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SCREENING FOR PLASMIDS IN HALOPHILES

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Seven strains of halophiles that are associated with organophosphorus acidic (OPA) anhydrase activity were screened for the presence of plasmid DNA. Only one out of seven showed evidence of plasmid DNA. The presence of this particular plasmid DNA was not correlated with antibiotic resistance. The results suggest that the gene that codes for OPA anhydrase is located on the chromosomal DNA. The information obtained in this study will be useful for the cloning of OPA anhydrase gene(s) of these halophiles. Keywords: Halophilic Bacteria. (AW)

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PREFACE

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CONTENTS

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SCREENING FOR PLASMIDS IN HALOPHILES

1. INTRODUCTION

Natural high-salt environments are widely distributed on the earth. Living bacteria are found in these environments where they often reproduce extremely well. Organisms capable of living at high salts are called halophiles. The most striking feature of the halophiles is their high requirement for NaCl. Their requirement for NaCl for growth is specific. No other salt or chemical can replace NaCl, except potassium.

Dr. DeFrank, Biotechnology Division, U.S. Army Chemical Research, Development and Engineering Center (CRDEC) isolated a number of halophiles from soil and water samples obtained from salt spring in Utah. Several of these strains possess high levels of organophosphorus acidic (OPA) anhydrase activity against the agent simulant diisopropyl fluorophosphate (DFP). Previously, work mainly concentrated on growth condition for maximum OPA anhydrase activity (1). Two of the strains in particular, designated 6-5 and 30-3, show considerable promise as sources of high activity enzymes for soman degradation. However, these strains have not been identified. Because of high salt (50-100 g/L) and magnesium (5-10 g/L) requirements of these strains, it is quite unlikely that they will prove to be pathogenic. So far, little has been done to elucidate the genetic or molecular aspects of these strains. The objective of this study was to grow these bacterial isolates and screen them to see whether any plasmids could be found. The strategy of cloning the genes, which code for production of these critical enzymes, will depend on whether the genes are located in plasmid or chromosomal DNA. This study could have considerable impact on the development of a enzyme-based detection and decontamination system by the U.S. Army in the future.

2. MATERIALS AND METHODS

2.1 *Bacterial Strains and Culture Media.*

Bacterial strains 6-5, 11-5, 11-6/2, 26-1, 28-2, 28-3, and 30-3 of halophiles were grown on salts medium (H-medium) consisting of 50 g NaCl, 10 g $MgSO_4$, 10 g Neopeptone, 6 g yeast extract, 5 g casamino acids, 2.5 g HEPES per liter, pH 6.8 (2). Usually, bacterial strains from a fresh plate culture were used to inoculate the medium, and the culture was grown overnight in an incubator shaker at 37 °C. To assay antibiotic resistance of halophilic strains, concentrations of antibiotics used per milliliter of H-broth were as follows: ampicillin, 25 μg; chloramphenicol, 25 μg; gentamycin, 25 μg; streptomycin, 20 μg; and tetracycline, 10 μg.

2.2 *Plasmid DNA Isolation.*

The following two methods were used for plasmid DNA isolation.

2.2.1 *SDS-Alkaline Method (3).*

Cells were grown in 10 mL of H-medium overnight at 37 °C and pelleted by centrifugation at 7500xg, 4 °C, for 5 min in a Beckman JA 20 rotor. The cell pellet was resuspended in 0.5 mL of buffer A (20 % sucrose, 20 mM EDTA, 25 mM Tris-HCl, pH 8). The cells were then lysed by adding 0.5 mL of lysing solution (4 mg/mL lysozyme in buffer A), which was mixed by brief suspension. The solution was incubated at room temperature for 10 min. Addition of 2.0 mL freshly prepared 1% SDS-0.2N NaOH solution was immediately followed by several inversions. The lysed solution was incubated in an ice-water bath for 10 min, and 1.5 mL of 3 M KOAc-1.8 M formic acid were added forming a white floc-like material. The solution was chilled in an ice-water bath for 15 min and centrifuged at 27,000xg for 15 min. The clear supernatant was transferred to new centrifuge tubes, and two volumes of phenol-chloroform solution (1:1 v/v) were added. The solution was emulsified by shaking briefly, and the emulsion was broken by centrifugation (7500xg, 5 min, 4 °C). The upper aqueous phase was transferred to new tubes, and the nucleic acids were precipitated by the adding 2.5 volumes of ice-cold ethanol. The tubes were placed at -20 °C for 30 min. The nucleic acids were pelleted by centrifugation at 12,000xg for 15 min at 4 °C, and the resulting pellet was dried under a vacuum for 10 min. The pellet was resuspended in 150 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

2.2.2 *SDS-Alkaline at Elevated Temperature.*

The modified procedure of Kado and Liu (4) was used. Briefly, the procedures for growing of halophiles were the same as those described above. The cells were lysed by adding 22 mL of lysing solution (3% SDS, 50 mM Tris, pH 12.6) and gently mixing. The solution was heated at 60 °C for 1 hr, and 2 volumes of phenol-chloroform (1:1 v/v) were added. After brief mixing by inversion, the emulsion was centrifuged in a microfuge for 10 min. The upper aqueous phase was transferred to a new tube. After phenol-chloroform treatment, the DNA samples were precipitated down as described above.

2.3 *Agarose Gel Electrophoresis.*

A sample of 10-30 μL of crude plasmid preparation was mixed on parafilm with 0.2 volume of 0.25% bromocresol purple in 50% glycerol-10 mM Tris-acetate (pH 8) and loaded on 0.7% agarose gel (14 x 15 cm) or alkaline agarose gel for electrophoresis following the procedure of Maniatis (5). Lambda bacteriophage DNA cut with Hind III served as the size marker. DNA bands were visualized under ultraviolet light after soaking gels in 0.4 μg of ethidium bromide per milliliter for 30 min and destained in water for 10 min.

3. RESULTS AND DISCUSSION

Seven different halophiles with OPA activity (assigned 6-5, 11-5, 11-6/2, 26-1, 28-2, 28-3, and 30-3) were used in the present study. Of these seven strains, 6-5 and 30-3 contain the highest level of OPA anhydrase activity (1). These bacterial strains are all halophilic, gram-negative, aerobic with an optimum growth temperature of 37 °C and a pH optimum between 6-8 (1). Previously, work has mainly concentrated on growth condition for maximum OPA anhydrase activity. Little has been done to elucidate the genetic or molecular aspects of these strains. To determine if a given bacterium harbors plasmids, it is necessary to isolate DNA by plasmid DNA isolation procedures. The most commonly used method to isolate plasmid DNA is the SDS-alkaline lysis procedure (3). Over the past few years, several techniques were also developed for isolating large plasmid DNAs (4,6-8). Among them, a protocol that used alkaline-SDS at elevated temperatures (55-65 °C) is a rapid and reproducible method applicable to many different bacteria (3). Plasmids of different sizes can be easily detected by both procedures. For comparison, the different halophiles were screened for the presence of plasmid DNA by these two different isolation procedures.

The DNA preparations obtained were analysed on 0.7% agarose gel electrophoresis. After gel staining with ethidium bromide, a distinct plasmid band was seen only in strain 28-2 preparation from both isolation procedures (see the Figure, A and C). No plasmid DNA was detected in the other halophilic strains. Both plasmid DNA preparations of 28-2 were also confirmed in a denatured alkaline agarose gel electrophoresis (see the Figure, B and D). This gel provided the denatured plasmid DNA band and gave a better resolution on the size of plasmid. The size of this plasmid DNA, by comparision with the known DNA size marker (λ/Hind III), was estimated ~20 kb (kilo basepairs). As described in "Methods," the K-acetate-formic acid addition (Procedure 1) and heat-treatment (Procedure 2) following the alkaline-SDS step are essential for the complete cell lysis and removal of chromosomal DNA. To facilitate the screening for the possible existence of plasmids in low copy number as seen in most of the soil bacteria, high concentration of DNA preparations were used in these gel electrophoresis. Consequently, a small amount of chromosomal DNA was detected in the DNA preparations. The plasmid in 28-2 had a low copy number, estimated at less than 5 copies per cell. It was reported that the extreme halophiles contain high percentage of plasmid (7). The finding that only one out of our seven strains contains a plasmid suggests that they may be different from the extreme halophiles. The lower requirement of salt for growth in the seven strains also confirms this observation.

Plasmids generally encode proteins that are not essential for the growth of the cell. However, many plasmids encode the proteins that are required for resistance to antibiotics, production of antibiotics or toxic materials, or degradation of complex organic compounds (9). All seven strains were tested for resistance to five antibiotics (ampicillin, chloramphenicol, gentamycin, streptomycin, and tetracycline) with a view to assigning functions to the plasmid detected. The concentration of antibiotics employed in these tests was the lowest possible to give complete inhibition for growth of the recipient cells and commonly used for the screening of plasmids present at low copy number (10). As shown in the table, all strains were sensitive to ampicillin and chloramphenicol but resistant to streptomycin and tetracycline. In addition, strains 6-5, 26-1, 28-3, and 30-3 were also resistant to gentamycin. The results showed considerable homogeneity between these strains, with little evidence to correlate resistance with the presence of plasmid DNA. These antibiotic markers, however, would be useful to grow or enrich the halophiles and make possible a selective tool. The results also suggest that the gene that codes for OPA is more likely located on the chromosomal instead of plasmid DNA; therefore, preparation of gene libraries should be of major use for cloning of these important decontamination enzymes.

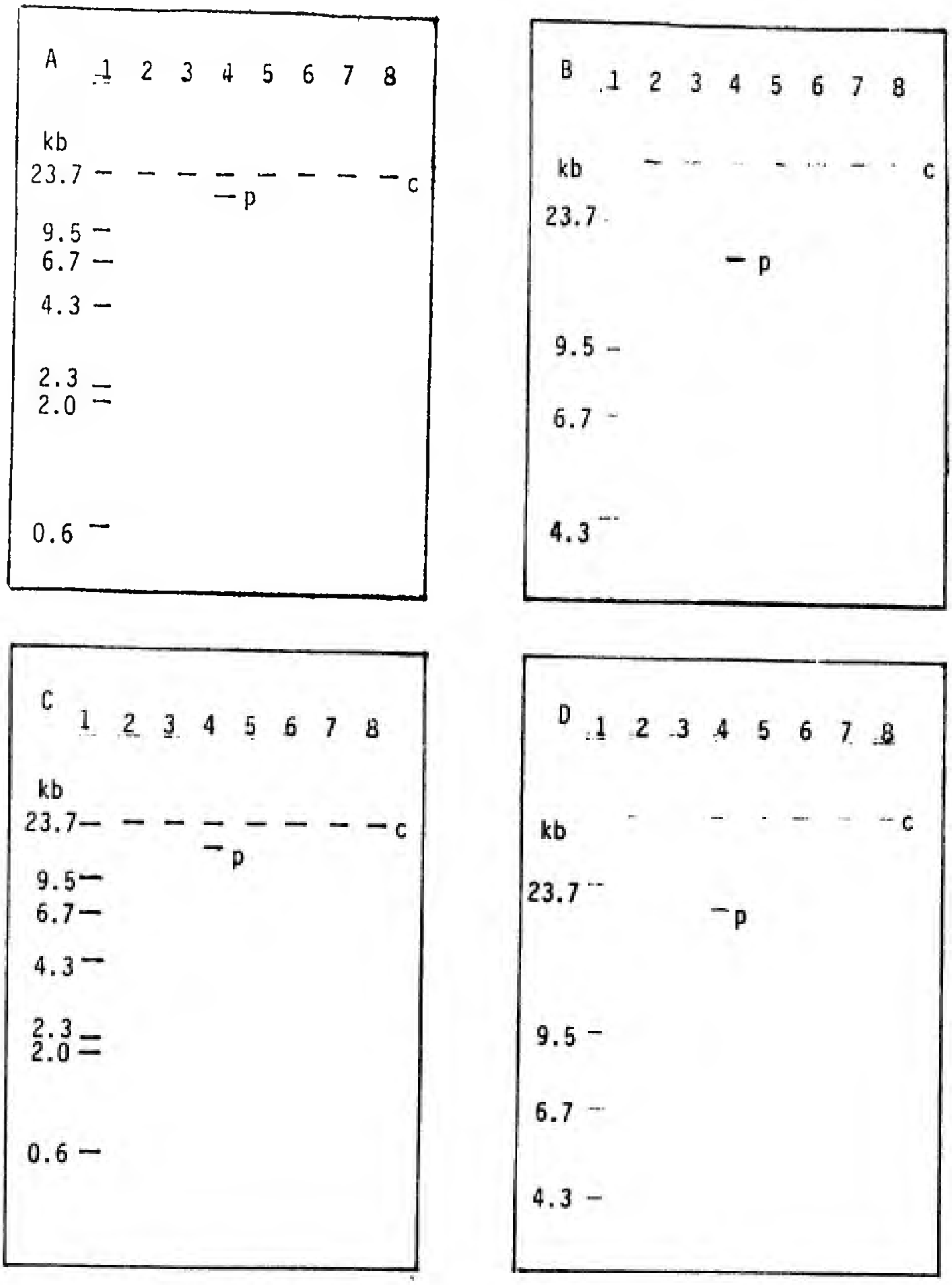

Figure. Plasmid DNA Identified by Diagrammatic Representation of Gel Electrophoresis. Plasmid DNA preparations of different halophilic strains were isolated by either alkaline-SDS (A and B) or alkaline-SDS with elevated temperature (C and D) and separated on 0.7% agarose gel (A and C) or 0.7% alkaline agarose gel (B and D). Lane 1, λ/Hind III DNA marker; lane 2, 6.5; lane 3, 11-5; lane 4, 11-6/2; lane 5, 26-1; lane 6, 28-2; lane 7, 28-3; lane 8, 30-3; c, chromosomal DNA; and p, plasmid DNA.

Table. Antibiotic Resistance of Halophiles

Bacterial Strains	Antibiotic Resistance Markers				
	Ampicillin	Chloramphenicol	Gentamycin	Streptomycin	Tetracyclin
6-5	-	-	+	+	+
11-5	-	-	-	+	+
11-6/2	-	-	-	+	+
26-1	-	-	+	+	+
28-2	-	-	-	+	+
28-3	-	-	+	+	+
30-3	-	-	+	+	+

4. CONCLUSION

Seven strains of halophiles were screened for the presence of plasmid DNA. Only one out of seven showed evidence of plasmid DNA (28-2 strain). The size of that particular plasmid is ~20 kb. The presence of this particular plasmid DNA was not correlated with antibiotic resistance. The information obtained in this study will be useful for the cloning of OPA anhydrase gene(s) that are associated with these halophiles. With the advent of recombinant DNA, we immediatly recognize the probability of converting bacteria and other easily cultured organisms into fermentors for the large-scale production of these valuable and important enzymes for decontamination.

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