells

Cells of *E. coli*, strain XL1-blue (Stratagene) were streaked onto a LB agar plate containing tetracyclin (12.5 µg/ml). The plate was incubated at 37°C overnight. A single colony of *E. coli* was picked from this plate into 10 ml of 2YT broth solution in a 50 ml flask and shaken overnight at 37°C. 250 µl of this culture was then added to 25 ml of Tf broth and incubated at 37°C until the OD_{600} reached 0.3-0.5. The cell pellet was harvested by centrifugation at 3,500x g for 6 minutes at 4°C and washed with 20 ml of ice-cold TfB_1. The cell suspension was incubated on ice for 5 minutes and centrifuged at 3,500x g for 6 minutes at 4°C. The pellets were resuspended in 4 ml of ice-cold TfB_2, then incubated on ice for at least 15 minutes to establish competency. The cell suspension was aliquoted in a volume of 100 µl per tube and kept frozen at -70°C.

2.5.7 Transformation into *E. coli* XL1-blue competent cells

A volume of 100 µl of *E. coli* competent cells was mixed gently with 0.1-1.0 µg of plasmid DNA. The mixture was left on ice for 30 minutes to give higher transformation frequency, next placed at 42°C for 90 seconds, and finally put on ice for an additional 5 minutes. The transformed cells were mixed with 500 µl of LB low salt broth and incubated at 37°C for 1 hour with constant shaking. Finally, 200 µl of transformed culture was spread onto LB low salt agar plates containing 50 µg/ml ampicillin and incubated at 37°C for 16 hours.

2.5.8 Plasmid DNA extraction from *E. coli* (Birnbolim and Doly, 1979)

A single bacterial colony was inoculated into 5.0 ml of LB medium containing 100 µl/ml ampicillin in a loosely capped 15 ml tube and incubated overnight at 37°C with vigorous shaking. The cell culture was collected by centrifugation at 12,000x g for 30 seconds at room temperature in a microcentrifuge tube. The supernatant was discarded and the bacterial pellet was resuspended in 80 µl lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0) containing 8 µl of a 10 mg/ml lysozyme solution. The liquid was mixed and incubated at 37°C for 10 minutes. After that, 200 µl of freshly prepared solution II (0.2 N NaOH and 1% (w/v) SDS) was added, gently mixed by hand, and placed on ice for 5 minutes. 180 µl of 3 M KOAc pH 4.8 was added to neutralize the base, then the tube was placed on ice for 15 minutes, and centrifuged at 12,000x g for 15 minutes at room temperature. The supernatant was transferred to a fresh tube and mixed with an equal volume of phenol/chloroform-isoamyl alcohol (25:24:1). The bacterial lysate was centrifuged at 12,000x g for 15 minutes at room temperature, and the clear aqueous phase was transferred to a clean tube. An equal volume of isopropanol was added into this tube and the mixture was incubated at -70°C for 10 minutes. After centrifugation at 12,000x g for 15 minutes at room temperature, the supernatant was discarded. The pellet was rinsed with 1,000 µl of 70% (v/v) ethanol and centrifuged at 12,000x g for 10 minutes at 4°C. The supernatant was discarded. Finally, the tubes were placed in an inverted position on a paper towel to allow all fluid to drain off. Plasmid DNA was resuspended in 30 µl of 10 mM Tris-HCl, pH 8.0 containing DNase free pancreatic RNase (2 mg/ml) and stored at -20°C.

2.5.9 DNA Sequencing

The inserts present in the clones obtained in this work were sequenced using an automated DNA (ABI PRISM^{TM}) sequencer (model 377, Perkin-Elmer Applied Biosystems, USA) based on PCR amplification of DNA in the presence of the fluorescent-labeled terminator (Smith *et al.*, 1986; Prober *et al.*, 1987). This protocol has been optimized for an automated DNA Thermal Cycler (Perkin-Elmer) model 2400 and completed 25 cycles in 3 hours. Each reaction was carried out in a labeled 0.2 ml microcentrifuge tube containing 8 µl of Terminator Premix, 0.5 µg of double-stranded DNA, 3.2 pmol of Primer and double-distilled water to bring the final volume to 20 µl. The tube was placed in the thermal cycler preheated to 96°C. After that the thermal cycler proceeded as follows (Table 12).

Table 12 PCR parameters for sequencing

Segment	Step	Temperature (°C)	Time	Number of cycles
1	Denaturing	96	5 minutes	1
2	Denaturing Annealing Extension	96 50 60	10 seconds 5 seconds 4 minutes	25
3	Holding	4	∞	1

The extension products were purified by ethanol precipitation. The entire contents of the reaction tube was transferred to the 1.5 ml microcentrifuge tube containing 2.0 µl of 3 M sodium acetate, pH 4.6 and 50 µl of 95% ethanol, vortexed and placed on ice for 10 minutes. The mixture was centrifuged at maximum speed for 15-30 minutes. The ethanol solution was removed as completely as possible, and the

pellet was washed by adding 250 µl of 70% isopropanol. The pellet was then dried in a vacuum centrifuge and resuspended in 4 µl of loading dye (see Appendix B). The samples were then loaded on a 6% polyacrylamide gel (see Appendix B).

2.5.10 Analysis of nucleotide sequencing

All inserts were sequenced on both strands. Computer analysis was performed using the DNASIS program (version 2.0, Hitachi, USA). The deduced amino acid sequences of the open reading frames were compared with GenBank databases using the BLAST program.

2.6 Lambda genomic cloning of the gene

2.6.1 Preparation of genomic DNA from bacteria

One loop of bacterial from each of the strain of interest was inoculated into 5 ml liquid culture and cultivated on a rotary shaker (200 rpm) at room temperature overnight. The culture broth was centrifuged in a microcentrifuge for 2 minutes and the supernatant was discarded. The pellet was resuspended in 576 µl TE buffer by repeated pipetting, and then mixed with 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added to give a final concentration of 100 µg/ml proteinase K in 0.5% SDS, and incubated 1 hour at 37°C. After that, 100 µl of 5 M NaCl was added, mixed thoroughly, mixed with 80 µl of CTAB/NaCl solution (10% CTAB in 0.7 M NaCl), and incubated 10 minutes at 65°C. The bacterial lysate was added to an equal volume of chloroform/isoamyl alcohol [24:1 (v/v)], mixed thoroughly and spun 4 to 5 minutes in a microcentrifuge. The aqueous, viscous supernatant was removed to a fresh microcentrifuge tube, extracted with an equal volume of phenol/chloroform/

isoamyl acohol (25:24:1), and spun in a microcentrifuge for 5 minutes. The supernatant was transferred to a fresh tube, together with 0.6 volume isopropanol to precipitate the nucleic acids, and shaken back and forth until a stringy white DNA precipitate became clearly visible. After centrifugation at 12,000x g for 5 minutes at room temperature, the supernatant was discarded. The DNA was washed with 70% ethanol to remove residual CTAB and spun 5 minutes at room temperature to pellet it. The supernatant was removed carefully and the pellet was dried before being resuspended in 100 µl TE buffer.

2.6.2 Restriction endonuclease digestion

Restriction endonucleases are groups of enzymes that bind and cleave double-stranded DNA at specific sequences. A typical enzyme reaction contains 1-2 µg of DNA, 1 µl of restriction enzyme (1-10 units), 1X reaction buffer and sterile distilled water to give a total volume of 20 µl. The restriction enzymes used in this study including their restriction sequences and optimal temperatures are shown in Table 13. After digestion had been completed, the digested products are analyzed by agarose gel electrophoresis.

Table 13 Restriction endonucleases with their recognition sequences and optimal temperatures

Restriction enzyme	Recognition sequences	Optimal temperature (°C)
*Acc*I	GT↓MKAC	37
*Apa*I	GGGCC↓C	37
*Bam*HI	G↓GATTC	37
*Bgl*II	A↓GATCT	37
*Eco*RI	G↓AATTC	37
*Eco*RV	GAT↓ATC	37
*Hin*cII	GTY↓RAC	37
*Hin*dIII	A↓AGCTT	37
*Hpa*I	GTT↓AAC	37
*Kpn*I	GGTAC↓C	37
*Nco*I	C↓CATGG	37
*Not*I	GC↓GGCCGC	37
*Pst*I	CTGCA↓G	37
*Pvu*II	CAG↓CTG	37
*Sac*I	GAGCT↓C	37
*Sal*I	G↓TCGAC	37
*Sau*3AI	↓GATC	37
*Sma*I	CCC↓GGG	25
*Xba*I	T↓CTAGA	37
*Xho*I	C↓TCGAG	37

2.6.3 Southern blotting and hybridization

The 10 µg of chromosomal AS11 DNA was digested using *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sau*3AI restriction enzymes and analyzed by Southern hybridization (Sambrook *et al.*, 1989) using a probe prepared from the PCR-amplified *aldh* gene (pGAS1101). First, an agarose gel was prepared to resolve the expected restriction fragments. The sample was mixed with gel loading buffer, loaded onto a 1.0% agarose gel along with DNA molecular weight markers (Lambda digested with *Hin*dII or *Pst*I) and electrophoresed for 1 hour. After electrophoresis, the gel was placed in the ethidium bromide staining solution for 30 minutes and photographed. The DNA was then blotted to Nylon STD, .4UM membrane (Pharmacia, USA, see Appendix B) and the DNA fixed by UV crosslinking for 90 seconds. The membrane was stored at 4°C until needed.

Before labeling, pGAS1101 was denatured by heating at 90°C for 3-5 minutes and then chilling the denatured DNA on ice for 5 minutes. The PCR-amplified *aldh* gene was then labeled with a Random Primer Fluorescein labeling Kit (DuPont®, NEN, USA) using 5 µl Random Primers and Reaction Buffer Mix, 5 µl of Fluorescein Nucleotide Mix and 1 µl Klenow fragment DNA polymerase. The mixture was incubated overnight at room temperature, and stopped by adding 5 µl of 100 mM EDTA (pH 8.0). After that the membrane was hybridized with Fluorescein-labeled pGAS1101 probes at 65°C overnight following the method of Sambrook and colleagues (1989). Any unhybridized probe was removed by washing the membrane in the salt/detergent mixture of 2X SSC, 1% SDS and the mixture of 0.2X SSC, 0.1% SDS for 15 minutes each at 65°C. The hybridized DNA on the membrane was

detected through use of CDP-Star™ Nucleic Acid Chemiluminescence Reagent (DuPont® NEN, USA, see Appendix B) with X-ray film (Fuji Rx, Japan).

2.6.4 Ligation and packaging for *Bam*HI arms of LambdaGEM-11 (Promega)

AS11 genomic DNA was digested with *Bam*HI and ligated to dephosphorylated *Bam*HI arms of LambdaGEM®-11 vector with T4 DNA ligase. The ligation mixture was performed in a final volume of 5 µl containing 0.5 µg of Lambda GEM®-11 *Bam*HI half-site arms; 0.5 µg of purified 10 kbp *Bam*HI-cut AS11 genomic DNA; 1X ligase buffer and 1 U of T4 DNA ligase. The reaction mixture was incubated for 3 hours at room temperature. The packaging extract was thawed on ice according to instructions of the manufacture, and then added to the entire ligation reaction, mixed by gently tapping the bottom of the tube and incubated at 22°C for 2 hours. The packaging mixture was mixed together with 450 µl of phage buffer and 25 µl of chloroform and stored at 4°C until needed. A single colony of *E. coli* was picked from a plate into 10 ml of LB broth solution supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ in a 50 ml flask. This was shaken overnight at 37°C. 250 µl of this culture was then added to 25 ml of LB broth supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ and incubated at 37°C until the OD_{600} reached 0.3-0.5. 100 µl of diluted phage was transfected to 100 µl of prepared *E. coli* LE392 bacteria as host. The phage was allowed to absorb for 30 minutes at 37°C without shaking. 3 ml molten TB top agar was added, vortexed gently and immediately poured onto 90 mm LB agar plates. The top agar was allowed to harden and incubated inverted at 37°C overnight. The plaques were transferred to nylon membranes, washed once with denaturing

solution (0.2 M NaOH, 1.5 M NaCl) for 7 minutes, twice with neutralizing solution (2X SSC, 0.4 M Tris-HCl, pH 7.6) for 3 minutes, and finally once with 2X SSC for 2 minutes. Subsequently, the membrane was fixed by UV crosslinking for 90 seconds. The library was screened by plaque hybridized (see Appendix B) using Fluorescein-labeled pGAS1101 probes leading to the identification of a positive clone.

2.6.5 Subcloning of the *aldh* gene for sequencing

The phage containing the 10 kbp insert was digested with *Acc*I, *Apa*I, *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*cII, *Hin*dIII, *Hpa*I, *Kpn*I, *Nco*I, *Not*I, *Pst*I, *Pvu*II, *Sac*I, *Sal*I, *Sma*I, *Xba*I and *Xho*I restriction enzymes and screened by Southern hybridization in order to determine the size of the DNA fragments encoding the *Aldh* gene. Based on these studies, a 7.5 kbp *Pst*I-restriction fragment from this phage was isolated from an agarose gel and cloned into pSPORT1 (Gibco BRL). Three *Bgl*II/*Pst*I fragments, as well as were *Xho*I, *Bgl*II/*Xho*I and *Eco*RV/*Bgl*II, were subsequently subcloned into pSPORT1 to obtain overlapping nucleotide sequences of the portion of the AS11 genomic in isolated phage.

2.7 Expression of the *aldh* gene in *E. coli*

2.7.1 Subcloning of the *aldh* gene into pQE40 vector

Both the pQE40 expression vector and the *aldh* gene were digested with *Kpn*I-*Hin*dIII and purified by using a QIAGEN™ kit. Then they were ligated in a 20 µl reaction mixture that contained the plasmid vector and *aldh* fragment with a molar ratio of 1:3, 1 unit of T4 DNA ligase and 1X ligase reaction buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT). The reaction mixture was incubated at 14°C for 16-24 h.

2.7.2 Growth of recombinant *E. coli*

A single colony of the recombinant *E. coli* M15 (QIAGEN) was inoculated in 10 ml of LB medium containing 100 μg/ml ampicillin and shaken overnight at 30°C at 250-300 rpm. Then the cell culture was transferred into either 25 ml (for small scale) or 100 ml (for large scale) fresh medium and cultured further until the cell suspension reached an of OD_{600} 0.1-0.2. In order to induce, cell cultures were supplemented with 1 mM IPTG for 3-6 hours. The cells were collected by centrifugation at maximum speed in a bench top microcentrifuge for 2-3 minutes at room temperature. Both cells and supernatant were stored at -80°C waiting further analysis.

2.7.3 Protein extraction from *E. coli*

For intracellular expression, cells were harvested at an OD_{600} of 2-4 by centrifugation at 12,000x g in a microcentrifuge tube for one minute. The cell pellet was resuspended in 100 μl of lysis buffer (see Appendix A). The mixture was vortexed gently and centrifuged at 12,000x g for 20-30 minutes to remove cellular debris. The supernatant was then transferred to a fresh tube. This supernatant was used for enzyme activity assays and SDS-PAGE analysis.

2.7.4 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular masses of denatured aldehyde dehydrogenases were determined by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Phosphorylase b (molecular mass 94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and trysin inhibitor (20 kDa) were used as molecular weight marker proteins. The gel solution was prepared as shown in Table 14. 10 μl of each sample

mixture was loaded onto a 12.5% SDS-Polyacrylamide gel and electrophoresis was carried out in the descending direction in a Tris-glycine buffer (25 mM Tris-HCl, pH 6.8, 192 mM glycine and 0.1% (w/v) SDS) using a constant 120 V for 90 minutes or until the tracking dye reached the edge of the gel. The protein patterns were visualized by Coomassie Brillant Blue staining.

After electrophoresis, the SDS-PA gel was stained with 0.2% (w/v) Coomassie Brillant Blue R-250 in 50% (v/v) methanol, 7% (v/v) acetic acid for 1-2 hours. Then the gel was destained in destaining buffer [25% (v/v) methanol, 12.5% (v/v) acetic acid in distilled water] overnight or until the background was clear. After that, the gel was rinsed with distilled water and air-dried between 2 sheets of cellophane paper.

Table 14 Preparation of SDS-Polyacrylamide Gel

Solution	Stacking Gel (4.5%)	Running gel (12.5%)
A [a] (ml)	1.13	6
1 M Tris-HCl, pH 8.7 (ml)	-	7.5
1 M Tris-HCl, pH 6.8 (ml)	1.25	-
H_2O (ml)	7.5	6.3
20% SDS [b] (µl)	50	100
10% APS [c] (µl)	50	100
TEMED [d] (µl)	8	15

[a]A = 40% Acrylamide (acrylamide : N,N'-methylenebisacrylamide, 37.5:1)

2.8 Expression of the *aldh* gene in rice

2.8.1 Sterilization and callus induction

Dehusked rice seeds were sterilized by first washing with 30% Domestos (see Appendix C) for 20 minutes, followed by one rinse with sterile water, 10 minutes in 70% ethanol and three rinses in sterilized water. The sterilized seeds were placed on callus inducing medium (CIM, see Appendix C) and grown at 30°C in the dark. Callus developed within one or two weeks from mature embryos and was subcultured every one to two weeks.

2.8.2 Tri-parental matting

Recombinant vector was mobilized from a donor strain, *E. coli* XL1-blue (2'-CALDH), a recipient strain (*Agrobacterium tumefaciens*, EHA105), and a helper strain, E. coli XL1-blue (pRK 2013). *A. tumefaciens*, EHA105 was inoculated into 25 ml YEB containing 100 mg/l rifampicin for 2 days at 30°C. *E. coli* harboring the plasmid pRK 2013 and *E. coli* containing 2'-CALDH was inoculated in LB supplemented with 25 mg/l kanamycin overnight at 37°C. All the cells pellet were collected by centrifugation at 4,000x g for 5 min. The pellet was suspended in 5 ml LB without antibiotic and centrifuged at 4,000x g for 5 minutes. The cells were suspended in 1 ml LB without antibiotic. The ratio of mix culture is 1: 1: 1 (EHA105: pRK2013: 2'-CALDH). 100 µl of the mixture was spoted on an agar LB plate without antibiotics overnight at 28-30°C. After at least 24 hours, the bacteria were streaked onto a YEB plate containing 50 mg/l kanamycin and 100 mg/l rifampicin. The inverted plates were left at 28-30°C for 2 days.

To check for the transferred plasmid, one colony of *Agrobacterium* transconjugant was cultured in YEB broth containing 50 mg/l kanamycin for 2 days.

The plasmid was extracted as described in Section 2.5.8 and transformed into competant *E. coli* cells. Then the plasmid was extracted from the transformed *E. coli*, run on a 1.0% agarose gel to verify that it was intact. The *Agrobacterium* harboring 2'-CALDH was ready for via transformation.

2.8.3 Transformation of rice callus (Toki, 1997)

Agrobacterium containing the plant expression vector with *aldh* gene (2'-CALDH) were grown overnight or two nights (for a slow growing strain) in YEB medium containing the appropriate antibiotic to select for the presence of the T-plasmid. A culture of EHA105/2'-CALDH in YEB supplemented with 100 mg/l rifampicin and 50 mg/l kanamycin at 30°C. The 10 ml of cultured bacteria was collected by centrifugation at approximately 1,500 to 2,000x g. The pellet was resuspended in 10 ml liquid CIM, pelleted once more by centrifugation. Bacteria were resuspended again in 2 ml liquid CIM. Transformation was carried out by filling a sterile petri plate with approximately 2 ml of saturated and washed *Agrobacterium.* Additional liquid CIM was added to bring the volume of the bacterial suspension to approximately 20 ml. The result was an approximately ten to twenty fold dilution of the bacterial cells to approximately 5 x 10^8 CFU/ml. Pieces of rice callus approximately one to two mm in diameter were placed in the bacterial suspension for approximately 10 minutes. The infected pieces of callus were then placed on CIM (at pH 5.2 instead of 5.8) containing 100 μM acetosyringone (AS) and 1% glucose. The infection was cocultivated for three days at 21°C in the dark. Following cocultivation rice callus was washed several times in liquid CIM containing 250 mg/l cefotaxime and 100 mg/l vancomycin before being placed on the solid CIM agar containing 250 mg/l cefotaxime and 100 mg/l vancomycin medium.

After four days on non-selective medium the callus was transferred to CIM agar containing 30 mg/l hygromycin, 250 mg/l cefotaxime and 100 mg/l vancomycin. Callus was transferred to CIM containing 50 mg/l hygromycin, 250 mg/l cefotaxime, and 100 mg/l vancomycin after one week.

2.8.4 Plant regeneration

Callus was left on pre-regeneration medium for one week before being transferred to regeneration medium (see Appendix C). The regeneration medium was the same as the pre-regeneration medium except that ABA and NAA were left out and the level of BAP was increased to 3 mg/l. Within one to two weeks on regeneration medium green spots on transgenic callus became apparent and shoots began to develop no later than three weeks after that. Callus and shoots were transferred to new medium every seven to ten days. Shoots were transferred to rooting medium after they were approximately 1 cm tall. Once roots developed and shoots were at least 3 to 4 cm tall. Plants were transferred to minimal medium without antibiotic.

2.8.5 Transferring to soil

Transformed shoots were transferred to soil after they were 4-6 cm tall by removing the entire plant (and root) from the sterile medium and placing it into a large pot (approximately six to eight inches in diameter) containing moist soil. A short stick approximately one inch taller than the plant was then inserted in that soil to prop up a sheet of plastic wrap. The plastic was then securely fasten to the pot to prevent loss of moisture, and the pot was placed in a shallow water bath in a lighted room or chamber at 30°C.

After two days, the plastic wrap was loosened to allow the plants to adjust to the ambient conditions. One day later, small holes were made in the plastic wrap to

allow for more air exchange between the pot and environment. After two additional days, the plastic wrap was completely removed and plants were moved to a green house growth room. Plants were watered at least every other day. Rice generally set flowers within two to three months and seeds were harvested and screened for hygromycin resistance or gene activity.

2.9 Nucleotide sequence accession number

The nucleotide sequence of the *aldh* and partial 16S rRNA gene has been submitted to the GenBank nucleotide sequence database under accession numbers AF284553 and AF301407 respectively.

3. Results and Discussion

3.1 Isolation of halophilic bacteria from sediment and wastewater

Shrimp production ponds produce large amounts of waste during each rearing cycle [185-199 t dry wt/ha or 139-150 m^3/ha (Funge-Smith and Briggs, 1994) 200-836 t dry wt/ha or 151-629 m^3/ha (Tunvilai *et al.,* 1993)]. Most of the pond sediments are composed of phosphorus, nitrogen, organic matter from uneaten feeds, dead organisms and metabolites, and inorganic substances leached from soil. These chemicals serve as nutrients to stimulate microorganism and algal growth. The metabolism of some compounds produces endogenous aldehydes that could be toxic to some organisms in high concentration.

Commonly in Thailand, much of this sediment is removed or allowed to oxidize. Unfortunately, when this is done in an illegal manner, it can sometimes lead to water pollution, salinization of soils and water, and can create a unique solid waste disposal problem. One way to correct this problem would be to seed the waters with microorganisms that would catabolize oragnic matter and eliminate undersirable waste compounds before they build up, but most of the well-known microorganisms that are responsible for breaking down wastes in fresh water or on dry land cannot survive in the high salt of these pond sediments. Instead, the predominant and most active organisms are extreme- and moderately- halophilic bacteria. Because of the unusual composition of those sediments, it is also likely that many of the bacteria that can thrive there have been required to develop ways to detoxify a broad range of chemicals that are not naturally found together or in such high amounts. Such

microorganisms might prove interesting for bioremediation of hypersaline wastewater resulting from the manufacture of pesticides pharmaceuticals, herbicides and petroleum by products.

In order to identify bacteria that are likely to have genes capable of breaking down the types of waste products produced in ponds devoted to shrimp culture, sediment with a high organic matter content was screened for the presence of halophilic bacteria. These sediments came from southern region (Songkhla) of Thailand. These ponds had been stocked for two years at a density of 50-60 shrimp m^{-2}. Measurements taken at the time of collection indicated that the sediment and wastewater temperatures were 33°C, and had a salinity level of 35 ppt. In general, more bacteria were isolated from sediment than from wastewater. This may be due to the fact that the feeding habits of the shrimp might remove many free bacteria from water while creating a solid substrate on the bottom of the pond to which other bacteria could attach.

A total of 36 halophilic bacteria were isolated on nutrient medium containing either 5 or 10% sodium chloride from samples taken from these prawn ponds using a standard spread plate technique as described in Chapter 2. Twenty-four of these were isolated from sediment, and twelve were obtained from wastewater. All 36 isolates were rounded, nonmucoid, opaque and cream colored colonies.

3.2 Selection of halophilic bacteria

All 36 bacterial isolates were inoculated onto nutrient medium supplemented with 0.5, 2.5, 5, 10, 15, 20, 22.5 and 25% sodium chloride in order to estimate whether they were dependent on high levels of salt for growth. Most isolates grew

well on medium containing between 2.5 and 20% sodium chloride and therefore could be classified as halophiles. However only five of the bacteria isolated were able to grow on 25% sodium chloride. Microorganisms growing optimally in media containing 5 to 15% salt are defined as moderately halophilic bacteria. They are inhabitants of saline environments. To cope with the high and often changing salinity of their environment, the halophilic bacteria, similar to all other microorganisms, need to balance the osmotic pressure of their cytoplasm with the osmotic pressure exerted by the external medium. At the cellular level, the most common type of osmotic adaptation involves the accumulation of compatible solutes such as amino acids, polyols, sugars, and quaternary amines in cytoplasm. Glycine betaine was recognized in the early 1980s as the most important organic osmotic solute in photosynthetic purple bacteria and halophilic cyanobacteria. When grown in complex growth media, most halophilic bacteria synthesize and accumulate glycine betaine or its precursor, choline, from the medium (Imhoff and Rodringuez-Valera, 1984).

The microbes collected from this high salinity area were screened for the presence of aldehyde dehydrogenase activity. In order to follow standard protocols for this enzyme assay (von Tigerstrom and Razzell, 1968), all 36 bacteria isolates were cultivated in both nutrient medium and modified ATCC medium 1270. No aldehyde dehydrogenase activity was detected in these bacteria grown in nutrient medium. However, twenty-two of thirty-six of the isolated halophilic bacteria showed aldehyde dehydrogenase activity on modified ATCC medium 1270 for *Halobacterium* (Table15).

The results revealed the following:

a) 3 of the isolated strains could not grow in high salt and thus only produced low aldehyde dehydrogenase activity.

b) 22 of the isolated strains did not produce aldehyde dehydrogenase activity in rich medium, but did in minimal medium (modified ATCC medium 1270).

c) Five of the isolated strains (isolates AS2, AS3, AS7, AS11 and AS14) grew and produced high levels (4.23-4.64 U/mg protein activity) of aldehyde dehydrogenase in minimal medium.

The strain AS11 was chosen for continued study because of its high growth rate even in 25% sodium chloride.

Table 15 Aldehyde dehydrogenase activity and growth of isolated strains

Strain	Concentration of sodium chloride (%) in medium					Aldehyde dehydrogenase activity (U/mg protein)
	5%	10%	15%	20%	25%	
AS1	3	3	3	2	1	1.23
AS2	3	3	3	2	1	4.64
AS3	3	3	3	2	1	4.44
AS4	3	3	3	2	1	3.81
AS5	3	3	3	2	1	3.30
AS6	3	3	3	2	1	1.22
AS7	3	3	3	2	1	4.37
AS8	3	2	1	0	0	0
AS9	3	3	3	1	0	0
AS10	3	3	3	2	1	2.59
AS11	3	3	3	2	2	4.25
AS12	3	3	3	2	2	3.12
AS13	3	2	1	0	0	0
AS14	3	3	3	2	1	4.23
AS15	3	3	3	2	1	1.05
AS16	3	3	3	2	1	1.02
AS17	3	3	3	2	2	2.92
AS18	3	3	3	2	2	2.21
AS19	3	3	3	2	1	2.56
AS20	3	3	3	2	1	3.81
AS21	3	3	2	1	1	0
AS22	3	3	3	2	2	3.15
AS23	3	2	1	0	0	0
AS24	3	3	2	2	1	0
AW1	3	3	3	2	1	0
AW2	3	3	3	2	0	0.46
AW3	3	3	3	2	0	0.17
AW4	3	3	2	1	1	0
AW5	3	1	1	1	0	0
AW6	3	3	1	1	1	0
AW7	3	3	2	1	1	0
AW8	3	3	3	1	0	0
AW9	3	3	3	2	1	2.35
AW10	3	3	2	1	0	1.79
AW11	3	2	0	0	0	0
AW12	3	2	0	0	0	0

Symbol: AS, aquatic sediment; AW, aquatic wastewater; 0, no growth; 1, slow growth; 2, average growth; 3, rapid growth

3.3 Identification of isolate strain AS11

I have begun to assess the ability of microorganisms isolated from saline environment to degrade some of the toxic by-products of prawn aquaculture. The first approach to this problem was to isolate some of the bacteria that live in this highly saline environment and characterize the breadth of their metabolic capabilities.

The isolate AS11 showed the carbohydrate utilization pattern, Gram reaction, and colony and cell morphology characteristic of previously published data for several *Halomonas* species (Ventosa *et al.*, 1998). The cells of strain AS11 were short gram negative, non-motile rods that were both oxidase and catalase-positive (Table 16). This strain was able to grow on arabinose, glucose, fructose, maltose, galactose, cellobiose, trehalose, sucrose and gluconate but not on mannitol and lactose as sole carbon sources in minimal media. AS11 produced creamy white circular colonies on nutrient media. This strain was studied for optimal salt concentration growth on YEB medium supplemented with 0.5-25% sodium chloride. The bacteria require at least 2.5% sodium chloride for growth in both minimal and rich medium. No growth was obtained in 0.5% sodium chloride when this was the sole salt, and growth was optimum at 5% (Figure 6). This result agreed with many earlier investigators who reported that *Halomonas* grew optimally in media containing at 2.0-8.0% sodium chloride at 30°C and but could grow in concentrations ranging from 2-32% (Vreeland and Martin, 1980; Ventosa *et al.*, 1998). These data together with those obtained by Biolog© GN microplate identification system (Table 17) indicated that AS11 belonged to the genus *Halomonas.*

Table 16 Morphological and biochemical characteristics of *Halomonas* and strain AS11

Feature	*Halomonas*	AS11
Gram stain	Negative	Negative
Morphology	Rod	Shot Rod
Motility	Non-Motile	Non-Motile
Pigmentation	White/Cream	Cream pigment
Oxidase	+	+
Catalase	+	+
Acid formation from		
Arabinose	-	-
Glucose	-	-
Lactose	-	-
Trehalose	-	-
Mannitol	-	-
β-galactosidase	-	-
Indole production	-	-
Nitrate reduction	+	+
Susceptible to:		
Geocillin 50 mg/l	d	-
Kanamycin 100mg/l	d	+
Neomycin 100mg/l	d	+
Streptomycin 100mg/l	-	+
Spectinomycin 50mg/l	d	+
Range of NaCl concentration (%) at which growth occurs	0.5-32.5	2.5-20.0
NaCl optimum (%) for optimum growth	3-8	5.0

Symbols: +, positive; -, negative; d, different in each strain

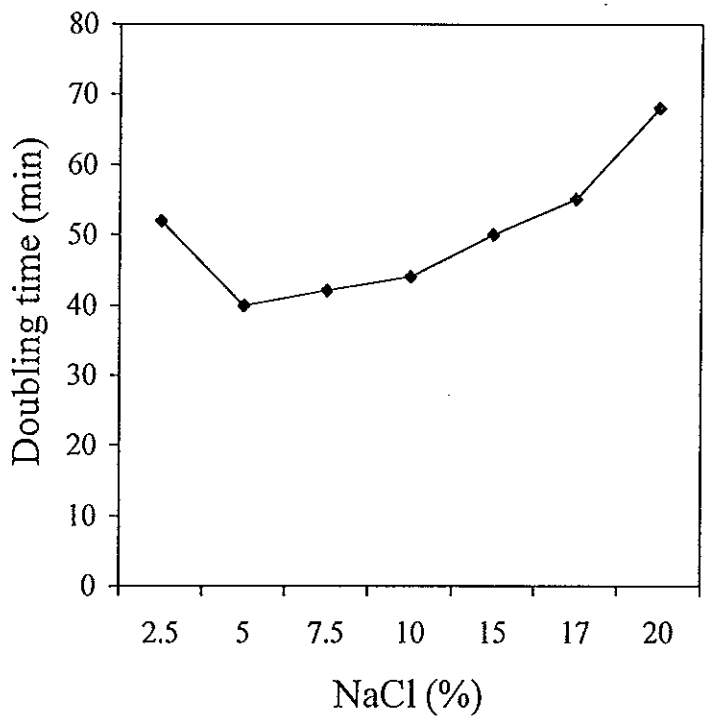


Figure 6 Effect of sodium chloride concentration on the doubling time of *Halomonas salina* AS11 The cells were grown in rich medium at 30°C. Doubling time were determined after inoculation of fresh medium with cells from a preculture grown on the same sodium chloride concentration. Each point represents the mean number from triplicate samples.

Table 17 The closest species of AS11 from GN microplate test

Closest species	Concentration of sodium chloride (%) in medium		
	0.9	5	10
1	*Xanthomonas oryzae* pv. Oryzae E	*Deleya cupida*	*Deleya marina*
2	*Brucella canis*	*Deleya pacifica*	*Pseudomonas sysingae* pv. phaseolicola
3	*Xanthomonas oryzae* pv. Oryzae B	*Pseudomonas sysingae* pv. phaseolicola	*Deleya aesta*
4	*Photobacterium phosphoreum*	*Halomonas elongata*	*Deleya cupida*
5	Kingella kingae	*Pseudomonas stutzeri*	*Sphingomonas paucimobilis* A

Note: Computer analysis was performed using Micrologᵀᴹ Release 3.5. The data compared with MicroLog GN database. The strain was cultured overnight in nutrient media with the indicated amount of salt.

The genus *Halomonas* was created to classify a group of moderately halophilic, gram negative, rod-shaped strains isolated from a saltern. These strains could tolerate salt concentrations between 20-32% (Vreeland *et al.*, 1980). Recent studies based on 16S rRNA sequence analysis have refined the assignments of the phylogenetic positions of most moderately halophilic bacteria. In particular, *Halomonas* species are currently recognized as members of the gamma subclass of the *Proteobacteria.*

Total DNA from strain AS11 was subjected to PCR amplification with the 16S rRNA forward and reverse primer to obtain a fragment from the 16S rRNA gene. The expected size of the PCR product obtained from the PCR reaction was 1,116 base pairs. The fragment was directly analyzed by using an automated sequencer. By means of PCR amplification, a nearly complete 16S rRNA sequence corresponding to *P. aeruginosa* 16S rRNA positions 207 through 1,320 was determined for AS11. The 16S rRNA sequence analysis of AS11 showed 99% identity to the rRNA sequence of *Halomonas salina* (ATCC 49509 accession no. AJ243447), 96% identity to the rRNA sequence of *Deleya salina* (EMBL accession no. X87217) which is a synonym for *H. salina* (Dobson *et al.*, 1993), and 89% identity to the rRNA sequence of *P. aeruginosa* (EMBL accession no. Z76651). A phylogenetic tree based on this information shows the relationships of strain AS11 to *H. salina* and several other species (Figure 7). On the basis of these data, the isolated strain is referred to as *H. salina* strain AS11. Studies based on 16S rRNA sequence analysis provided additional proof of the phylogenetic position of the strain AS11 to *H. salina.*

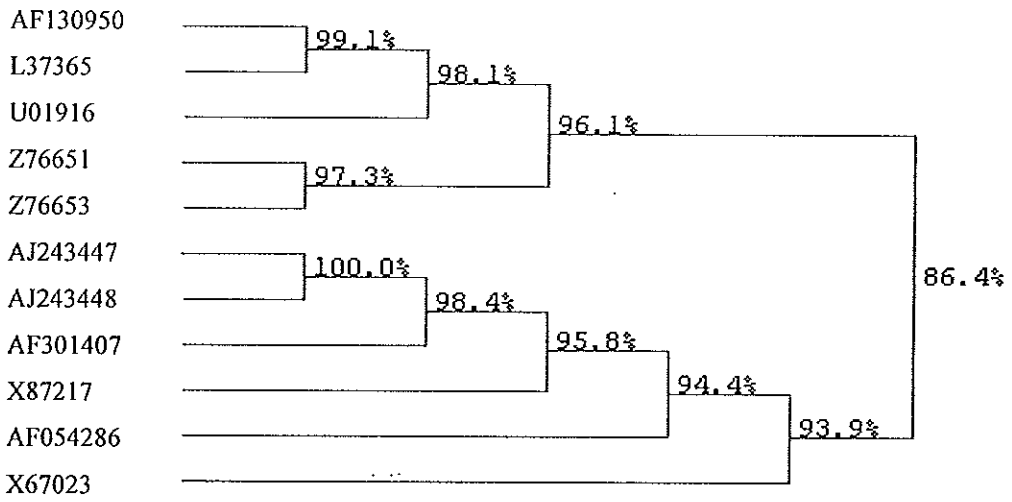


Figure 7 Phylogenetic tree showing the relationship of *Halomonas salina* to other species AF301407; 16S ribosomal RNA partial sequence strain AS11 AJ243447, AJ243448; *H. salina* AF054286; *H. campisalis* X67023; *H. elongata* X87217; *D. salina* AF130950; *P. syringae* L37365; *P. putida* U01916; *P. flavescens* Z76651; *P. aeruginosa* Z76653; *P. alcaligenes*

3.4 Growth of *Halomonas salina* strain AS11 and the production of enzymes

In addition to extreme tolerance to salt, AS11 also proved able to catabolize a number of simple alcohols. Although it grew best using glucose as a sole carbon source, it could also utilize isopropanol, ethanol, or polyethylene glycol. On the other hand, it could not utilize methanol as a sole carbon source.

No aldehyde dehydrogenase activity was detected in AS11 grown in rich medium to either early or late stationary phase. On the other hand, both aldehyde dehydrogenase and a second enzyme involved in detoxifying certain metabolites, alcohol dehydrogenase, were produced in a minimal medium (modified ATCC culture medium 1270) supplemented with different alcohols (Table 18). The aldehyde dehydrogenase enzyme was observed in the cells at the exponential phase and continued to increase until it reached its maximum when cells entered stationary phase. When this experiment was repeated with cells grown to late stationary phase, similar levels of activity were obtained.

Up to now, the capability to degrade toxic compounds has been mainly observed among members of *Pseudomonas* (Van Ginkel *et al.*, 1992; Vandenbergh and Kunka, 1988), *Ancylobactor* (van den Wijngaard *et al.*, 1992; van den Wijngaard *et al.*, 1993) and *Xanthobacter* (Janssen *et al.*, 1985; Tardif *et al.*, 1991; van den Ploeg *et al.*, 1994) groups, and has not been described from halophilic bacteria. The *H. salina* strain AS11 was found to produce aldehyde dehydrogenase and alcohol dehydrogenase on minimal media containing isopropanol or ethanol. Since the expression of aldehyde dehydrogenase was specifically induced only during growth on these substrates, this enzyme seems to be responsible for the oxidation of aldehyde to the corresponding carboxylic acid in both catabolic pathways.

Table 18 Specific activity of aldehyde dehydrogenase and alcohol dehydrogenase in crude extracts of AS11

Medium	OD 600 nm	Activity (U/mg protein)	
		Aldehyde dehydrogenase	Alcohol dehydrogenase
A	1.235	0	0
B	0.958	10.46	51.70
B+0.2% PEG	0.980	8.46	168.6
B+3% ethanol	0.548	10.85	179.3
B+0.2% glucose	1.214	5.98	126.89
B+5% isopropanol	0.931	15.18	141.1

Cells were grown on medium at 30°C.

A; Nutrient medium with 8% sodium chloride (see Appendix A)

B; Mineral salt medium (see Appendix A)

3.5 Amplification of aldehyde dehydrogenase gene

In order to screen for genes involved in the metabolism of xenobiotic compounds, two degenerate DNA primers were synthesized based on the conserved ELGGKS and EEIFGP peptide motifs of *aldh* sequences from *Vibrio cholerae* (P23240), *P. putida* (U24215), *P. aeruginosa* (AF068264) (Schobert and Gorisch, 1999), *Escherichia coli* (S47809), *Alteromonase* sp. (AB009654), and *Alcaligenase eutrophus* (P46368) (Priefert *et al.*, 1992). Within the *P. aeruginosa* AcDH sequence, these peptides are located at amino acids 262-268 and 401-406 respectively (Hidalgo *et al.*, 1991).

Genomic DNA from *H. salina* strain AS11 was subjected to PCR amplification as described in Chapter 2, Section 2.5.2 with the designed primers to obtain a fragment from the aldehyde dehydrogenase gene. The expected size of the product obtained from the PCR reaction was 435 base pairs. After purification using a QIAquick kit, the amplified fragment was inserted into the plasmid pGEM®-T, resulting in a construct named pGAS1101 (3.43 kbp). The construction of recombinant plasmids is shown in Figure 8. The ligation mixture was transformed into *E. coli* XL1-blue using calcium chloride (Chapter 2, Section 2.5.6). *E. coli* transformants were then screened on LB plates containing 100 μg/ml ampicillin. Twelve ampicillin-resistant transformants from each experiment were selected, and plasmid DNA was prepared from the selected colonies as described in Chapter 2, Section 2.5.8. The DNA patterns of recombinant clones were analyzed by using 1.0% (w/v) agarose gel electrophoresis and visualized under the UV light and compared with that of pGEM®-T. All of them were subsequently analyzed by digestion with *Pst*I and *Nco*I-*Pst*I. A total of twelve transformants containing the *aldh* amplified

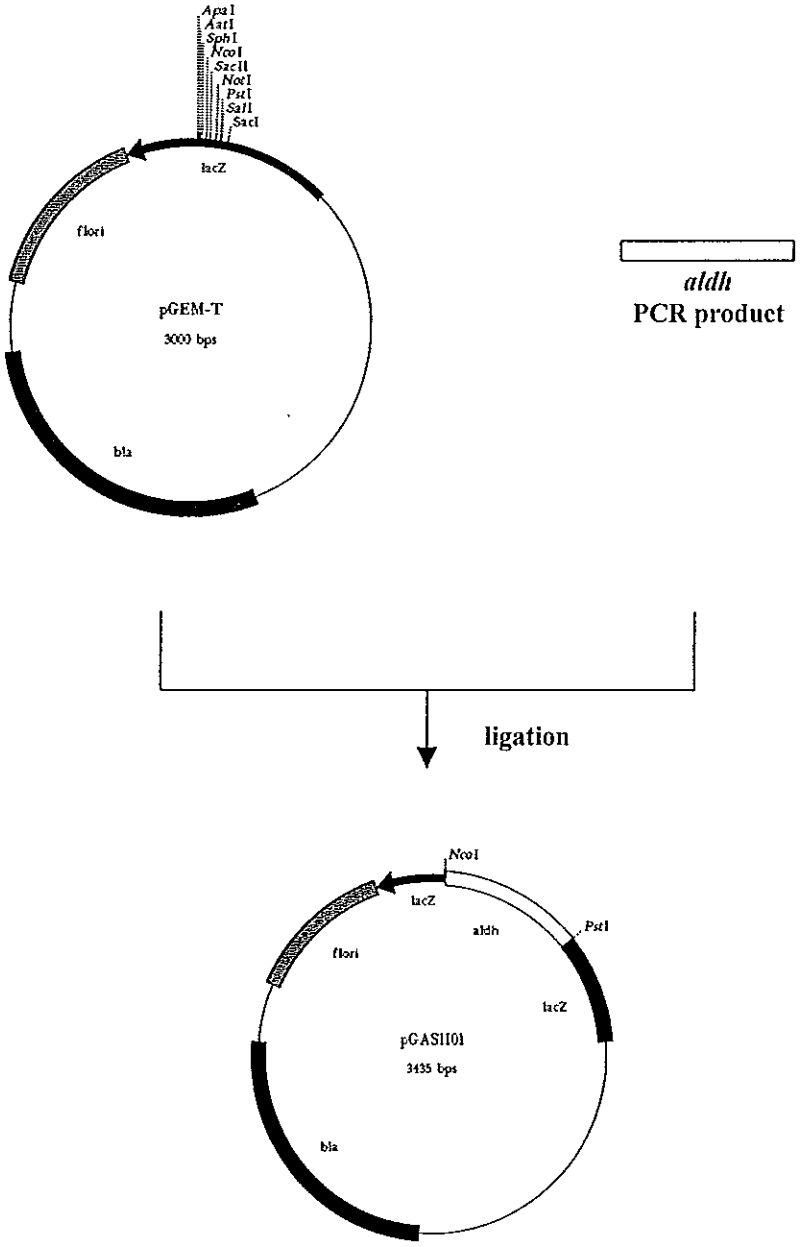


Figure 8 Construction of recombinant plasmid pGAS1101

The figure illustrates the construction of recombinant plasmids containing *aldh* gene PCR products. The 435 bp of amplified *aldh* gene by PCR was cloned into pGEM-T yielding a construct named pGAS1101.

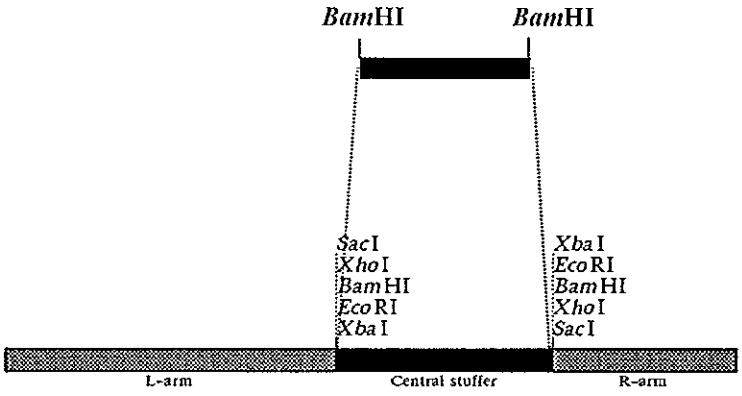
fragment was obtained. Plasmid DNA from transformants containing these *aldh* amplified fragments were extracted and sequenced on an automated sequencer (ABI PRISM^{TM} 377 DNA sequencer).

The amino acid sequence deduced from the nucleotide sequence of the cloned fragment was found to be 73.0% identical to that of the aldehyde dehydrogenase of *Alteromonas* sp. and the NAD^+ dependent acetaldehyde dehydrogenase of *Pseudomonas aeruginosa* from the NCBI database.

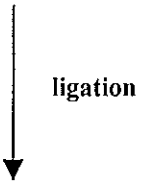
3.6 Cloning of the aldehyde dehydrogenase gene cluster from *Halomonas salina* strain AS11

In order to construct a genomic library enriched for this gene, Southern blot analysis of chromosomal DNA from AS11 that had been digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Sau*3AI restriction enzymes was carried out using the cloned PCR fragment as a probe. These analyses demonstrated that the homologous region of the AS11 genome could be isolated on a *Bam*HI fragment of 10 kbp. This region of a *Bam*HI digest of genomic DNA was then isolated from an agarose gel, purified using the QIAquick kit and packaged into the LambdaGEM®-11 bacteriophage. The LambdaGEM®-11 bacteriophage was then transfected into *E. coli* LE392. The library of 5.34 x 10^5 plaque-forming units was evaluated and screened. Ten positive plaques, each containing a 10 kbp fragment, were identified by plaque hybridization using pGAS1101 as a probe. One of these phages was named lambdaA21 (Figure 9) and chosen for further analysis. In order to identify the smallest region of the clone likely to encode the entire *aldh* gene, the insert was prepared by agarose gel electrophoresis from *Bam*HI digested phage DNA. This DNA fragment was then digested with *Acc*I,

*Apa*I, *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*cII, *Hin*dIII, *Hpa*I, *Kpn*I, *Nco*I, *Not*I, *Pst*I, *Pvu*II, *Sac*I, *Sal*I, *Sma*I, *Xba*I and *Xho*I restriction enzymes and the resulting fragments were separated by 1% agarose gel electrophoresis. The result of Southern blotting and hybridization against the pGAS1101 probe showed that a portion of the gene was located in a single 7.5 kbp *Pst*I restriction fragment. In order to sequence this fragment, the *Bam*HI fragment was digested with *Pst*I, subcloned into a plasmid-based vector called pSPORT1 (Gibco BRL, 4.11 kbp) and transformed into *E coli* XL1-blue. The pSPORT1 harboring the full length of *aldh* gene was designated pPP75. The construction of plasmid pPP75 is shown in Figure 10. In order to facilitate sequencing further, this fragment was cut into three smaller pieces using *Pst*I and *Bgl*II and each was subcloned into pSPORT1 to produce the plasmids, pPGP1, pPGP8 and pPB1. The pPBX1 was modified further by deleting a 600 bp *Xho*I fragment from pPB1. Sequence analysis of the entire fragment present in pPB1, together with partial sequences obtained from the 5' and 3' ends from PGP1 and PGP8 fragments, revealed three open reading frames (Figure 11) corresponding to an aldehyde dehydrogenase, an alcohol dehydrogenase and possibly a transcriptional regulatory protein. The regulatory and *aldh* genes lie upstream and downstream of *aldh*, respectively. Although neither the promoter nor the start of transcription have been identified, it is possible that the *aldh* reading frame and the *adh* reading frame immediately downstream of it, are cotranscribed as part of the same operon.



LambdaGEM-11 (43000 bps)



LambdaA21 (39000 bps)

Figure 9 Construction of recombinant LambdaA21

The figure illustrates the construction of lambdaA21 bacteriophage containing the 10 kbp *Bam*HI fragment of the chromosomal DNA from *H. salina* AS11.

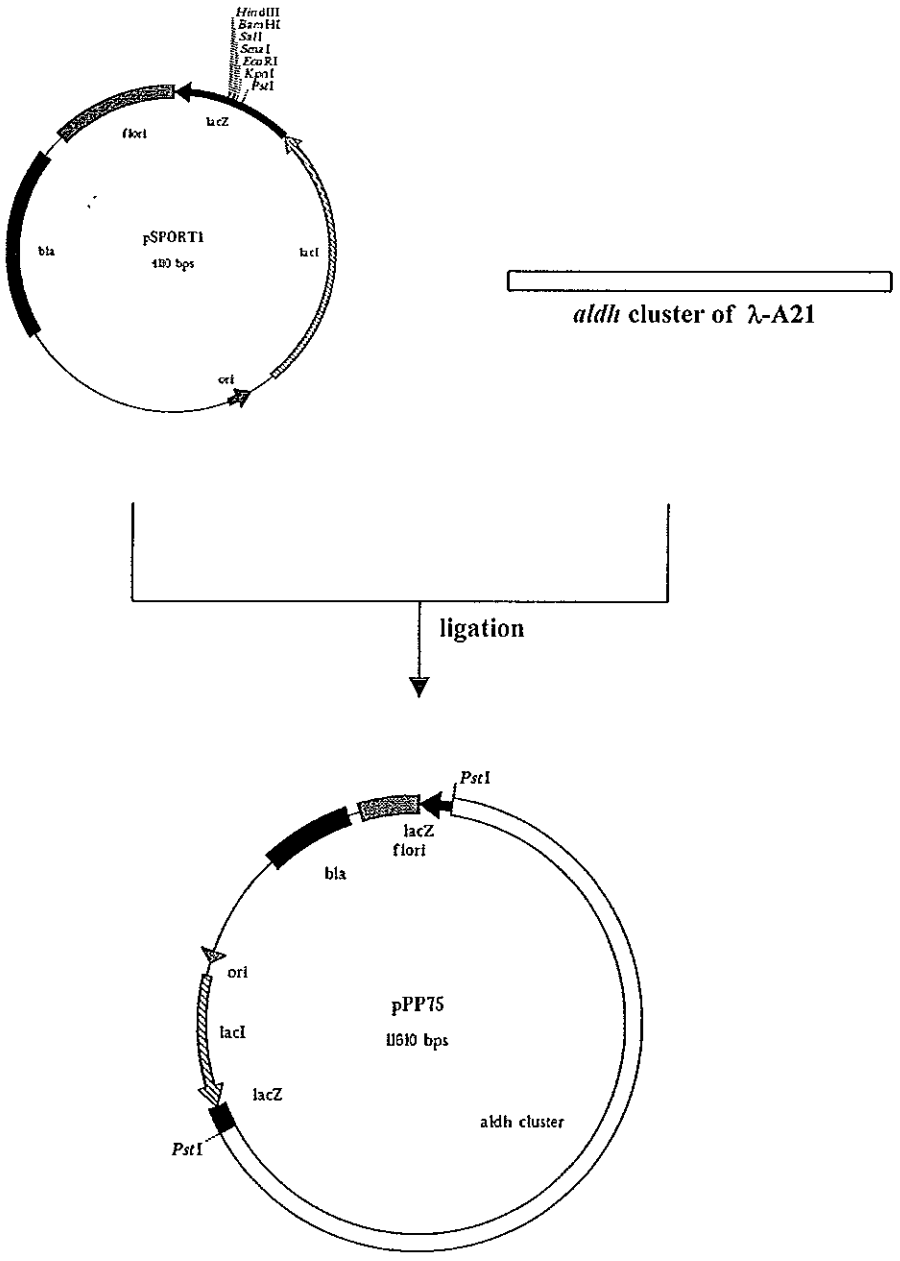


Figure 10 Construction of recombinant plasmid pPP75

Plasmid pPP75 was a hybrid plasmid consisting of pSPORT1 (4.1 kbp) and the *Pst*I fragment (7.5 kbp) containing the *aldh* cluster gene of *H. salina* strain AS11.

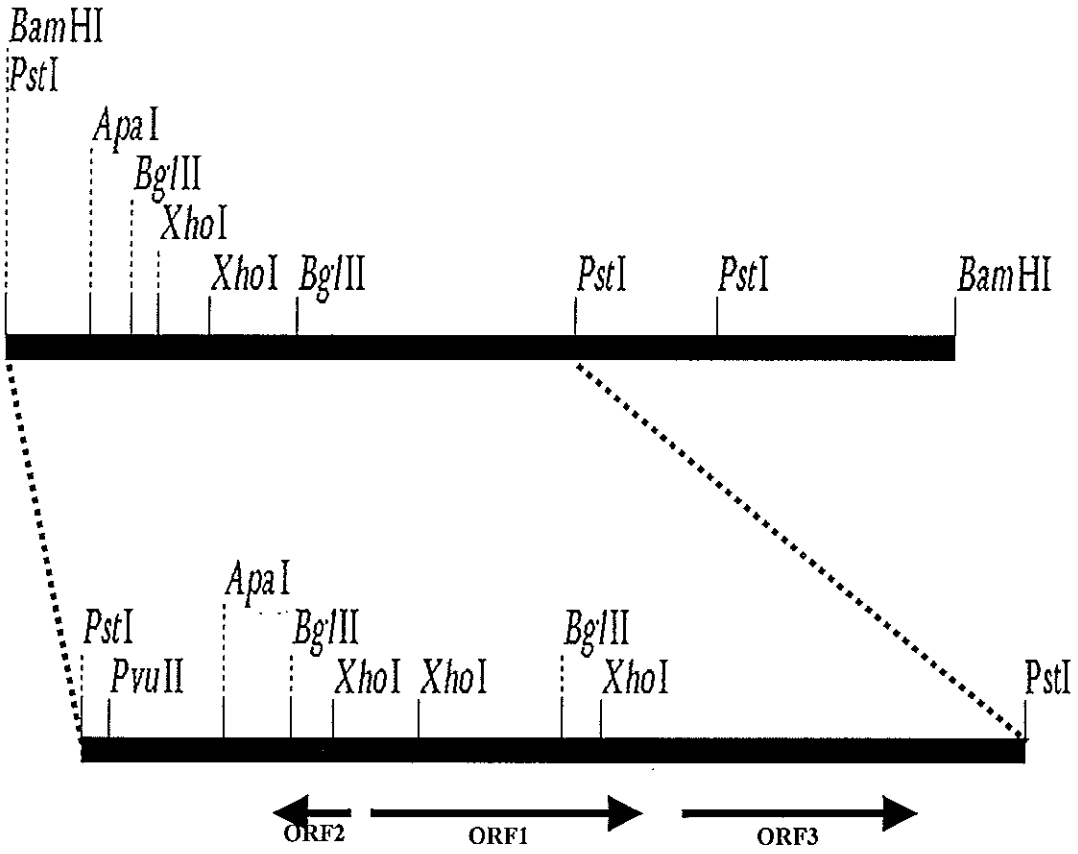


Figure 11 Physical and genetic map of 10 kb DNA fragment containing the cluster gene in *Halomonas salina* chromosome The arrows shown directly below the physical map denote the open reading frames and the direction of transcription.

3.7 Analysis of the nucleotide sequence

The region of the AS11 genome that has been analyzed here contained three ORFs as indicated in Figure 12. The 3,227 bp sequence region (from PB1, and PGP8 fragments) spanned one large reading frame of 1,521 bp or 506 amino acids that extended from a potential ATG initiation codon at nucleotide 1,173 to a TAG stop codon at nucleotide 2,694. ORF1 was preceded by a putative Shine-Dalgano sequence seven nucleotides upstream of the translational start codon. The deduced amino acid sequence of the ORF1 sequence exhibited a high degree of similarity to aldehyde dehydrogenases of other bacteria. The gene has an average G+C content of 66.7%, which was in the upper end of the range of the values reported for the genomic DNA of *Halomonas* (52 to 68%) by Dobson and Franzmann (1996).

ORF2, which was approximately 705 bp long, was identified upstream to ORF1 but oriented in the opposite direction on the complementary strand, and was not preceded by a Shine-Dalgano sequence. The encoded protein was similar to the glycerol regulatory protein (M60805) from *P. aeruginosa* (44% identical amino acid), and the putative two-component system transcriptional response regulator *tcsR* (U33883) from *Bradyrhizobium japonicum* (36%). The glycerol regulatory protein from *P. aeruginosa* is the regulatory component of a multi-component regulatory system. It regulates glycerol metabolism and causes perturbations of the cytoplasmic membrane, thus allowing glycerol at high concentrations to diffuse passively into cell. Intracellular glycerol can then be metabolized via action of a glycerol dehydrogenase activity (Schweizer, 1991).

```
   1  CTGGAGGGCTGGGGCTGTCGGGTGCGCTGCGCGGCCACGCCGGCGGCGCTGGAG
  55  GCCGCCGGCCGGCGCGAGCGCGCCGACCTGCTGCTGGTCGACTACCAGCTGGCG
 109  CCAGGCGACCCGGACGGGCTCAGCGTGGCGGACCGGCTGCGCCGACGCCATCCC
 163  GGGCTCGGCGTGGTGGTGATCTCCGCTCATCAGGATGGCGAGCTCACGCAGCGG
 217  GCCCGGGAGCGCGGCTACGACTGCCTGCTCAAGCCGGTGAAGCCGCTGCGGCTG
 271  AGGCTGTTGATTGCCCATCGGCTGGAGGCGGGCGGCGACTAGTCGTGCTGTGGC
 325  GTCGTGGGCGACAGGCGTGCCAGCCGGGCGGCCAGTTCACCCTGGTCGGCGGCC
 379  AGGATGGCCTGGACCCGGCTGCCGACGTTGAGCTTGCGCAGGATCGCCGAGACA
 433  TGCGTCTTGACCGTGGTCTCGGCGATGTCGAGATCCCGGGCGATCACCTTGTTG
 487  GCGTCGCCCCGCACCAGGCGGGCGAGCACCTCGAGCTGCTTGTCGGTGAGGGCG
 541  TCCAGCGGCGAGGGCGCCTCCGGGGCGGGGGGCGCGGCGAACGAGGCATGATCC
 595  TCCAGCGTGGGCGAAGGCGGACGGCGCATGATGTCCGGGGGCAGGTAGATCTGG
 649  CCGCTCAGCACCTGGCGCAGCGCCTCGAGCATCGCGCGGCGCGGCGTCGACTTG
 703  GGAATGTAGCCCACCGCGCCCAGGGCGATGGACTCGAGGATCAGGTTGCGTTCG
 757  CGCTCGGCGGAGACCACCACCACCGGCAGCGCCGGACAGGCCTCGCGCAGCCGC
 811  TCGAGCCCGGCCAGCCCGGCGGCATCGGGCAGCCCCAGGTCGAGCAGCAGCAGG
 865  TCGAGCTCTTCGCTCTCCTCGGCGATGCCCTTCAGGGCATCCTCGAGGCTGCCG
 919  GCCTCGAGCAGCCGGCAGCTGGGCAGGCCGGCACGGATCGCCGCGCCGATCGCC
 973  TCGCGAAACAGTGGGTGGTCGTCGGCCACCATCAGGGTGTTCATGGGGGACTCC
1027  TTGGAACGCTCGGGCGCCGGTCCGTCTTTCCCTGGGCCCTCCTACCGAAGGAGG
1081  AGGCGTTCTCCTGCCATCGCAGTATGTCCTAGACGTCGGGGCTGGCCAAGACTG
1135  GGGTGGCAGTCACATAACCACAACAGCGGGAGACACGCCATGATCTACGCCAAC
                                          M  I  Y  A  N
1189  CCCGGAAGCCCCGAGTCCGTGGTGAGCTTCGACGCGCGCTACGGCAACTACATC
      P  G  S  P  E  S  V  V  S  F  D  A  R  Y  G  N  Y  I
1243  GGCGGCGAGTTCGTGCCGCCGGTCAAGGGCCAGTATTTCGACAACGTCAGCCCG
      G  G  E  F  V  P  P  V  K  G  Q  Y  F  D  N  V  S  P
1297  GTCAACGGCAAGCCGTTCTGCGAGATTCCCCGTTCCACCGCCGAGGACATCGAC
      V  N  G  K  P  F  C  E  I  P  R  S  T  A  E  D  I  D
1351  AAGGCGCTGGACGCCGCCCACGCCGCGGCACCGGCCTGGGGCAAGACCTCGGCC
      K  A  L  D  A  A  H  A  A  A  P  A  W  G  K  T  S  A
1405  GCCGAGCGCGGCAATATCCTGCTGAGGATCGCCGACCGCATCGAGCAGAACCTC
      A  E  R  G  N  I  L  L  R  I  A  D  R  I  E  Q  N  L
1459  GAGCTGCTGGCCGTGGCCGAGACCTGGGACAACGGCAAGGCGGTGCGCGAGACC
      E  L  L  A  V  A  E  T  W  D  N  G  K  A  V  R  E  T
1513  CTGAACGCCGACCTGCCGCTGGCGGTGGACCACTTCCGCTACTTCGCCGGCTGC
      L  N  A  D  L  P  L  A  V  D  H  F  R  Y  F  A  G  C
1567  ATCCGCGCCCAGGAAGGCACCGCGGCGGACATCGACGCCAACACCGTGGCCTAT
      I  R  A  Q  E  G  T  A  A  D  I  D  A  N  T  V  A  Y
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1621  CACTTCCACGAGCCGCTGGGCGTGGTGGGGCAGATCATTCCCTGGAACTTCCCG
      H  F  H  E  P  L  G  V  V  G  Q  I  I  P  W  N  F  P
1675  ATCCTGATGGCGGTGTGGAAGCTGGCGCCGGCCCTGGCCGCGGGCAACTGCGTG
      I  L  M  A  V  W  K  L  A  P  A  L  A  A  G  N  C  V
1729  GTGCTCAAGCCCGCCGAGCAGACCCCGGCCTCGATCCTCAAGCTGATGGAGCTG
      V  L  K  P  A  E  Q  T  P  A  S  I  L  K  L  M  E  L
1783  GTCGGCGACCTGCTGCCGCCGGGCGTGGTCAACGTCGTCAACGGCTACGGCGCC
      V  G  D  L  L  P  P  G  V  V  N  V  V  N  G  Y  G  A
1837  GAGGCCGGCCAGGCGCTGGCCACCAGCACCCGGATCGCCAAGATCGCCTTCACC
      E  A  G  Q  A  L  A  T  S  T  R  I  A  K  I  A  F  T
1891  GGCTCGACCCCGGTGGGCTCGCACATCCTCAAGTGCGCCGCCGAGAACATCATT
      G  S  T  P  V  G  S  H  I  L  K  C  A  A  E  N  I  I
1945  CCCTCCACCGTGGAGCTGGGTGGCAAGTCGCCGAACATCTACTTCGCCGACATC
      P  S  T  V  E  L  G  G  K  S  P  N  I  Y  F  A  D  I
1999  ATGGACGCCGAGCCCGAGTTCATCGACAAGGCCGCCGAGGGCCTGGTGCTGGCC
      M  D  A  E  P  E  F  I  D  K  A  A  E  G  L  V  L  A
2053  TTCTTCAACCAGGGCGAGGTGTGCACCTGCCCGTCGCGGGCGCTGATCCAGGAA
      F  F  N  Q  G  E  V  C  T  C  P  S  R  A  L  I  Q  E
2107  GACATCTACGACGCCTTCATGGCCAAGGTGATGGAGAAGGTCGCGACCATCACT
      D  I  Y  D  A  F  M  A  K  V  M  E  K  V  A  T  I  T
2161  CGCGGCAACCCGCTGGACACCGACGTCAAGGTCGGCGCCCAGGCCTCCCAGGAG
      R  G  N  P  L  D  T  D  V  K  V  G  A  Q  A  S  Q  E
2215  CAGTTCGACAAGATCATGTCGTACATGGAGGTGGCCCGCGAGGAGGGCGCCGAG
      Q  F  D  K  I  M  S  Y  M  E  V  A  R  E  E  G  A  E
2269  TTCCTGACCGGCGGCGACAAGGAGCACTTCGATCCGGCCTATGACAGCGGCTAT
      F  L  T  G  G  D  K  E  H  F  D  P  A  Y  D  S  G  Y
2323  TACATCCAGCCGACCCTGCTCAAGGGCCACAACAAGATGCGCGTGTTCCAGGAA
      Y  I  Q  P  T  L  L  K  G  H  N  K  M  R  V  F  Q  E
2377  GAGATCTTCGGCCCGGTGGTGGCGGTGACCACCTTCAAGGACGAGGAAGAGGCG
      E  I  F  G  P  V  V  A  V  T  T  F  K  D  E  E  E  A
2431  CTGGCCATCGCCAACGACACCGAGTTCGGCCTCGGCGCCGGGGTCTGGAGCCGC
      L  A  I  A  N  D  T  E  F  G  L  G  A  G  V  W  S  R
2485  GACATCAACGTCGCCTTCCGCATGGGCCGCGGCATCCAGGCCGGCCGGGTGTGG
      D  I  N  V  A  F  R  M  G  R  G  I  Q  A  G  R  V  W
2539  ACCAACTGCTACCACCAGTATCCGGCCCACGCCGCCTTCGGCGGTTACAAGAAG
      T  N  C  Y  H  Q  Y  P  A  H  A  A  F  G  G  Y  K  K
2593  TCCGGCGTCGGCCGCGAGACCCACAAGGTCGCGCTCGAGCACTACCAGCAGACC
      S  G  V  G  R  E  T  H  K  V  A  L  E  H  Y  Q  Q  T
```

```
2647  AAGAACCTGCTGGTCAGCTATGACACCAATCCCCTCGGCTTCTTCTAGGACAGA
      K  N  L  L  V  S  Y  D  T  N  P  L  G  F  F  *
2701  CTGACGTGTCGCTGCGCCAGTGACATGGCGCCGTCATTCCCGGAAAGCCCCGAC
2755  CCCGGGCATCGAACCCGGGGCATTCCCTCCCTGACGCAGCCTGCTCCGTCGCCT
2809  CCGGCGACGGGGCTCCCCTACCCGTTGTTGAGGAGAAAGCACATGGACAGCACG
                                                M  D  S  T
2863  ATGCAAGCCGCCGTGGTGCGGGCCTTCGGTGAGCCGCTGAGCCTCGAGGAGGTC
      M  Q  A  A  V  V  R  A  F  G  E  P  L  S  L  E  E  V
2917  GAGGTGCCGCGGCCCGGGCGCGGCGAGATCCTGGTCAAGGTGGCCGCCTCCGGC
      E  V  P  R  P  G  R  G  E  I  L  V  K  V  A  A  S  G
2971  GTCTGCCACACCGATCTGCACGCCGCCCACGGCGACTGGCCGGTCAAGCCCGAG
      V  C  H  T  D  L  H  A  A  H  G  D  W  P  V  K  P  E
3025  CCACCCTTCATCCCGGGGCACGAGGGCGTCGGCCACGTGGCCGCCGTGGGCGAG
      P  P  F  I  P  G  H  E  G  V  G  H  V  A  A  V  G  E
3079  GGCGTGACCCATCTCAGGGAAGGCGACCGGGTCGGCGTGCCCTGGCTTTATTCC
      G  V  T  H  L  R  E  G  D  R  V  G  V  P  W  L  Y  S
3133  GCCTGCGGCCACTGCGAGCACTTGCCTGGGCGGCTGGGAGACCCTTTTGCGAAT
      A  C  G  H  C  E  H  L  P  G  R  L  G  D  P  F  A  N
3187  CGCAGCAAGAACAACCGGGCTACTTCGGTGAAACGGGCGGG
      R  S  K  N  N  R  A  T  S  V  K  R  A  G
```

Figure 12 Nucleotide sequence of the aldehyde dehydrogenase and adjacent genes from *Halomonas salina* AS11 Amino acids deduced from the nucleotide sequence are specified by standard one-letter abbreviations. Putative Shine-Dalgano sequences are underlined.

Analysis of the DNA sequence downstream of the *aldh* gene found a partial reading frame that was in the same orientation as *aldh*. The deduced amino acid sequence (122 amino acids) of this partial reading frame displayed between 55.0% and 58.0% similarity to the N-termini of a group of alcohol dehydrogenases including those encoded by *Sinorhizobium moliloti adhA* gene (AF031940), *Bacillus stearothermophilus adh-HT* gene (Z27089), and a putative *Streptomyces coelicolor* alcohol dehydrogenase (AL109989).

Translation of the *aldh* DNA sequence yields a protein of 506 amino acid residues with a calculated molecular mass of 54,919 Da. The deduced amino acid sequence of the identified *aldh* gene shows all characteristics common to a dehydrogenase including the motif VELGGKSP beginning at amino acid position 261, and FENGGEVCTCPS at amino acid position 294, that are essential for the activity of this family of proteins (Von Bahr-Linstorm *et al.*, 1985). This protein also has the sequence GYGAEAG at amino acid position 218 to 224 that resembles the G-X-G-X_3-G motif believed to be involved in NAD^+ binding to aldehyde dehydrogenases catalyzing irreversible reactions (Hidalgo *et al.*, 1991). By comparison, enzymes such as alcohol dehydrogenase that catalyze reversible reactions have the alternative motif, G-X-G-X_2-G (Scrutton *et al.*, 1990). Comparison of the amino acid sequence of ALDH with those of proteins collected in the NCBI database showed extended homologies with ALDH from other sources. The primary structure of ALDH was compared with that of ALDH from other bacteria; however, no relevant homologies were obtained from the halophilic bacteria. The phylogenetic relationship of the AS11 *aldh* gene to other bacterial *aldh* genes is shown in Figure 13. The alignment of the amino acid sequence of ALDH and of eight representative ALDHs;

an NAD^+-dependent acetaldehyde dehydrogenase gene from *Pseudomonas aeruginosa* (76.0% identical amino acids), *Alcaligenase eutrophus* (65.0%); aldehyde dehydrogenase gene from *Alteromonas* sp. (72.0%), *Deinococcus radiodurans* (66.0%), *Vibrio cholerae* (61.0%), *Rhodospirillum rubrum* (62.0%) and Chloroacetaldehyde dehydrogenase from Xanthobacter autotrophicus (65.0%) is shown in Figure 14.

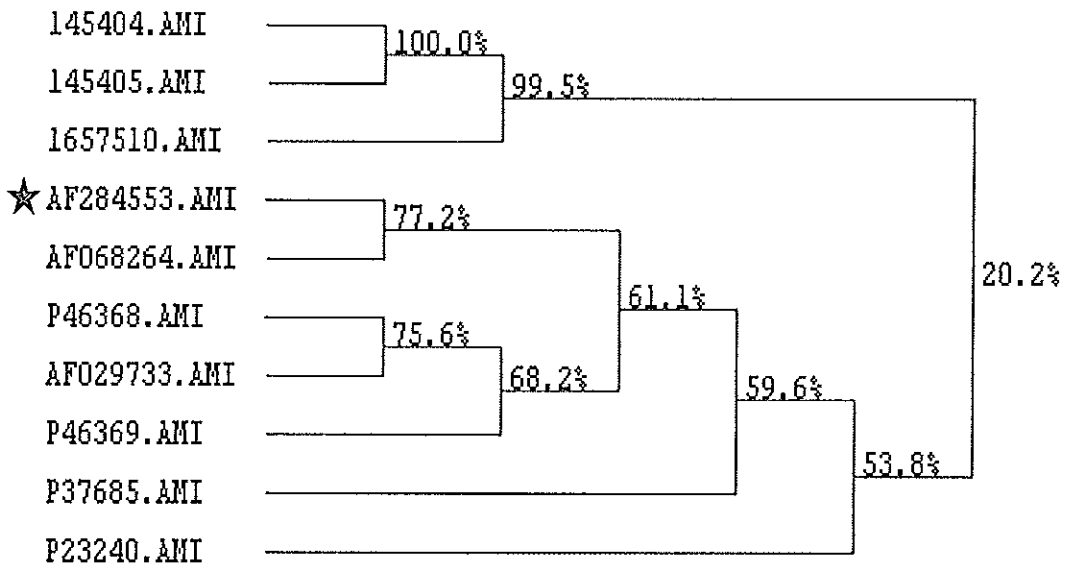

Figure 13 Phylogenetic relationship of bacteria AS11 *aldh* gene and representative bacterial aldehyde dehydrogenase. 145404, 145405, 1657510; *E. coli* betaine aldehyde dehydrogenase AF284553; *H. salina* AS11 aldehyde dehydrogenase AF068264; *P. aeruginosa* NAD^+ dependent acetaldehyde dehydrogenase P46368; *Alcaligenase eutrophus* acetaldehyde dehydrogenase AF029733; *X. autotrophicus* chloroacetaldehyde dehydrogenase, P46369; *Rhodococcus erythropolis* EPTC-inducible aldehyde dehydrogenase P37685; *E. coli* lactaldehyde dehydrogenase P23240; *V. cholerae* aldehyde dehydrogenase

```
                    *         20          *         40          *
AF284553 : -MIYA------NPGSPESVVSFDARYGNYIGGEFVPPVKGQYFDNVSPVNG :  44
AF068264 : -MIYA-----APGTPGAVVTFKPRYGNYIGGEFVPPVKGQYFTNTSPVNG :  44
P46368   : -MNMA-----EIAQLGVSNPYKQQYENYIGGAWVPPAGGEYFESTTPITG :  44
AF029733 : -MNKP-----EIAITKQS-PFKARYGNFIGGKFVEPVGGRYFDNTSPVTG :  43
P46369   : MTKYA-----RPGTADAIMSFQSRYDNWIGNEWVAPVKGQYFENPTPVTG :  45
P37685   : MTNNPPSAQIKPGEYGFPLKLKARYDNFIGGEWVAPADGEYYQNLTPVTG :  50
P23240   : -MIYP-----IPNSETSTVHFKDVYDNYIGGQWMKPHSGEYFSNTSPVNG :  44
145404   : ----------------------MMSRMAEQQLYIHGGYTSATSGRTFETINPANG :  33
145405   : ----------------------MSRMAEQQLYIHGGYTSATSGRTFETINPANG :  32
1657510  : ----------------------MMSRMAEQQLYIHGGYTSATSGRTFETINPANG :  33
                                      5I g 5     G  5     P  G

                   60          *         80          *         100
AF284553 : KPFCEIPRSTAEDIDKALDAAHAAAPAWGKTSAAERGNILLRIADRIEQN :  94
AF068264 : QPIAEFPRSTAEDIDKALDAAHAAADAWGRTSVQERSNILLKIADRIEQN :  94
P46368   : KPFTRVPRSGQQDVDAALDAAHAAKAAWARTSTTERANILNRIADRIEAN :  94
AF029733 : GKICEIARSDADDIEKALDAAHAAKDAWGKTSAAVRALILNRIADRMEEN :  93
P46369   : QNFCDVARSTAEDIELALDAAHAAAPAWGKTSVAERAIILNKIADRMEEN :  95
P37685   : QLLCEVASSGKRDIDLALDAAHKVKDKWAHTSVQDRAAILFKIADRMEQN : 100
P23240   : LVFCRVARSSSQDVELALDAAHNALESWSTTSAVERSNILLRIADRIESN :  94
145404   : NVLATVQAAGREDVDRAVKSAQQGQKIWASMTAMERSRILRRAVDILRER :  83
145405   : NVLATVQAAGREDVDRAVKSAQQGQKIWASMTAMERSRILRRAVDILRER :  82
1657510  : NVLATVQAAGREDVDRAVKSAQQGQKIWASMTAMERSRILRRAVDILRER :  83
                     D6  A6  A      W   3  eR  IL 4  D 6

                    *        120         *        140         *
AF284553 : LELLAVAETWDNGKAVRETLNADLPLAVDHFRYFAGCIRAQEGTAADIDA : 144
AF068264 : LELLAVTETWDNGKAVRETLNADIPLAADHFRYFAGCIRAQEGSAAEIND : 144
P46368   : LKLLAVAESIDNGKPVRETTAADLPLAVDHFRYFAGCIRAQEGGISEIDA : 144
AF029733 : LDLLALAETWDNGKPIRETTAADMPLAIDHFRYFAGAVRAQEGGISEIDH : 143
P46369   : LESIALAESWDNGKPIRETLNADIPLAIDHFRYFAGAIRAQEGSLSEINS : 145
P37685   : LELLATAETWDNGKPIRETSAADVPLAIDHFRYFASCIRAQEGGISEVDS : 150
P23240   : LETLAIVESWDNGKPIRETLAADLPLTIDHFRYFAACIRSQEGAASELDS : 144
145404   : NDELAKLETLDTGKAYSETSTVDIVTGADVLEYYAGLIPALEGSQIPLRE : 133
145405   : NDELAKLETLDTGKAYSETSTVDIVTGADVLEYYAGLIPALEGSQIPLRE : 132
1657510  : NDELAKLETLDTGKAYSETSTVDIVTGADVLEYYAGLIPALEGSQIPLRE : 133
              6A  E3 D GK   ET   D6    D   Y5Ag 6 a EG    6

                   160         *        180         *        200
AF284553 : NTVAYHFHEPLGVVGQIIPWNFPILMAVWKLAPALAAGNCVVLKPAEQTP : 194
AF068264 : STVAYHIHEPLGVVGQIIPWNFPLLMAAWKLAPALAAGNCVVLKPAEQTP : 194
P46368   : DTIAYHFHEPLGVVGQIIPWNFPLLMATWKLAPALAAGNCVVLKPAEQTP : 194
AF029733 : DTVAYHFHEPLGVVGQIIPWNFPILMAVWKLAPALAAGNCVVLKPAEQTP : 193
P46369   : DTVAYHFHEPLGVVGQIIPWNFPILMAVWKLAPALAAGNAIVLKPAEQTP : 195
P37685   : ETVAYHFHEPLGVVGQIIPWNFPLLMASWKMAPALAAGNCVVLKPARLTP : 200
P23240   : RTLTYHLPEPIGVVGQIIPWNFPLLMAAWKLAPALAAGCTVVLKPAEQTP : 194
145404   : TSFVYTRREPLGVVAGIGAWNYPIQIALWKSAPALAAGNAMIFKPSEVTP : 183
145405   : TSFVYTRREPLGVVAGIGAWNYPIQIALWKSAPALAAGNAMIFKPSEVTP : 182
1657510  : TSFVYTRREPLGVVAGIGAWNYPIQIALWKSAPALAAGNAMIFKPSEVTP : 183
             3  Y   EP6GVV  I  WN5P6 6A WK APALAAGn 66 KP e TP
```

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                    *       220         *       240         *
AF284553 : ASILKLMELVGDL-LPPGVVNVVNGYGAEAGQALATSTRIAKIAFTGSTP : 243
AF068264 : LGICVLLELIGDL-LPPGVLNVVQGFGREAGEALATSKRIAKIAFTGSTP : 243
P46368   : ASILVLMEVIGDL-LPPGVVNVINGFGLEAGKPLASSPRISKVAFTGETT : 243
AF029733 : ASILVLAELIADL-LPPGVLNIVNGFGLEAGKPLASSSRIAKIAFTGETT : 242
P46369   : VSILHLIGIIGDL-LPAGVLNIVNGFGVEAGKPLASSPRIKKIAFTGETT : 244
P37685   : LSVLLLMEIVGDL-LPPGVVNVVNGAGGVIGEYLATSKRIAKVAFTGSTE : 249
P23240   : VSILFLMEIIGDL-IPAGVINVVNGFGSEAGNALATSQRIDKLAFTGSTE : 243
145404   : LTALKLAEIYSEAGLPDGVFNVLPGVGAETGQYLTEHPGIAKVSFTGGVR : 233
145405   : LTALKLAEIYSEAGLPDGVFNVLPGVGAETGQYLTEHPGIAKVSFTGGVR : 232
1657510  : LTALKLAEIYSEAGLPDGVFNVLPGVGAETGQYLTEHPGIAKVSFTGGVA : 233
              l L e6     6P GV N66 G G e G  L     I K6 FTG

                  260         *       280         *       300
AF284553 : VGSHILK-CAAENIIPSTVELGGKSPNIYFADIMDAEPEFIDKAAEGLVL : 292
AF068264 : VGSHILK-CAAESIIPSTVELGGKSPNIYFEDIMQAEPAFIEKAAEGLVL : 292
P46368   : TGRLIMQ-YASQNLIPVTLELGGKSPNIFFEDVLAADDAFFDKALEGFAM : 292
AF029733 : TGRLIMQ-YASQNLIPVTLELGGKSPNIFFADVVAEDDDFFDKAVEGFVM : 291
P46369   : TGRLIMQ-YASQNLIPVTLELGGKSPNVFFSDVLASNDDYQDKALEGFTM : 293
P37685   : VGQQIMQ-YATQNIIPVTLELGGKSPNIFFADVMDEEDAFFDKALEGFAL : 298
P23240   : IGNHILK-CAADNLIPSTIELGGKSPNIYFPDIFSHEDQYLDKCIEGALL : 292
145404   : SGKKVMANSAASSLKEVTMELGGKSPLIVFDDA-D-----LDLAADIAMM : 277
145405   : SGKKVMANSAASSLKEVTMELGGKSPLIVFDDA-D-----LDLAADIAMM : 276
1657510  : SGKKVMANSAASSLKEVTMELGGKSPLIVFDDA-D-----LDLAADIAMM : 277
              G  66   A   6   T6ELGGKSP 6 F D         d a     6

                    *       320         *       340         *
AF284553 : A-FFNQGEVCTCPSRALIQEDIYDAFMAKVMEKVATITRGNPLDTDVKVG : 341
AF068264 : A-FFNQGEVCTCPSRALVQESIYPAFMEEVLKKVRAIKRGDPLDTETMVG : 341
P46368   : F-ALNQGEVCTCPSRALIQESIYDRFMERALKRVAAIRQGHPLDTGTMIG : 341
AF029733 : F-ALNQGEVCTCPSRALIQESIYDRFMEKALKRVAAIVQGSPLDPATMIG : 340
P46369   : F-ALNQGEVCTAPSRALIQEDIFDEFLAMAAIRTKAVRQGDPLDTDTMIG : 342
P37685   : F-AFNQGEVCTCPSRALVQESIYERFMERAIRRVESIRSGNPLDSVTQMG : 347
P23240   : A-FFNQGEVCTCPSRILVHESIYEKFIAKIIERVALIKQGNPLDTETQIG : 341
145404   : ANFFSSGQVCTNGTRVFVPAKCKAAFEQKILARVEPIRAGDVFDPQTNFG : 327
145405   : ANFFSSGQVCTNGTRVFVPAKCKAAFEQKILARVEPIRAGDVFDPQTNFG : 326
1657510  : ANFFSSGQVCTNGTRVFVPAKCKAAFEQKILARVERIRAGDVFDPQTNFG : 327
                 G2VCT  3R  6       F      4v  6  G   D  t  G

                  360         *       380         *       400
AF284553 : AQASQEQFDKIMSYMEVAREEGAEFLTGGDKEHFDPAYDSGYYIQPTLLK : 391
AF068264 : AQASQQQYEKILSYLDIAQQEGAELLAGGSVEKLEGNLASGYYIQPTLLK : 391
P46368   : AQASAEQLEKILSYIDLGRKEGAQCLTGGERNVLDGDLAGGYYVKPTVFA : 391
AF029733 : AQASSEQLEKILSYIDIGKQEGAEVLIGGERNTFGGDLAGGYYVKPTVFK : 390
P46369   : AQASNDQLEKILSYIEIGKAEGAKVITGGERAELGGDLSGGYYVQPTVFT : 392
P37685   : AQVSHGQLETILNYIDIGKKEGADVLTGGRRKLLEGELKDGYYLEPTILF : 397
P23240   : AQVSKEQYDKILGYIQIGKDEGAELIFGGHPNNQENYLSGGYYIKPTLFF : 391
145404   : PLVSFPHRDNVLRYIAKGKEEGARVLCGGDVLKGDG-FDNGAWVAPTVFT : 376
145405   : PLVSFPHRDNVLRYIAKGKEEGARVLCGGDVLKGDG-FDNGAWVAPTVFT : 375
1657510  : PLVSFPHRDNVLRYIAKGKEEGARVLCGGDVLKGDG-FDNGAWVAPTVFT : 376
              S       66 Y6  g  EGA  6 GG       g    G 56 PT6
```

```
                    *       420         *       440         *
AF284553 : G-HNKMRVFQEEIFGPVVAVTTFKDEEEALAIANDTEFGLGAGVWSRDIN : 440
AF068264 : G-HNGMRVFQEEIFGPVVGVTTFKDEAEALAIANDTEYGLGAGLWTRDIN : 440
P46368   : G-HNKMRIFQEEIFGPVVSVTTFKDEEEALAIANDTLYGLGAGVWTRDGA : 440
AF029733 : G-HNKMRIFQEEIFGPVVSVTTFTDDAEALHIANDTLYGLGAGVWTRDGN : 439
P46369   : G-NNKMRIFQ-EIFGPVVSVTSFKDYDEAIEIANDTLYGLGAGVWSRDGG : 440
P37685   : G-QNNMRVFQEEIFGPVLAVTTFKTMEEALELANDTQYGLGAGVWSRNGN : 446
P23240   : G-HNQMHIFQEEIFGPVIAITKFKDEIEALHLANDTVYGLGAGVWTRDIN : 440
145404   : DCSDDMTIVREEIFGPVMSILTYESEDEVIRRANDTDYGLAAGIVTADLN : 426
145405   : DCSDDMTIVREEIFGPVMSILTYESEDEVIRRANDTDYGLAAGIVTADLN : 425
1657510  : DCSDDMTIVREEIFGPVMSILTYESEDEVIRRANDTDYGLAAGIVTADLN : 426
             1 M 6  eEIFGPV6 6  5    E 6  ANDT 5GL AG6 3 1 n


                  460         *       480         *       500
AF284553 : VAFRMGRGIQAGRVWTNCYHQYPAHAAFGGYKKSGVGRETHKVALEHYQQ : 490
AF068264 : RAYRMGRGIKAGRVWTNCYHLYPAHAAFGGYKKSGVGRETHKMMLDHYQQ : 490
P46368   : RAFRMGRGIQAGRVWTNCYHAYPAHAAFGGYKQSGIGRENHRMMLDHYQQ : 490
AF029733 : RAYRFGRAIQAGRVWTNCYHAYPAHAAFGGYKQSGIGRENHKMMLDHYQQ : 489
P46369   : VAYRAGRDIQAGRVWTNTYHQYPAHAAFGGYKQSGIGRENHLMMLSHYQQ : 490
P37685   : LAYKMGRGIQAGRVWTNCYHAYPAHAAFGGYKQSGIGRETHKMMLEHYQQ : 496
P23240   : IAHRMAKNIKAGRVWVNCYHAYPAHAAFGGYKKSGIGRETHKLTLSHYQN : 490
145404   : RAHRVIHQLEAGICWINTWGESPAEMPVGGYKHSGIGRENGVMTLQSYTQ : 476
145405   : RAHRVIHQLEAGICWINTWGESPAEMPVGGYKHSGIGRENGVMTLQSYTQ : 475
1657510  : RAHRVIHQLEAGICWINTWGESPAEMPVGGYKHSGIGRENGVMTLQSYTQ : 476
             A 4    6 AG  W N 5   PA    GGYK SG6GRE   6 L  Y q


                   *
AF284553 : TKNLLVSYDTNPLGFF : 506
AF068264 : TKNLLVSYDIDPLGFF : 506
P46368   : TKNLLVSYSPNALGFF : 506
AF029733 : TKNMLVSYSPKKLGFF : 505
P46369   : TKNLLVSYAQKAQGFF : 506
P37685   : TKCLLVSYSDKPLGLF : 512
P23240   : IKNVLISHEIHPLGLF : 506
145404   : VKSIQVEMA-KFQSIF : 491
145405   : VKSIQVEMA-KFQSIF : 490
1657510  : VKSIQVEMA-KFQSIF : 491
             K 6 6         F
```

Figure 14 **Amino acid sequence alignment of ALDH from *Halomonas salina* AS11 and ALDHs from other microorganisms** Sequence abbreviations are as given in the legend to figure 13. Symbols are described in Appendix E.

3.8 Expression of ALDH in *E. coli*

To verify the enzymatic activity of the ORF1 gene product, it was expressed in *E. coli*. The 1.7 kbp *Kpn*I-*Hin*dIII fragment containing the *aldh* gene was obtained by using ex *aldh* forward and reverse primers for a PCR amplification of the entire open reading frame as described in Chapter 2, Section 2.5.2. After purification by using a QIAquick kit, the PCR products were cleaved with *Kpn*I and *Hin*dIII, and repurified using the QIAquick kit. The construction of plasmid pQA 802 is shown in Figure 15. An *E. coli aldh*-producing vector for expression, pQA 802 (5.7 kbp) was constructed by cloning a *Kpn*I-*Hin*dIII fragment containing the isolated *aldh* gene into the expression vector pQE40 (QIAGEN, 4.0 kbp), downstream from the T5 promoter, 6x His tag and DHFR (Chapter 2, Section 2.7.1). After the constructed plasmid had been transformed into *E. coli* XL1-blue, the transformants were screened using ampicillin as a selectable marker. Twelve ampicillin resistant clones were selected by comparing the plasmid DNA restriction pattern with the parental plasmid vector pQE40. Clones no. 2, 6 and 8 were subsequently analyzed by digestion with *Bam*HI-*Hin*dIII, *Kpn*I-*Hin*dIII, *Eco*RI, *Bam*HI and *Bgl*II. The results showed that all three clones contained the *aldh* gene. In order to confirm that the insertion was in the correct reading frame relative to the start of transcription, this region of the plasmid was again sequenced. Once the clone was verified, the plasmid DNA was transformed into *E coli* M15 for studies of the encoded protein.

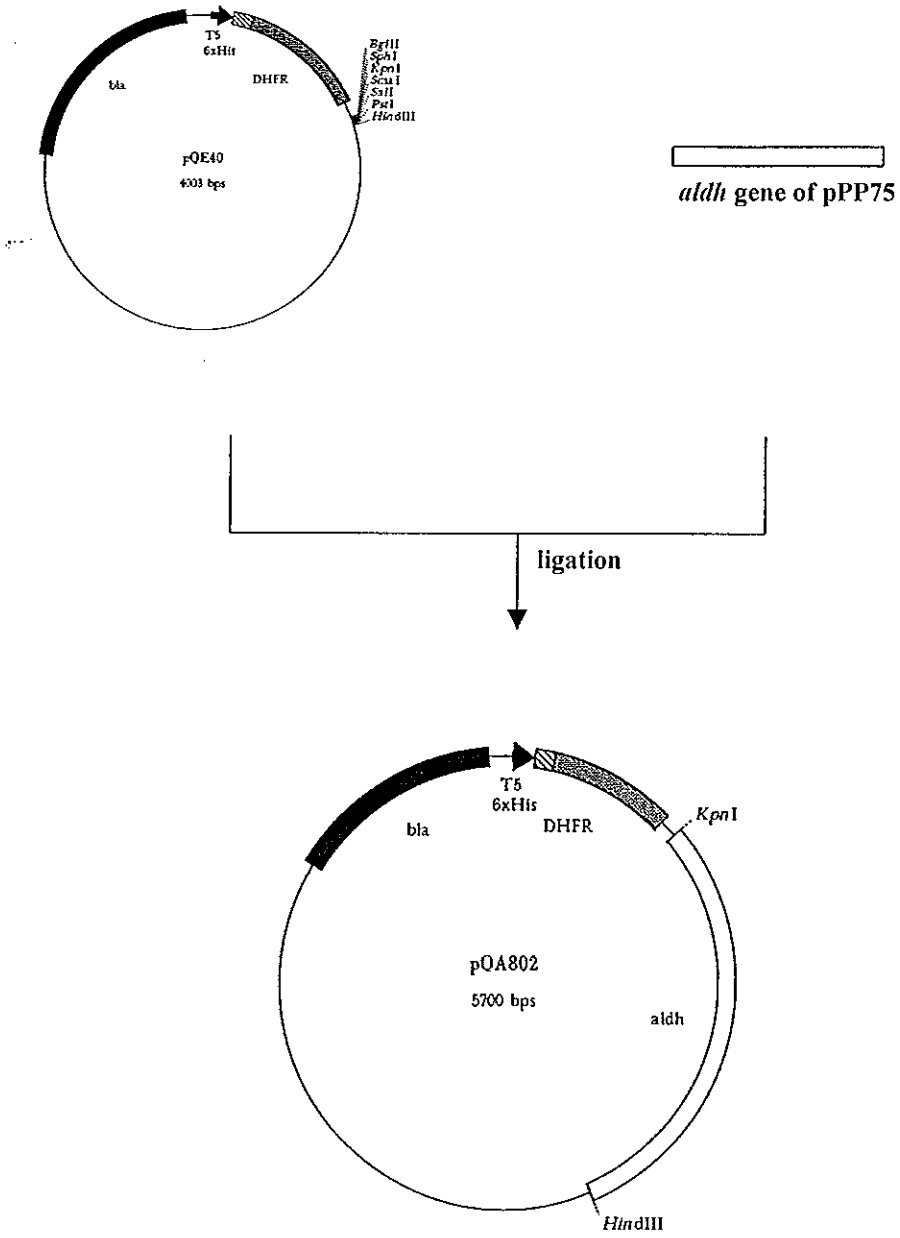

Figure 15 Construction of recombinant plasmid pQA802

Plasmid pQA802 was a hybrid plasmid consisting of pQE40 (4 kbp) and the *Kpn*I-*Hin*dIII fragment (1.7 kbp) containing the *aldh* gene of *H. salina* strain AS11.

Two positive clones were chosen for small-scale expression in shaker flasks as described in Chapter 2, Section 2.7.2. Individual clones were grown in LB until an OD_{600} had reached 0.1-0.2. To induce expression, the culture was supplemented by the addition of IPTG to a final concentration of 1 mM. Samples were collected after 3 hours of induction. The accumulation of the *aldh* protein was detected by SDS-PAGE analysis. The result showed that *aldh* was successfully produced by *E. coli* and the level of recombinant *aldh* obtained from clone no. 2 was higher than that produced by the other clones. This clone was therefore selected to determine the effect of growth condition on the level of product.

In this study, the bacteria harboring pQA802 were first grown in LB, then induced by treatment with 1 mM IPTG. Samples were taken every hour for 5 hours, cells were concentrated and processed as described in Chapter 2, Section 2.7.3, and a volume of 10 µl of supernatant from each sample was analyzed by SDS-PAGE analysis. Although SDS-PAGE analysis revealed a number of proteins, only one of these bands was unique to cells containing pQA802 and had a MW corresponding to 81 kDa. This protein most likely corresponded to the 26 kDa 6xHis-DHFR tag fused to the expected 55 kDa ALDH gene product (Figure16). The results also showed that the 81 kDa protein was produced at a high level after 3 hours of induction and increased thereafter. On the other hand, no such protein was detected from *E. coli* harboring the parental plasmid, pQE40. In order to determine whether this protein had the expected enzyme activity, an experiment was conducted to identify the optimal concentration for induction by IPTG. The recombinant bacterium was grown in LB and induced by adding 5 µM, 50 µM, 500 µM and 1 mM IPTG and cultured at 30°C overnight. Expression of ALDH was stimulated by treatment with 5 µM IPTG. An

overnight culture of *E. coli* harboring pQA802 produced 2.43 U ALDH activity/mg protein while enzyme levels in bacteria containing pQE40 were undetectable. These results proved that the *aldh* gene of *H. salina* AS11 was producing an enzymatically active protein when expressed in *E. coli*. This result is similar to the expressed product of *aldh* gene from *Xanthobacter autotrophicus* GJ10 in *E. coli* XL1-blue (Bergeron *et al.*, 1998).

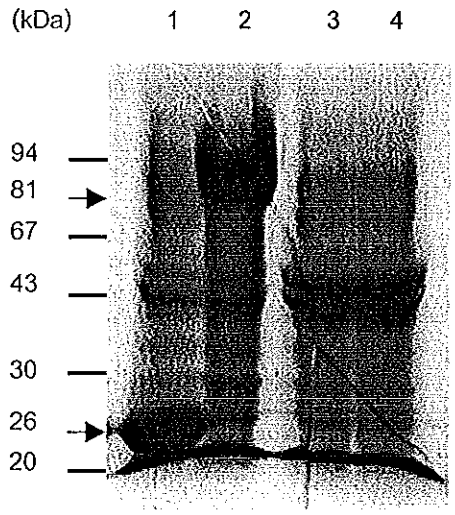

Figure 16 Production of aldehyde dehydrogenase from *H. salina* AS11 in recombinant *E. coli* XL-1 blue The gene expression was induced with 500 μM IPTG in LB medium. Lane 1 pQE40 in LB with 500 μM IPTG Lane 2 pQA802 in LB with 500 μM IPTG Lane 3 pQE40 in LB Lane 4 pQA802 in LB

3.9 Genetic transformation of *Oryza sativa* by *Agrobacterium tumefaciens*

In order to study the effect of the *aldh* gene on the growth of plants, the 1.8 kbp fragment containing the *aldh* gene from pPP75 was digested with *Sma*I and *Pvu*II and inserted into plasmid 2'-CAMBIA in the *Sma*I restriction site, resulting in the construct called 2'-CALDH (Figure 17). The ligation mixture was transformed into *E. coli* XL1-blue using calcium chloride (Cohen *et al.*, 1972). *E. coli* transformants were first screened on LB plates containing 25 μg/ml kanamycin. Nine kanamycin-resistant transformants were selected and plasmid DNA was prepared from the colonies as described in Chapter 2, Section 2.5.8. The DNA patterns of the recombinant clones were analyzed on a 1.0% (w/v) agarose gel and compared with similarly prepared digests of 2'-CAMBIA. Eight of the nine transformants contained the *aldh* fragment. By analyzing digests of these clones made with *Bam*HI-*Eco*RI, *Bgl*II and *Pvu*II, it was found that half contained the *aldh* gene in the sense orientation relative to the promoter. This plasmid was introduced into *A. tumefaciens* EHA105 (Hood *et al.*, 1986) by a tri-parental mating procedure (Chapter 2, Section 2.8.2) using pRK 2013 (Ditta *et al.*, 1980) to provide the mobilization function. These bacteria were then used to transform rice callus.

The procedure that was used to transform rice required newly generated callus (Hiei *et al.*, 1994). In order to initiate the growth of this callus, rice seeds were dehusked and sterilized as describe in Chapter 2, Section 2.8.1. Dehusked greenhouse grown seeds have proven to be the best for tissue culture. Field grown seeds often show heavy fungal and bacterial contamination that cannot be eliminated efficiently by seed sterilization. 80% of the sterilized seed from the japonica variety Kitaake began to produce callus within one week, and each seed produced approximately 3

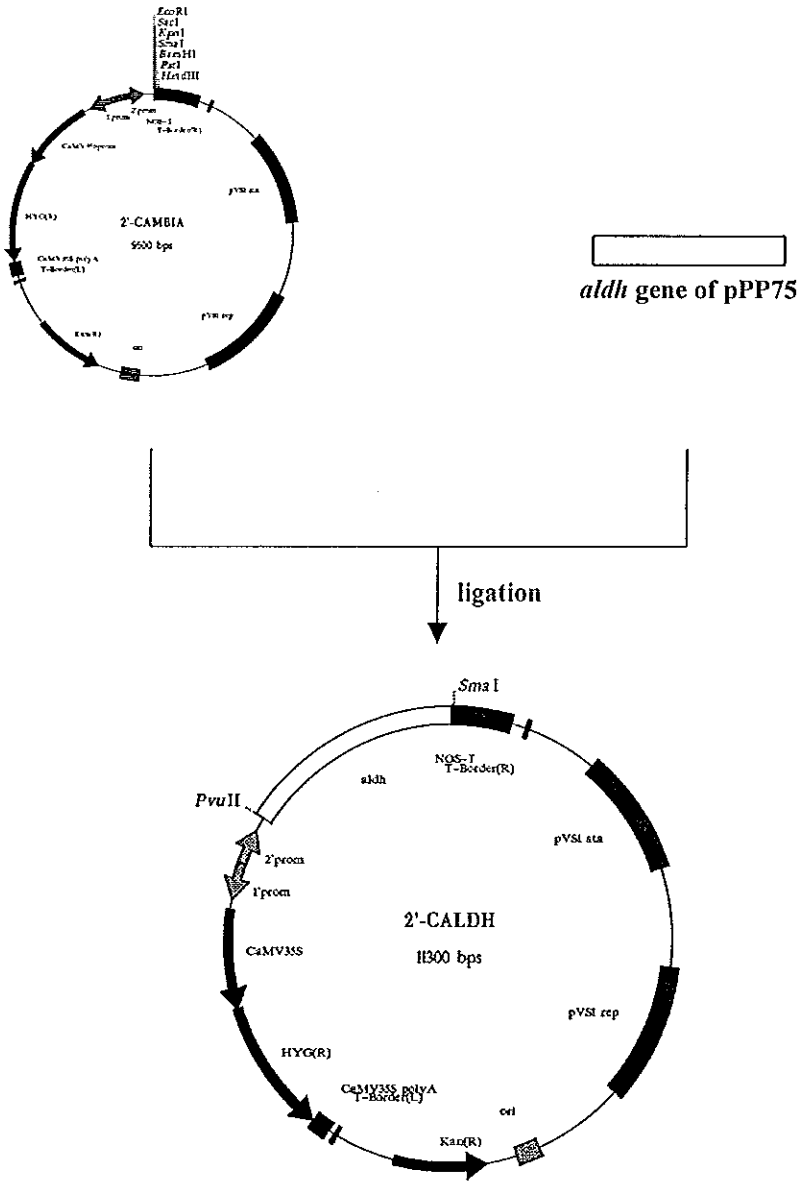

Figure 17 Construction of recombinant plasmid 2'-CALDH

This plasmid was a hybrid plasmid consisting 2'-CAMBIA (9.5 kbp) and the *Pvu*II-*Sma*I fragment (1.8 kbp) containing the *aldh* gene from pPP75.

pieces of callus of the correct size for transformation within one month. By comparison, only 2% of sterilized seeds of the indica variety Cypress produced callus that could be used for transformation.

Once callus began to form, it was necessary to remove it from the seed and developing plumule (seedling). The callus was cut into pieces that were approximately 1 mm in diameter or smaller. Within one to two weeks these small pieces of callus reached a useful size for transformation. From that time on, the callus had to be subcultured every one to two weeks. Callus older than two months could not be used for transformation.

Dense, creamy-white callus (Figure 18) was selected for rice transformation via *A. tumefaciens* as described in Chapter 2, Section 2.8.3. Pieces of rice callus approximately 1-2 mm in diameter were preferred. Pieces larger than 3 mm in diameter tended to become contaminated later. *Agrobacterium* containing 2'-CALDH was grown in YEB medium containing 50 μg/ml rifampicin and 25 μg/ml kanamycin for transformation. Pieces of rice callus were submerged in the EHA105/2'-CALDH for 10 minutes (Chapter 2, Section 2.8.3). After a total of ninety-six calli were infected, pieces were placed on callus inducing medium (CIM) containing 100 μM acetosyringone and 1% glucose.

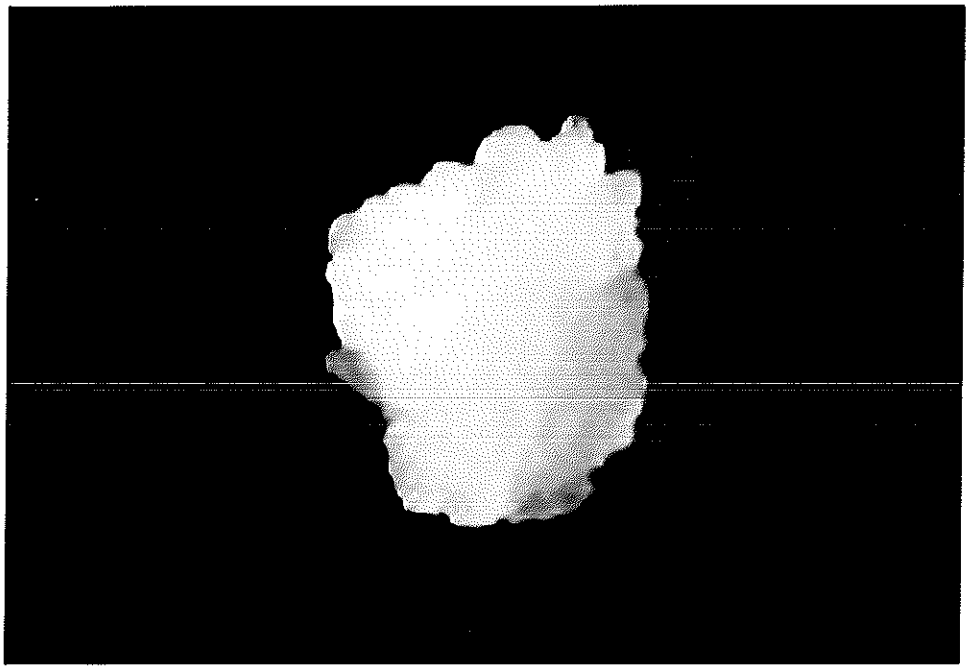

Figure 18 Callus formation from mature embryos rice

Callus was induced and subcultured on callus inducing medium in the dark.

The addition of acetosyringone and glucose was believed to increase transformation efficiency through induction of *Agrobacterium vir* genes. Following co-cultivation with *A. tumefaciens*, after three days at 22°C in the dark, rice callus was washed at least two hours in CIM containing 250 mg/l cefotaxine and 100 mg/l vancomycin, and changed the solution every 30 minutes. The washing step is intended to remove any excess bacteria. Selection is not imposed upon the callus at this time in order to allow any transformed cells to proliferate prior to the stress of selection. After four days on non-selective medium (see Appendix C), the callus was transferred to selective medium (see Appendix C). Initially a low level of selection was used to allow transferred callus a little more time to adjust and continue to divide rapidly while the growth of non-transformed callus was slowing. At this point most of the non-transformed callus began to turn brown (Figure 19). The eighteen hygromycin-resistance calli were transferred to CIM containing 50 mg/l hygromycin, 250 mg/l cefotaxime, and 100 mg/l vancomycin. Vancomycin was left out if the callus was bacteria free. The higher level of selection at this step ensured that transformed cells would survive long enough to be transferred to pre-regeneration medium (see Appendix C). During this culture period, non-transformed, dying callus was removed from any healthy tissue since chemicals and enzymes released from dead cells inhibit the growth and regeneration of transformed cells.

Prior to being placed on regeneration medium, Toki (1997) suggested placing callus on pre-regeneration medium. The low levels of BAP and NAA were believed to allow the callus to adjust to regeneration hormones while the ABA was believed to help callus adjust to changes in osmotic potential through the induction of osmotic defense genes.

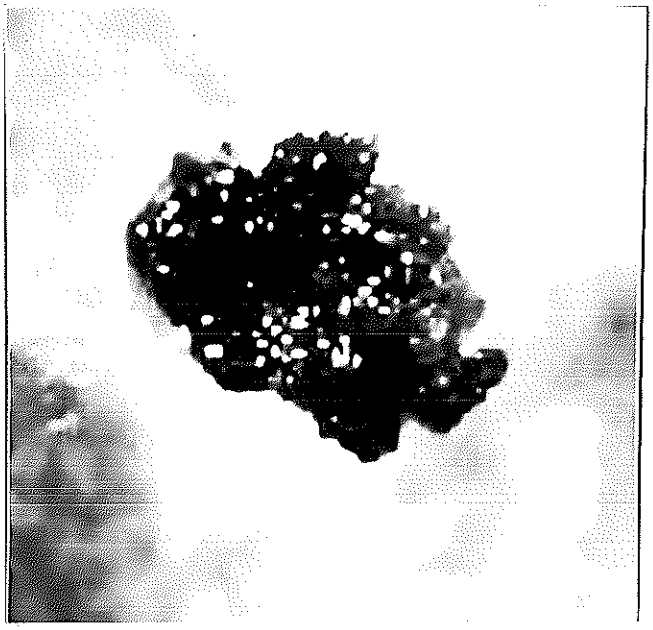

Figure 19 Non-transformed callus on selective medium

After one week, non-transformed callus turned brown on callus inducing medium supplemented with 30 mg/l hygromycin, 250 mg/l cefotaxine and 100 mg/l vancomycin.

Following these recommendations, callus was left on pre-regeneration medium for one week before being transferred to regeneration medium (see Appendix C). Within one to two weeks on regeneration medium green spots began to develop on transgenic callus and shoots began to arise three weeks later. Following removal of callus, six of eighteen hygromycin-resistance callus proliferated and developed green spots on regeneration medium. Four clonal shoots remained green and actively proliferated following transfer of callus to the regeneration medium. Callus and shoots (Figure 20) were transferred to new medium every ten days. After they reached one cm tall, shoots were transferred to rooting medium in Magenta Boxes. After two months, four individual shoots were three to four cm tall, these were transferred to minimal medium until they were at least six to nine cm tall (Figure 21). They were then transferred to soil by removing the entire plant and roots from the sterile medium and placing them into plenty of moist soil (Figure 22). The plants were phenotypically normal. After one month in soil, transgenic rice grew 30cm tall and produced seeds. Unfortunately all seed were sterile.

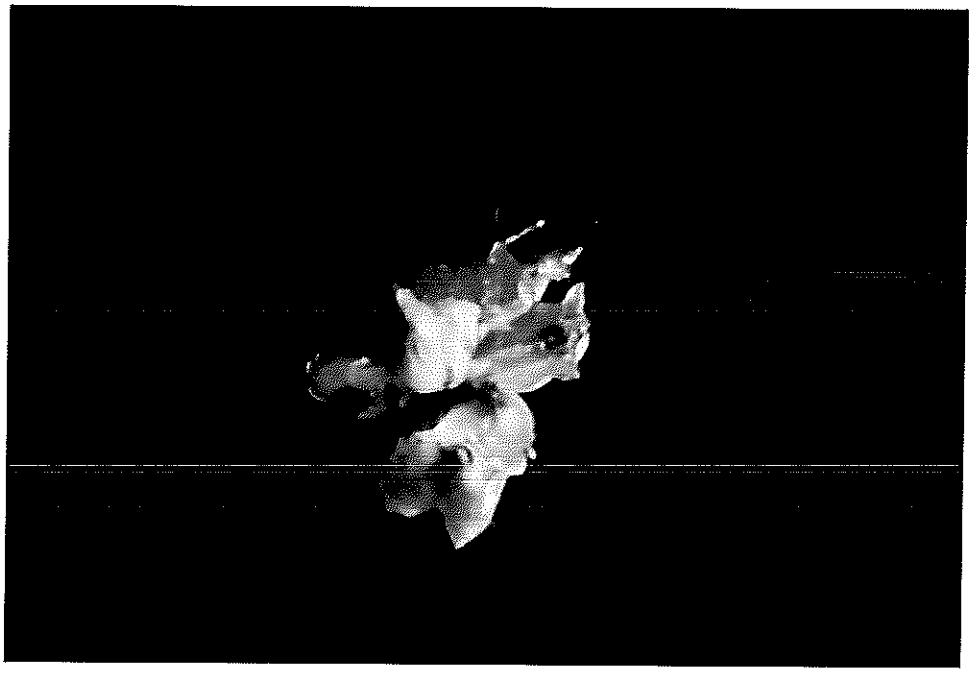

Figure 20 Shoot formation in co-cultivated callus on regeneration medium

Transgenic callus and shoots developed green spots on regeneration medium.

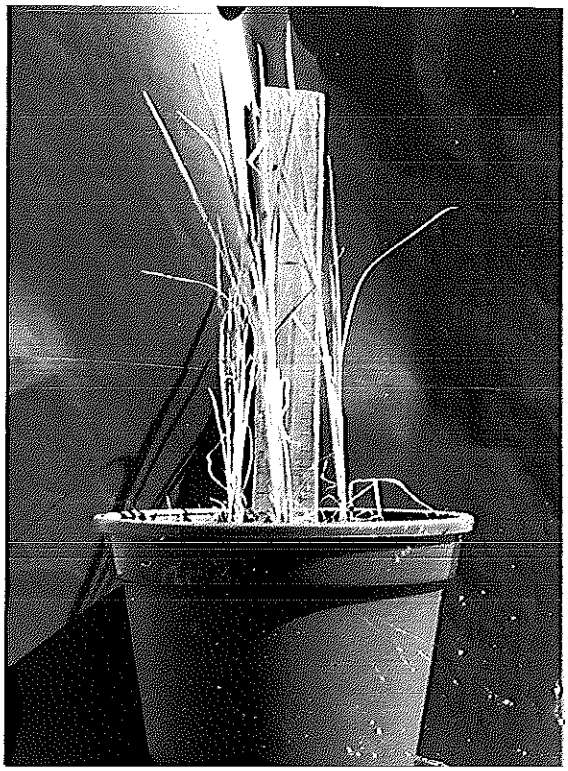

Figure 21 Transgenic rice two months after transfer to the soil

4. Conclusions

A total of 36 strains of halophilic bacteria were isolated from sediment and wastewater of prawn pond. Five of the isolated strain grew and produced high levels of aldehyde dehydrogenase in modified ATCC medium 1270. In comparing the ability of the produced aldehyde dehydrogense activity, strain AS11 was selected because of its high growth rate even in 25% sodium chloride. It is able to catabolize a number of simple alcohols including isopropanol, ethanol and polyethylene glycol. Based on it Gram reaction, colony and cell morphology, biochemical characterization, and utilization of carbohydrate, AS11 has been identified as belonging to the genus *Halomonas*. Studies based on 16S ribosomal RNA sequence analysis of chromosomal DNA provided additional proof of the phylogenetic relationship of the strain AS11 to *Halomonas salina*.

The *H. salina* AS11 was found to produce aldehyde dehydrogenase and alcohol dehydrogenase on production media containing isopropanal or ethanol. Since the expression of ALDH is specifically induced only during growth on these substrates, it is highly likely that these enzymes are at least in part responsible for growth in these media, however additional studies are needed to demonstrate this. Towards this end, the structural gene of *aldh* was localized and sequenced. The 3,227 bp of the *aldh* cluster sequence contains a putative regulatory protein, alcohol dehydrogenase and aldehyde dehydrogenase gene. The open reading frame of the *aldh*

gene could encode a 506-amino acid polypeptide with a calculated molecular mass of 54,919 Da.

The deduced primary structure is similar to aldehyde dehydrogenase genes that have been isolated from other sources. The resulting protein would contain the conserved catalytic domains VELGGKSP and FENGGEVCTCPS that are essential for the dehydrogenase activity of this protein family. This protein also contains a GYGAEAG motif that is believed to be involved in NAD^+ binding.

The expression of *aldh* gene was performed in *E. coli*. The recombinant aldehyde dehydrogenase protein was subjected to SDS-PAGE analysis. This analysis revealed that transgenic bacteria producing ALDH activity produced an extra protein of approximately at 81 kDa consisting of a 26 kDa 6xHis-DHFR tag and the 55 kDa ALDH gene product. The *E. coli* harboring pQA802 produced 2.43 U aldehyde dehydrogenase activity per mg protein after being induced by treatment with 5 μM IPTG overnight at 30°C.

In order to establish the technique of gene manipulation in rice, an *Agrobacterium* -mediated transformation system were used to transfer the AS11 *aldh* gene into rice callus. Four clonal shoots were obtained from regeneration medium and subsequently transferred to soil. The transgenic plants were grown for 2 months, they appeared phenotypically normal that they produced sterile seeds. Future repetitions of this work would be needed to determine if this was the result of the constitutive expression of *aldh* during plant development. However, the result demonstrated the potential of future development of the transgenic rice. The rice that harbored a suitably regulated gene might be better adapted for growth in the extreme conditions such as are found in soil containing prawn pond sediment.

Studies in the future will focus on the identification of the promoter region, and isolation and characterization of the regulatory protein(s), which is required for transcription of *aldh* and *adh*. The knowledge will contribute to the understanding of controlled gene expression in halophile *Halomonas* sp.

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WWW sites

Bio-Rad Laboratories:

http://www.bio-rad.com/cgi-bin/tango.cgi/application bits/literature.query

GenBank

http://ncbi.nlm.nih.gov/entrez/query

PE Applied Biosystems:

http://www.perkin-elmer.com

QIAGEN

http://www.qiagen.com

Science direct

http://www.sciencedirect.com

University of Idaho

http:///www.uidaho.edu

University of Washington Genome Center:

http://www.genome.washington.edu/uwge

Appendix A

Media for microorganism

Media for petri plates are prepared in 1-liter flasks, with each flask containing half liter of medium, which is sufficient for approximately 20 plates. Unless stated otherwise, all components are autoclaved together for 20 minutes at 121°C and 15 pounds pressure. The plates should be allowed to dry at room temperature for 10-15 minutes after pouring. The plates can be stored in sealed plastic bags for over three months. The media were add 1.5% agar for solidify cultivation. The agar is omitted for liquid media.

LB (Luria-Bertani) medium: This medium is used for *E. coli*.

1% (w/v) tryptone or peptone	10	g
0.5% (w/v) yeast extract	5	g
NaCl	5	g
Distilled water	1000	ml

The pH of medium was adjusted to 7.0 with 5 N NaOH. The *E. coli* transformants were grown in LB medium containing 50 μg/ml Amplicilin as a selectable marker.

Mineral salts medium: The modified ATCC culture medium 1270 is suitable for *Halobacterium*.

$CaCl_2$	1	g
KCl	5	g
$MgCl_2$	16	g

$MgSO_4$	24	g
NaBr	0.5	g
NaCl	80	g
$NaHCO_3$	0.2	g
yeast extract	0.5	g
Distilled water	1000	ml

The yeast extract and salt solution were sterilized separately, after which the solutions were aseptically combined. Before sterilization the pH of each solutions was adjusted to 7.3 with 2 M NaOH and optimal temperature is 30°C.

Nutrient medium: This medium is used for screening bacteria.

bacto beef extract	3	g
bacto peptone	5	g
NaCl	50-200	g
Distilled water	1000	ml

The medium was adjusted to pH 7.0 with 1 M HCl and 1 M NaOH and optimal temperature is 30°C.

Tf broth: This medium used for preparation of competent cells.

$MgSO_4$	2	g
bacto-tryptone	10	g
yeast extract	2.5	g
KCl	0.3725	g
Distilled water	500	ml

Resuspend in about 350 ml of distilled water. The pH of medium was adjusted to 8.0 with 1 M NaOH. Bring volume up to 500 ml with distilled water.

YEB medium: This medium used for *Agrobacterium*.

$MgSO_4$	240.7	mg
sucrose	5	g
peptone	5	g
beef extract	5	g
yeast extract	19	g
Distilled water	1000	ml

The medium was adjusted to pH 7.5 with 1 N NaOH.

2YT Medium: This medium used for bacteria.

NaCl	5	g
yeast extract	10	g
tryptone	16	g
Distilled water	1000	ml

The medium was adjusted to pH 7.0 with 1 N NaOH.

Solutions for preparing competent cells

TfB_1 solution

Potasium Acetate	0.44	g
$MnCl_2.4H_2O$	1.484	g
RbCl	1.814	g
$CaCl_2.2H_2O$	0.22	g
glycerol	15	%

Resuspend in 60 ml of deionized water. Adjust the pH to 5.8 with 0.2 M acetic acid. Filter sterilized into a sterile water to 150 ml. Mix the solution and aliquot 30 ml. Store at 4°C.

TfB_2 solution

Sodium MOPS	0.042	g
$CaCl_2.2H_2O$	0.22	g
RbCl	0.024	g
glycerol	15	%

Resuspend in 60 ml of deionized water. Adjust the pH to 6.8 with KOH. Filter sterilized into a sterile water to 150 ml. Mix the solution and aliquot 30 ml. Store at 4°C.

Solutions for electrophoresis

50X TAE: Electrophoresis buffer

Tris Base	242	g
Glacial acetic acid	57.1	ml
0.5 M EDTA pH 8.0	100	ml
Distilled water	1000	ml

Combine the ingredient with distilled water. Bring up to volume. Can be stored for a couple of months. When using this buffer in a gel, its concentration in the gel and the buffer should be 1X. During the run, the voltage should be kept constant and at a level so that the current stays below 70 m Amps (About 60 V is usually safe).

10xTBE: Electrophoresis buffer

Tris Base	107.8	g
Boric acid	55.0	g
Na_2EDTA	8.2	g
Distilled water	1000	ml

The pH should be approximately 8.3 at room temperature. Filter using Whatman #1 filter paper and store at 4°C up to two weeks.

Sample buffer: 30% (v/v) gel loading buffer

25% (v/v)	glycerol
60 mM	EDTA
0.25% (w/v)	Bromophenol blue

Make up in sterile water. Store at room temperature. Combined with samples for electrophoresis.

Solutions for Lambda genomic cloning

Phage buffer:

Tris-HCl, pH 7.4	20	mM
NaCl	100	mM
$MgSO_4$	10	mM

TB top agar: This medium used for preparation of phage culture.

bacto-tryptone	1.0	g
yeast extract	0.5	g

NaCl	0.8	g
Distilled water	100	ml

Microwave or autoclave to melt the agar. When the solution has cooled to 60°C, add 1 ml of 1 M $MgSO_4$.

Solution for purification under denaturing conditions

Lysis buffer

Urea	8	M
NaH_2PO_4	100	mM
Tris-HCl, pH 8.0	10	mM

Wash buffer

Urea	8	M
NaH_2PO_4	100	mM
Tris-HCl, pH 6.3	10	mM

Elution buffers

Urea	8	M
NaH_2PO_4	100	mM
Tris-HCl, pH 5.9 or 4.5	10	mM

Note: Due to the dissociation of urea, the pH of buffer should be adjusted immediately prior to use. Do not autoclave.

Solutions for purification under native conditions

Lysis buffer:

	NaH_2PO_4, pH 8.0	50 mM
	NaCl	300 mM
	Imidazole	10 mM
Wash buffer		
	NaH_2PO_4, pH 8.0	50 mM
	NaCl	300 mM
	Imidazole	20 mM
Elution buffers		
	NaH_2PO_4, pH 8.0	50 mM
	NaCl	300 mM
	Imidazole	250 mM

Buffers and Solutions

Buffer/Solution	Method of preparation/Comment
0.5 M EDTA (pH 8.0)	Add 186.1 g of disodium ethylene diamine tetraacetate.$2H_2O$ to 800 ml of H_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving. (The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approximately 8.0 by the addition of NaOH)

Ethidium bromide (10 mg/ml)	Add 1 g of ethidium bromide to 100 ml of H_2O. Stir on magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer the solution to a dark bottle and store at room temperature. Caution: Ethidium bromide is a mutagen and is toxic. Wear gloves when working with ethidium bromide solutions and a mask when weighing the power.
3 M Sodium Acetate (pH 5.2)	Dissolve 408.1 g of sodium acetate.$3H_2O$ in 800 ml of H_2O. Adjust the pH to 5.2 with glacial acetic acid. Adjust the volume to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving.
5 M sodium chloride	Dissolve 292.2 g of NaCl in 800 ml of H_2O. Adjust the volume to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving.
10% sodium dodecyl sulfate (SDS)	Dissolve 100 g of electrophoresis-grade SDS in 900 ml of H_2O. Heat to 68°C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 liter with H_2O. Dispense into aliquots.
20X SSC	Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of H_2O. Adjust the pH to 7.0 with 10 N solution of NaOH. Adjust the volume to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving.

Hybridization buffer	2X SSC; 1% (w/v) Blocking reagent; 5% (w/v) Dextran sulfate; 0.1% (w/v) SDS Add 10 ml of 20X SSC and 0.5 g Blocking Reagent to 50 ml of distilled H_2O. Before adding the dextran sulphate, heat gradually (up to 60°C) with continuous stirring to dissolve the Blocking Reagent. Slowly add 5 g dextran sulphate (Molecular weight ≅ 500,000) and stir until dissolved. Add 1 ml of a 10% solution of SDS and dH_2O to a final volume of 100 ml. Store at –20°C when not in use.
TE pH 8.0	10 mM Tris.HCl (pH 8.0); 1 mM EDTA (pH 8.0) Combine 1.22 g of Tris and 0.4 g of Na_2-EDTA-$2H_2O$ with 500 ml of dH_2O. Adjust the pH to 8.0 with HCl while stirring. Bring to a final volume of 1,000 ml with dH_2O.

Appendix B

Protocol for Fluorescence-Based Automated DNA sequencing

Template preparation

1. Double-stranded DNA templates from plasmid-based vector containing the aldehyde dehydrogenase gene are used in this study.
2. Purification of plasmid DNA from each clone
3. Removal of RNA from Preparation of plasmid DNA

Protocol for cycle sequencing

This protocol has been optimized for an automated DNA Thermal cycler (Perkin-Elmer) model 2400. The total time required for 25 cycles should be 3 hours.

1. Mix the following reagents in a labeled 0.2 ml microcentrifuge tube

Terminator Premix	8	µl
double-stranded DNA, 0.2 µg/µl	1.5-2.5	µl
Primer	3.2	pmol
DDW	q.s.	µl
Final Reaction Volume	20	µl

2. Cycling the reactions:

2.1 Place the tubes in a thermal cycler preheated to 96 °C

2.2 Immediately after placing the tubes in the thermal cycler, begin thermal cycler as follows:

Segment	Step	Temperature (°C)	Time	Number of cycles
1	Denaturing	96	5 minutes	1
2	Denaturing Annealing Extension	96 50 60	10 seconds 5 seconds 4 minutes	25
3	Holding	4	∞	1

2.3 Proceed with "Purifying Extension Products"

3. Purifying Extension Products by Ethanol Precipitation Protocol

3.1 For each reaction, prepare a 0.5 ml microcentrifuge tube by adding the following: 2.0 µl 3 M Sodium acetate, pH 4.8; 50 µl 95% ethanol

3.2 Transfer the entire 20 µl contents of the reaction tubes to the microcentrifuge tubes containing the ethanol solution, vertex and place on ice for 10 minutes.

3.3 Centrifuge in a microcentrifuge at 12;000 rpm for 15-30 minutes.

3.4 Carefully aspirate the ethanol solution with a micropipetter. Remove as completely as possible.

3.5 Rinse the pellet by adding 250 µl 70% ethanol. At this point it is not necessary to centrifuge.

3.6 Carefully aspirate all the alcohol solution with a micropipetter. Use a Kim Wipe to remove any alcohol from the side of the tube. Be careful not to disturb the pellet, which may or may not be visible.

3.7 Dry the pellet in a vacuum centrifuge.

4. Preparing and loading the samples

4.1 Preparing a mixture of the following reagents:

5 µl deionized formamide (1 g Amberlite MB-1/1 ml formamide)

1 µl 50 mM EDTA (pH 8.0) with blue dextran (50 mg/ml)

4.2 Add 6 µl of this mixture to each tube and agitate vigorously to dissolve the dry residue. Centrifuge the solution briefly to collect all the liquid at the bottom of the tube.

4.3 When the gel is ready for loading, heat the samples at 90°C for 2 minutes to denature, then transfer them immediately onto ice.

4.4 Load the 1.5 µl of samples into a separate lane of the acrylamide gel.

4.5 Electrophoresis parameter; voltage is 1,000 V, current is 35 mA, power 50 W, gel temperature 51°C and Laser power 40 mW. Run time is 7 hours and run speed is 1200 scans/hour of 36-cm well-to-read distance.

Preparation of 4.5% acrylamide gel solution

Buffer/Solution	Method of preparation
1X TBE buffer (per run)	Add 150 ml of 10X TBE stock solution to 1350 ml of dH_2O. Stir for 5 min. Make fresh daily.
40% Acrylamide stock solution	Add 48 ml dH_2O directly to the bottle containing, preweighed 29:1 acrylamide/Bis-acrylamide powder. Stir for 30 min. Store at 4°C up to a month.
10% Ammonium Persulfate (W/V)	Add 2 ml of dH_2O to 0.2 g APS and stir or shake until dissolved. This solution can be stored at 4°C for 5 days.

1. Casting the gel

2. Combine 18 g of urea and 5.63 ml of 40% acrylamide stock solution in a 150 ml erlenmeyer flask. Add dH_2O to bring the volume up to 25 ml.

3. Stir and gently heat the solution until the crystals just begin to dissolve. Turn off the heat and continue to stir until the crystals have dissolved completely.

4. Add 5 ml of filtered 10X TBE stock solution and add dH_2O to bring the volume to 50 ml.

5. Vacuum filter the acrylamide solution using a 0.2 µm until for 5 minutes.

6. Add 250 µl of 10% APS to the 50 ml filtered gel solution and swirl for a few seconds. Note : Make sure all gel casting reagents, gel supplies and the laboratory are at 20-23°C

7. Add 30 µl TEMED to the acrylamide solution and gently swirl for a few seconds.

8. Gently pour the solution between the glass plates until the solution is up to the notch. To avoid air bubbles, do not interrupt the flow once you have started to pour.

9. Let the gel polymerize undistured at room temperature for at least 2 hours, if storing the gel for >24 hours. pour 1X TBE buffer over the comp area, cover with plastic wrap and store at room temperature. (should be used within 48 hours)

Southern Blotting and Hybridization (Sambrook *et al.*, 1989)

1 Southern Blotting

Reagents and solutions

- 0.2 N HCl
- Denaturation solution (1.5 M NaCl, 0.5 N NaOH)
- Neutralization solution (3 M NaCl, 0.5 M Tris-HCl pH 8.0)

- 20X SSC (3 M NaCl, 0.3 M Sodium citrate, adjust the pH to 7.0)
- 2X SSC
- 0.1% SDS

Procedure

1. After electrophoresis, transfer the gel to a glass baking dish and trim away any unused areas of the gel with a razor blade. Cut off the bottom left-hand corner of the gel; this serves to orient the gel during the succeeding operations.
2. Soak the gel for 10 minutes in 500 ml of 0.2 N HCl. The acid solution was decant and rinsed the gel briefly with deionized water several times.
3. Denature the DNA by soaking the gel for 45 minutes in several volumes of denaturation solution with constant, gentle agitation.
4. Rinsed the gel briefly in deionized water and then neutralize it by soaking for 30 minutes in several volumes of neutralization solution at room temperature with constant, gently agitation. Change the neutralization solution and continue soaking the gel for a further 15 minutes.
5. While the gel is in the neutralization solution, wrap a piece of Whatman 3MM paper around a stack of glass plates to form a support that is longer and wider than the gel. Place the wrapped support inside a large baking dish. Fill the dish with transfer buffer (20X SSC) until the level of the liquid reaches almost to the top of the support. When the 3MM paper on the top of the support is thoroughly wet, smooth out all air bubbles with a glass rod.
6. Using a fresh scalpel or a paper cutter, cut a piece of nylon filter about 1 mm larger than the gel in both dimensions. Use gloves and blunt-ended forceps to handle the filter. A nylon filter that has been touched by greasy hands will not wet.

7. Float the nylon filter on the surface of a dish of deionized water until it wets completely from beneath, and then immerse the filter in transfer buffer for at least 5 minutes. Using a clean scalpel blade cut a corner from the nylon filter to match the corner cut from the gel.

8. Remove the gel from the neutralization solution and invert it so that its underside is now uppermost. Place the inverted gel on the support so that it is centered on the wet 3MM papers. Make sure that there are no air bubbles between the 3MM paper and the gel.

9. Surround, but do not cover, the gel with Saran Wrap or Parafilm. This serves as a barrier to prevent liquid from flowing directly from the reservoir to paper towels placed on the top of the gel. If these towels are not precisely stacked, they tend to droop over the edge of the gel and may touch the support. This type of short-circuiting is a major cause of inefficient transfer of DNA from the gel to the filter.

10. Place the wet nylon filter on top of the gel so that the cut corners are aligned. One edge of the filter should extend just over the edge of the line of slots at the top of the gel. Do not move the filter once it has been applied to the surface of the gel. Make sure that there are no air bubbles between the filter and the gel.

11. Wet two pieces of 3MM paper (cut to exactly the same size as the gel) in 2X SSC and place them on top of the wet nylon filter. Smooth out any air bubbles with a glass rod.

12. Cut a stack of paper towels (5-8 cm high) just smaller than the 3MM papers. Place the towels on the 3MM papers. Put a glass plate on top of the stack and weigh it down with a 500 g weight. The objective is to set up a flow of liquid from the reservoir

through the gel and the nylon filter, so that the fragments of denatured DNA are eluted from the gel and are deposited on the nylon filter.

13. Allowed the transfer of DNA to proceed for 8 to 24 hours. As the paper towels become wet, they should be replaced.

14. Remove the paper towels and the 3MM papers above the gel. Turn over the gel and nylon filter and lay them, gel side up, on a dry sheet of 3Mmpaper. Mark the positions of the gel slots on the filter with a very-soft-lead pencil.

15. Peel the gel from the filter and discard it. Soak the filter in 2X SSC for 5 minutes at room temperature. This removes any pieces of agarose sticking to the filter.

16. Remove the filter from 2X SSC and allow excess fluid to drain away. Placed the filter flat on a paper towel to dry for at least 30 minutes at room temperature.

17. Sandwich the filter between two new sheets of dry 3MM paper. Fix the DNA to the filter by UV crosslinking.

2 DNA hydridization precedure

Reagents and solutions

- Prehybridization Buffer

- Hybridization Buffer (Add Fluorescein-labeled pGAS1101 probes to Prehybridization Buffer)

- Carrier DNA (e.g. sheared sonicated salmon sperm DNA)

- Labeled probe

- Stringency wash buffer; a) 2.0X SSC and 1.0% SDS; b) 0.2X SSC and 0.1% SDS

Procedure

1. Equilibrate the membrane with the crosslinked, denatured target in 2X SSC. Ensure that the entire membrane is wetted before proceeding.

2. Insert membrane into an appropriate hybridization pouch or tube. Add Prehybridization Buffer supplement with 50 µg/ml Carrier DNA. For standard heat sealable polyester pouches, a minimum volume of 0.1 ml/cm^2 of membrane is recommended.

3. Incubate at 65°C, in a hybridization oven, for at least one hour.

4. Combine the probe with 200-300 µl of Hybridization Buffer and Carrier DNA. The final concentration of Carrier DNA in the Hybridization step should be 50 µg/ml. Heat 95°C for 3-5 minutes and immediately place on ice for 5 minutes.

5. Add Hybridization Buffer, equilibrated at 65°C, to the denatured probe mix. The recommended hybridization volume for standard polyester pouches is 0.05-0.1 ml/cm^2 of membrane. The recommended concentration of labeled probe in Hybridization Buffer is 20 ng/ml.

6. Empty the Hybridization Buffer from the pouch or tube and add the Hybridization Buffer with probe and Carrier DNA.

7. For maximum probe to target base paring, hybridize overnight at 65°C, in a hybridization oven. If the template DNA for the probe labeling reaction was less than 500 base pairs then, hybridize at 55-60°C instead of 65°C.

Detection-Nucleic Acid Chemiluminescence Reagent (DuPont NEN®, USA)

All step are carried out at room temperature.

Reagents and solutions

- Buffer 1; 150 mM NaCl and 100 mM Tris-HCl pH 7.5 (Note: To minimize background, pass through a 0.22 μm filter before use)
- Buffer 2; 150 mM NaCl, 100 mM Tris-HCl pH 7.5 and 0.5% blocking reagent (Note: To minimize background, pass through a 0.22 μm filter before use. Add blocking reagent after filtration. Prepare the buffer an hour in advance by dissolving at 50-70°C)
- Antibody Conjugate Solution; 150 mM NaCl, 100 mM Tris-HCl pH 7.5, 0.5% blocking reagent and 1/1000 (v/v) Antifluorescein-AP Conjugate
- Buffer 3; 100 mM NaCl and 100 mM Tris-HCl pH 9.5

Procedure

1. Immerse and vigorously agitate the membrane in Buffer 1 for 5 minutes. Use at least 1 ml of buffer per cm^2 of membrane. Ensure that all SDS (indicated by soap-like foam in wash buffer) has been washed from the membrane before proceeding.
2. Block the membrane in Buffer 2 for an hour with gentle agitation. Use at least 0.1 ml of buffer per cm^2 of membrane.
3. Place the membrane in the Antibody Conjugate Solution and incubate for an hour with gentle agitation. Use at least 0.1 ml of solution per cm^2 of membrane.
4. Vigorously wash the membrane 4X 5minutes in Buffer 1.
5. Vigorously wash the membrane 2X 5minutes in Buffer 3.

Chemiluminescence substrate incubation (DuPont NEN®, USA)

Reagents and solutions

- CDP-Star™ Nucleic Acid Chemiluminescence Reagent [(Ready-to-Use), NEL601]
- Blotting paper

- Polypropylene Sheet Protectors or plastic wrap

Procedure

1. Transfer the membrane from the final Antibody Conjugate wash solution to a clean container.

2. Completely cover the membrane with CDP-Star™ (0.01-0.05 ml/cm^2) .

3. Incubate the substrate on the membrane for 5 minutes.

4. Gently removed excess solution with blotting paper.

5. Place the damp membrane inside a polypropylene sheet protector with the blank interleaf remove. Plastic wrap can be used in place of a sheet protector. The plastic sheet protects the film from the wet membrane. Ensure that there is no moisture on the outside of the sheet protector before overlaying with film.

Film Exposure (Fuji ^{Rx}, Japan)

Reagents and solutions

- Autoradiography film

- Film Expose Cassette

Procedure

1. Place the prepared membrane, DNA side up, in the film cassette.

2. Expose the film for 5-10 minutes and then develop.

3. Repeat the film exposure, varying the exposure time as needed for optimal sensitivity. A 5-10 minutes exposure results in adequate signal detection for many applications (e.g., single gene copy). However, greater signal detection can be achieved when film exposures begin at least 1 hour after incubation with CDP-Star™.

Appendix C

Solution for seed sterilization

Domestos solution

bleach	10.5	%
Na_2CO_3	0.3	%
NaCl	10.0	%
NaOH	0.5	%
SDS	0.1	%

Make fresh before use.

Media and solution for rice transformation

Media for petri plates are prepared in 1-liter flasks, with each flask containing half liter of medium. All components are autoclaved together for 20 minutes at 121°C and 15 pounds pressure. The plates should be allowed to dry at room temperature for 10-15 minutes after pouring. The plates can be stored in sealed plastic bags for a month. The agar is omitted for liquid media.

Basic Medium:

N6 salts	1	X
Kao-Michayluk vitamins	1	X
proline	500	mg/l
glutamine	500	mg/l

Casein Enzymatic Hydrolysate	300	mg/l
Phytogel	3.0	g/l

The medium was adjusted to pH 5.8.

Callus inducing Medium (CIM):

Basic Medium

sucrose (or maltose)	3.0	%
2,4-D	2.5	mg/l

Co-cultivation Medium CIM-AS):

Basic Medium

sucrose (or maltose)	3.0	%
glucose	1.0	%
Acetosyringone	100	µM
2,4-D	2.5	mg/l

The medium was adjusted to pH 5.2

Pre-Selection Medium (CIM-C/V):

Basic Medium

sucrose (or maltose)	3.0	%
2,4-D	2.5	mg/l
Cefotaxime (Claforin®)	250	mg/l
Vancomycin	100	mg/l

Selection Medium (CIM-30H, or CIM-50H):

Basic Medium

sucrose (or maltose)	3.0	%

2,4-D	2.5	Mg/l
Cefotaxime (Claforin®)	250	mg/l
Vancomycin	100	mg/l
Hygromycin	30 or 50	mg/l

Pre-regeneration Medium (Pre-reg-H):

Basic Medium		
maltose	2.0	%
Sorbitol	3.0	%
Hygromycin	50	mg/l
Cefotaxime (Claforin®)	250	mg/l
BAP	2	mg/l
NAA	1	mg/l
ABA	5	mg/l

Regeneration Medium:

Basic Medium		
maltose	2.0	%
Sorbitol	3.0	%
Hygromycin	50	mg/l
Cefotaxime (Claforin®)	250	mg/l
BAP	3	mg/l

Rooting Medium:

Chu N6 Salt		
Kao-Michayluk vitamins	1	X

Sucrose	2.0	%
Hygromycin	50	mg/l
Cefotaxime (Claforin®)	250	mg/l
Agar	0.8	%

Mineral Medium:

Muskiringe-Skoog Salts	½	X
sucrose	1	%
Agar	0.8	%

Appendix D

IUB codes

A = adenosine	S = G or C (Strong-3H bonds)
C = cytosine	W = A or T (Weak-2H bonds)
G = guanosine	Y = C or T (pYrimidine)
T = thumidine	B = C, G or T
U = uracil	D = A, G or T
K = G or T (Keto)	H = A, C or T
M = A or C (aMino)	V = A, C or G
R = A or G (puRine)	N = aNy base

Appendix E

Abbreviations and molecular weight for Amino acids

Amino acid	Three-letter abbreviation	One-letter symbol	Molecular weight
Alanine	Ala	A	89
Arginine	Arg	R	174
Asparagine	Asn	N	132
Aspartic acid	Asp	D	133
Asparagine or Aspartic acid	Asx	B	-
Cysteine	Cys	C	121
Glutamine	Gln	Q	146
Glutamic acid	Glu	E	147
Glutamine or Glutamic acid	Glx	Z	-
Glycine	Gly	G	75
Histidine	His	H	155
Isoleucine	Lle	I	131
Leucine	Leu	L	131
Lysine	Lys	K	146
Methionine	Met	M	149
Phenylalanine	Phe	F	165
Proline	Pro	P	115
Serine	Ser	S	105
Threonine	Thr	T	119
Tryptophan	Try	W	204
Tyrosine	Tyr	Y	181
Valine	Val	V	117

Similality group

Group number	Amino acid
1	D N
2	E Q
3	S T
4	K R
5	F Y W
6	L I V M

Symbol for Amino acid alignment

Symbol	
Capital letter	Conserved 100 percent
Small letter	Conserved 80-60 percent
*	10, 30, 50, 70, 90, 110, 130, 150, 170, etc.

Publications

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