ISOLATION OF MARINE TOLUATE-OXIDIZING BACTERIA

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MATERIALS AND METHODS

Bacterial strains. The two TOL plasmid-containing organisms that were used in this study were Pseudomonas arvilla (PaW) and Pseudomonas putida (arvilla) mt-2.

The organisms, designated XF8, SF9 and XF15 were isolated from the Great Sippewissett Salt Marsh, Woods Hole, Mass. Initially, two types of marsh plots were used to isolate the organisms. One type of marsh plot was a heavily fertilized plot (designated XF) and the second was an unfertilized, control marsh plot (designated C). Samples of the marsh plots were taken from only the top 2 cm of each plot. These 2 cm core samples were made using a cork borer. Two XF plots were sampled and 2 C plots were sampled. Three core samples were taken from each individual plot. The core samples were collected in large beakers designated either XF or C, and the contents in each beaker were then mixed together with a spatula. A sample weighing 1 gram was then removed from each beaker and placed in 250 ml Erlenmeyer flasks containing 100 ml of a modified Baumann and Baumann (1981) medium containing 5 mM m-toluate (MBT medium). The flasks were incubated on a shaker for 4 days at 30°C. After incubation, 1 ml aliquots were taken from the above flasks and diluted 10^1, 10^2 and 10^3 using 9 ml artificial seawater (ASW) dilution blanks. Next, 0.1 ml was removed from each dilution blank and transferred to MBT agar medium using the spread plate technique.

After incubation, the plates were examined with the aid of a stereo dissecting microscope. Based on differences observed in the morphological characteristics of the colonies on the above plates, 15 isolates were obtained from the XF plots and 6 from the C plots. Two colony types were readily distinguished, they produced a diffusable pigment which spread through the surrounding agar. One colony type produced a brownish-black pigment, while the second colony type produced a yellow pigment. Neither colony type fluoresced when exposed to long wavelength ultraviolet light. Spread plates designated XF contained both types of pigment producing colonies. Only the brownish-black pigment-producers were observed on the C plates.

As many different colonies as possible were picked from the XF and C spread plates. The colonies were streaked on fresh MBT plates and incubated for 48 h at 30°C. This process was repeated until the 15 XF isolates and 6 C isolates were purified. Out of the above XF isolates, all but XF8 XF9 and XF15 were discarded because they grew either as well or better on MB agar (minus m-toluate) as on MBT agar. These colonies also did not turn liquid MBT shake tubes turbid. In comparison, XF8, XF9 and XF15 grew very poorly on MB agar (minus m-toluate) when compared with growth on MBT agar. These 3 isolates also produced turbid MBT liquid shake tubes after incubation for 48 h at 30°C. The isolates XF8, XF9 and XF15 are yellow pigment-producing colony types. They are given different designations due to the different colonial appearance that each displays on MBT agar.

produced a diffusable bronish-black pigment; however, no yellow pigment producers were observed on the original C-spread plates.

Media. Enrichment cultures were prepared from a modified Baumann (1981) medium, designated MB, containing 5 mM toluic acid. The medium was solidified with 2% Agar (Difco) for the initial isolation and purification of the toluate-degrading microorganisms. The composition of MBT medium is given in Appendix 1. The composition of MBT medium is given in Appendix 1. The basal medium is prepared from 10X stock solutions; these are combined and diluted to 2X and autoclaved. If a solid medium is prepared then the agar is combined with the basal medium and autoclaved. Wolfe's mineral solution was added to the 2X basal medium before it was autoclaved. For every liter of complete medium, 10 ml of the mineral solution was added. The artificial Sterile Seawater (Gerhardt, 1981) is prepared from 10X stock solutions of each of the salts. A 1X solution is prepared after combining and diluting the individual stock solutions. This 1X solution of Artificial Sterile Seawater, ASW, is then autoclaved separately. A precipitate develops in this solution upon autoclaving; the precipitate should redissolve if the solution is brought to room temperature and mixed. Equal volumes of the 1X ASW and the 2X basal medium are combined and stirred, the medium is then ready for use. When preparing solid MBT medium, the basal solution containing agar is first held at 60°C while the ASW (which is held at room temperature) is added. The complete medium is then stirred on a hot plate -- magnetic stirring device.

Enrichment cultures were diluted with sterile ASW (1X) before being used for spread plates. The spread plates contained MBT agar. Colonies were isolated from these plates and purified as well using MBT agar.

Determination of Growth Substrates. The screening of compounds which could serve as growth substrates was carried out using MB medium solidified with 2% agar. To test for growth with the heat-stable substrates, these compounds were supplied at 5 mM final concentration and autoclaved along with the basal salts of the MB medium. The heat-stable substrates which were also soluble in the basal medium were: sodium benzoate; sodium succinate; m-, o-, p- toluic acid; and p-methyl benzyl alcohol. The liquid hydrocarbons that were tested as growth substrates were supplied to the medium using the vapor phase. The liquid compound was placed in a tapered glass tube that was cut from a Pasteur pipette and sealed at the narrow end. The filled tubes were taped to the lids of the petri plates. The liquid hydrocarbons used were: m-, o- and p- xylene; benzaldehyde; m- and p- tolualdehyde; toluene; benzyl alcohol; and m-methyl benzyl alcohol.

Screening of Isolates for Plasmid DNA. Cells used for plasmid extraction and isolation procedures were taken from MBT liquid shake tubes and used to inoculate shake tubes containing either Luria broth (Luria and Burrows, 1957) or Zobell's medium. The isolates XF8, XF9 and XF15 were grown in Zobell's medium, and P. arvilla (PaW) and P. putida (arvilla) mt-2 were grown in Luria broth. The above shake tubes were all incubated at 30°C for 16 h and then harvested for plasmid detection.

The first procedure used for isolation and detection of TOL plasmids was one described by Kado and Liu (1981). There were two modifications made in the procedure. The three isolates, XF8, XF9 and XF25 were harvested and resuspended

in electrophoresis buffer (E buffer) with 0.5% NaCl added. In addition, the lysis step was carried out at 65°C for only 30 min.

The second procedure used to isolate and detect plasmid DNA was the Birnboim and Doly procedure. Gel electrophoresis was carried out for both of the above procedures using 0.6% agarose.

Curing Experiments. The curing procedure used in this report was based on similar experiments described by Kunz and Chapman (1981). Liquid MBT shake cultures of Pseudomonas arvilla (PaW), P. putida (arvilla) mt-2, XF8, XF9 and XF15 were used to inocultate 50 ml Erlenmeyer flasks containing 10 of MB medium with 0.05% benzyote as the sole carbon source. These flasks were incubated for 24 h at 30°C on a shaker. After incubation the medium became yellow and turbid. If the cells were incubated for longer periods of time they began to form macroscopic clumps and the medium lost its turbidity.

Following 24 h incubation, a loopful of suspension was removed from each of the shake flasks and streaked onto MB agar medium containing 0.05% m-toluate plus 0.005% succinate. At the same time, enough culture suspension was removed from each of the flasks to provide a 1% inoculum to flasks containing fresh benzoate-MB medium. The five cultures listed above were repeatedly transferred in liquid benzoate-MB medium and 24 h culture samples were plated on m-toluate-succinate MB agar. This procedure was repeated until the colonies formed on the above plates were distinct from the wild-type colonies. Kunz and Chapman (1981) have reported finding small colonies surrounded by a brown halo in addition to the larger, wild-type colonies using the same procedure. Cured strains were found among the smaller, pigmented colonies (Kunz and Chapman, 1981).

RESULTS AND DISCUSSION

Characterization of Isolates. XF8, XF9 and XF15 are all able to utilize m-toluate as the sole carbon source for growth. The three isolates produce a yellow diffusable pigment that is visible when the cells are grown in either solid or liquid medium containing m-toluate. Unlike P. putida (arvilla) mt-2 and P. arvilla (PaW), which grow well in Luria broth, all 3 of the isolates were unable to grow in this broth and were cultured in Zobell's medium instead.

SB8 is a long Gram negative rod containing a single polar flagellum. XF9 produces long to short coccobacillus-type cells which stain Gram negative and have single, polar flagella. XF15 cells are long Gram negative rods that are slightly larger in diameter than XF8. These cells also contain single, polar flagella. XF8 is catalase positive and yields a weak oxidase positive reaction. XF9 is catalase positive and yields a very strong oxidase positive reaction. All three isolates produced negative reactions in the modified oxidative-fermentative test using glucose as the carbon source.

Determination of Growth Substrates. Preliminary experiments indicated that P. arvilla (PaW) and P. putida (arvilla) mt-2 were able to grow in liquid and solid MBT. Growth in this medium was, however, not as good as in MBT medium minus ASW. However, in order to facilitate media preparation, both of the above organisms were cultured in liquid and solid medium with ASW.

P. putida and P. arvilla were able to grow well using many of the compounds tested. Only three of the compounds -- o-toluate, o-xylene and

benzaldehyde -- resulted in poor growth when tested with each of the above organisms. In addition, m-methyl benzyl alcohol resulted in poor growth for only P. arvilla. With the exception of m-methyl benzyl alcohol all the compounds tested yielded identical results for both organisms. In contrast, the isolates grew well with few of the compounds tested. The isolates grew well with only m-tolualdehyde, benzoate and succinate. Growth was either poor or not observed at all with all the other compounds tested for all three isolates.

Screening of Isolates for Pladmid DNA. Tol plasmids were detected in both P. putida and P. arvilla using either the Kado and Liu procedure or the Birnboim and Doly procedure. The plasmid migrates a very short distance with either procedure and the 0.6% agarose gel system. Using the Kado and Liu procedure two closely-spaced ethidium bromide-staining bands were repeatedly observed for XF15. No bands were observed after staining for either XF8 or XF9. The two bands observed in the XF15 extract migrated much further than did the band corresponding to the Tol plasmid.

Two bands which were not observed with the Kado and Liu procedure were detected using the Birnboim and Doly procedure. In addition to the TOL plasmid, both bands were present in extracts of either P. putida or P. arvilla. Both bands were also detected in XF8. One of the two bands was detected in XF9 and the other was detected in XF15. Both bands probably represent chromosomal DNA. None of the isolates had TOL plasmid bands.

Curing Experiments. Curing of P. putida, P. arvilla, XF8, XF9 and XF15 was accomplished according to the procedure described by Kunz and Chapman (1981). After several transfers on liquid benzoate MB medium, samples plated onto solid MB agar containing m-toluate and succinate produced both large and tiny cream colored colonies. Brown halos were not formed around these colonies. Kunz and Chapman, however, have reported the formation of brown halos around supposedly cured strains. The tiny colonies that formed on the m-toluate-succinate plates were picked and streaked onto MB benzoate medium.

P. arvilla produced tiny, transparent colonies on the above benzoate medium. Approximately 1% of the colonies were represented by a second colony type. The latter colony type was small, round and cream colored. Both colony types were significantly smaller than the colonies formed by the strain before the curing experiments.

P. putida also produced small, round, cream colored colonies. Approximately 10% of the colonies belonged to a second colony type whose size was similar to the wild type colonies when they were grown on benzoate.

When cured colonies were streaked on MB benzoate medium, XF8 formed mostly small, round cream colored colonies. Approximately 10% of the colonies were also cream colored, but they were smaller than the first type. Both types of colonies were smaller than the original XF8 growing on benzoate-MB medium.

Cured XF9 colonies formed only 1 colony type when streaked on benzoate MB medium. The colonies were small, round and cream colored.

Cured XF15 colonies formed mostly small, round, cream colored colonies when streaked on MB benzoate medium. Approximately 1% of the colonies were larger, resembling the size of the wild type XF15 on MB benzoate medium. A less frequently occuring colony type was represented by tiny, transparent colonies.

THE DESIGN AND CONSTRUCTION OF A REACTOR SYSTEM TO MODEL THE ANAEROBIC/OXIC WASTEWATER TREATMENT SYSTEM

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Secondary wastewater treatment systems are designed to remove suspended solids, soluble organics, and nutrients from wastewater, resulting in an effluent acceptable for disposal into receiving waters. Soluble organics are converted into biomass under aerobic conditions, resulting in a diverse microbial community known as activated sludge. Modifications to the totally aerobic method of treatment have been made in recent years by exposing the activated sludge to an initial anaerobic treatment, followed by an aerobic treatment zone. This modified process has been shown to remove excess phosphate from the wastewater above the amounts needed for microbial growth, and also results in the production of a sludge with improved settling qualities. The basic biological phenomena occuring in this treatment system are poorly understood, due to both the complex interactions taking place in mixed microbial populations and also to the lack of an accurate model of the system for use in the laboratory. We have designed an inexpensive benchtop reactor system that will allow investigation of the microbiology of the anaerobic/oxic treatment system under controlled conditions.

The reactor is constructed of interlocking circular plastic Tupperware™ hamburger freezing containers, each 2 cm deep and 10.5 cm wide, modified to serve as individual chambers in a plug flow reactor. Passages were cut in the plastic using cork borers and razor blades. Nitrogen gas or compressed air was bubbled into the bottom of each chamber through tubing connected to modified plastic bulkhead fittings. Bubbling gasses through the liquids served to mix the contents of the reactor and also was a means of keeping the environment anaerobic or aerobic. Sampling ports in each chamber were made from the same plastic fittings modified to hold a rubber septum, allowing liquids to be added or removed from each chamber with a syringe. A clarifier, also constructed or six plastic sections, was attached to the end of the reactor to provide for settling and recycle of the biomass. The chambers were modified to provide common channels for the sludge to settle and for clarified water to overflow. Settled sludge was removed through glass rods held in modified bulkhead fittings at the top of two of the six chambers. Flow rates of artificial wastewater to the reactor and recycle flow rates were controlled using a peristalic pump, and were determined based on the desired hydraulic retention time within each chamber and the volume of the reactor. The total system, consisting of three anaerobic chambers, five aerobic chambers, and the clarifier, was held together and off of the benchtop by a clamping device made of plastic and threaded rods with thumbscrews. The support devise allowed easy access to all fittings in the reactor as well as helped to prevent leaks from developing at the seals of the chambers.

The reactor design will accomodate experiments that require the use of pure cultures or call for the use of isotopes in identifying cell populations and/or metabolic products. It also allows for the collection and analysis of volatile or soluble producs produced anaerobically or aerobically, offers flexibility in changing chemical and hydraulic characteristics by varying the number of chambers used, and allows the system to be perturbed chemically or biologically via the influent feed or the sampling ports. The reactor can be

cheaply and easily constructed, sterilized in a microwave oven, and conserves medium due to its small volume (1.5 liters, clarifier inlcuded).

While the reactor was designed to model waste water treatment systems, slight modifications to the design may make it applicable for use in a variety of systems requiring the use of a plug flow reactor .

FACTORS AFFECTING GROWTH INHIBITION OF ENTERIC BACTERIA BY METHYL α-D-GLUCOSIDE

Dale Sutherland

INTRODUCTION

In previous work done by D.J. Schnell and colleagues (1982) it was reported that a marine enteric bacterium, Vibrio parahaemolyticus, responded in a peculiar fashion to analogs of glucose. When methyl α-D-glucoside (αMG) was added to cultures of the organism growing on either mannose or fructose, there was an initial period of inhibition, two to four hrs in low phosphate media (0.6M), which was rapidly overcome and subsequent growth was no longer affected by αMG. This phenomenon has since been termed an "escape" from inhibition. Also, this work raised the question as to whether this "escape" differed from that observed in E. coli. (Figure 1).

The purpose of our experimental work this summer was to determine the escape behavior in E. coli, and to describe the mechanism of escape from the inhibition of αMG. In our studies this year, we decided to work with the more extreme halophilic marine bacterium Vibrio harveyi rather the Vibrio parahaemolyticus.

METHODS

Vibrio harveyi cells were grown on AK/0.4M NaCl media containing 10 mM of a carbon source, such as glucose, fructose, or mannose. For strains of E. coli, basal medium (BM) plus essential amino acids (tHALT) was used, as well as 10 mM of a carbon source. When analyzing the escape phenomenon, 2 mM αMG was added to the above media. For growth, all cultures were incubated at 30°C and left to shake. 2 mM [^{14}C] glucose, mannose, and αMG were used in our uptake studies. The final strength of the isotopes was 6 μCi/ml when the assay was performed with cells at an optical density of 0.15. Also sampling was done over a ten min period at two min intervals beginning one min after the addition of the isotope. To determine the phosphotransferase activity of these organisms, a technique developed by Kornberg and Reeves (1976), with slight modifications, was used. In this cells were harvested via centrifugation and then resuspended in PT/0.4M NaCl to an optical density of 2.0. The cells were then toluenized with a 10% solution of toluene and ethanol for exactly one min on the vortex mixer. From this point on, the method of Kornberg and Reeves was followed. The modifications were necessary because the cells require a salt concentration to lower than 0.08M. Without this the Vibrios will lyse due to an increased osmotic pressure. It should also be noted that too high a concentration of salt will hamper the phosphotransferase activity in the cells. Thus, a final salt concentration of 0.08M is the optimum condition for the phosphotransferase assay. The toluenization of the cells renders them permeable and through the addition of PEP, lactate dehydrogenase (LDH), reduced nicotinamide adenine denucleotide ($NADH_2^+$), and a carbon source; the phosphorylation of the carbon source can be measured directly by recording the decrease in optical density of $NIDH_2^+$ at 340nm (Figure 4).