A GENETIC STUDY OF

ACINETOBACTER PHOSPHADEVORUS

by

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A Thesis Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE

In the Graduate College

The University of Arizona

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ACKNOWLEDGMENTS

I would like to thank Dr. Irving Yall for his support and counsel in this endeavor. Appreciation is also expressed to my committee members Dr. R. Pritchett and Dr. D. Zink for their advice and the use of their equipment.

This research was supported in part by a fellowship from the National Wildlife Federation and the American Petroleum Institute.

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ABSTRACT

Acinetobacter phosphadevorus was originally isolated from activated sludge because of its phosphate uptake capabilities. The organism is able to grow on a variety of carbon compounds of chain length ranging from C-1 to C-40, but does not catabolize sugars. The plasmid content of A. phosphadevorus was compared to that of other Acinetobacter sp. A single plasmid (29 Mdal) was found in A. phosphadevorus. When cured of the 29 Mdal plasmid (pYG1), A. phosphadevorus was unable to grow on the n-alkanes C_8 through C_{10}. Growth on the other alkanes tested (C_{11} through C_{18} and C_{20}) was normal. Two other Acinetobacter sp. were also found to harbor plasmids.

Several growth substrate mutants of A. phosphadevorus were also isolated and characterized in this study, including two classes of n-alkane mutants. One strain, HP-6, was unable to grow on C_8 through C_{12}, exhibited slight growth on C_{13} and C_{14}, and grew well on C_{15} through C_{18} and C_{20}. The other strain, HP-2, was unable to grow on C_{15} through C_{17}, exhibited slight growth on C_{13}, C_{14}, C_{18}, and C_{20}, but grew well on C_8 through C_{12}.

Two other growth substrate mutants isolated were a citrate mutant and an acetate mutant. These were characterized using radiolabeled substrates. It was found that the citrate mutant was defective in its citrate transport system, and the acetate mutant was able to oxidize ^{14}C-labeled acetate but unable to grow on that compound.

CHAPTER 1

INTRODUCTION

Acinetobacter phosphadevorus (P-7) is a gram-negative coccobacillus approximately 1 μm in diameter, occurring in clusters. It is a strict aerobe and is non-motile. When grown in culture, nitrites are not oxidized to nitrates, it is oxidase negative and catalase positive, it is resistant to penicillin and forms large volutin granules (42).

A. phosphadevorus was originally isolated from activated sludge because of its phosphate uptake characteristics (42). Initial studies demonstrated that developed Tucson sludge containing *A. phosphadevorus* could reduce the concentration of dissolved phosphates in Tucson sewage to 20% of that normally present (43). Volatile acids such as acetate were found to be the primary growth substrate for this organism in sewage (43).

Ferguson (13) demonstrated that a wide range of substrates would support growth of *A. phosphadevorus*. The substrates supporting growth included one-carbon compounds such as formate and methanol, as well as hexadecane and motor oil. All carbohydrates and most aromatic compounds tested did not support growth.

The hydrocarbon growth characteristics of *A. phosphadevorus* were examined by Nash (34). It was shown that the organism could degrade n-parafins as large as tetracontane, but the organism preferred lower molecular weight hydrocarbons, such as dodecane. Electronmicrographs of

hexadecane grown cells revealed the presence of large hexadecane inclusions in the cells.

The characteristic traits of some bacterial species have been shown to be plasmid encoded. Citrate utilization by Escherichia coli strains (23), lactose fermentation by Streptococcus lactis subsp. diacetylactis (28), and hydrocarbon degradation by pseudomonads (5) are all examples of such plasmid mediated functions. Other phenotypes encoded by plasmids include drug resistance, colicin production, virulence, resistance to metal ions, tumorgenesis in plants, production of DNA restriction and modification enzymes, and synthesis of organic chemicals (9, 20, 12, 37, 46, 24, 8).

Publications describing plasmids in Acinetobacter sp. have been scant. The first mention of plasmids in Acinetobacter sp. was by Christiansen (7). The functions of these plasmids were not determined. A mercury resistance plasmid in an Acinetobacter sp., isolated from Chesapeake Bay sediment, has recently been described (38). Hinchcliffe and Vivian (21) have characterized a native plasmid of Acinetobacter calcoaceticus. The plasmid (pAV1) is a self-transmissible sulfonamide resistance factor of limited host range and belonging to the P incompatibility group.

The Acinetobacter sp. resemble the pseudomonads in being able to utilize a variety of organic compounds as growth substrates (18). In Pseudomonas sp., some of the hydrocarbon growth traits have been shown to be plasmid mediated (5). Two significant features of these plasmids are: 1) some of them belong to different incompatibility

groups (5); and 2) it has been shown that some of these hydrocarbon degrading functions are transposable (6). These features can greatly enhance the versatility of the host organism; i.e., an organism containing several such degradative plasmids may be useful in cleaning up oil spills or the ballast holds of oil tankers (2, 19).

Most of the hydrocarbon degradative plasmids in Pseudomonas sp. mediate catabolism of aromatic compounds. The SAL (salicylate) and NAH (naphthalene) plasmids code for the entire degradative pathways for their respective substrates, including a functional meta pathway (4, 11). Other plasmids specify enzymes for only a portion of the degradative pathway. For example, the CAM (camphor) plasmid codes for the degradation of camphor to isobutyrate; therefore, the host must be able to metabolize isobutyrate if it is to be able to grow on camphor (41).

The TOL and XYL plasmids specify the same degradative functions, that is, the ability to catabolize p- or m-toluene and the corresponding xylenes (48). The plasmids appear to have the same xylene degradative genes, the expression of which are regulated in similar manners. The only apparent difference is that TOL is self-transmissible and XYL is not (16).

Other aromatic hydrocarbon degradation functions that are plasmid mediated in Pseudomonas sp. include: nicotine and nicotinate degradation; 3,5 xylenol and p-cresol degradation; and possible plasmid involvement in steroid degradation (45, 22, 44).

An n-alkane degrading plasmid (OCT) was found in a Pseudomonas oleorovans strain (36). Host strains are capable of growing on the

hydrocarbons n-hexane through n-decane via monoterminal oxidation (36). The plasmid codes for the inducible alkane hydroxylating and primary alcohol dehydrogenating activities; the enzymes of the fatty acid cycle are coded for by the host chromosome (18).

The hydrocarbon degradative plasmids discussed so far have been exclusively isolated from Pseudomonas; however, such plasmids have recently been shown to be present in other bacteria. The functions mediated by these plasmids include: degradation of pesticides by Alcaligenes paradoxus (15); 2-hydroxypyridine utilization by Arthrobacter crystallopoites (47); and degradation of the unnatural synthetic substrate 6-aminohexanoic acid by a Flavobacterium sp. (35).

The isolation and characterization of the plasmid observed by Kronland (29) in A. phosphadevorus was the main purpose of this study.

This was accomplished by examining mini lysates of A. phosphadevorus for the presence of extrachromosomal DNA. The approximate size of the plasmid was determined by comparison with plasmids of known size. Ethidium bromide curing resulted in the isolation of a plasmidless strain of A. phosphadevorus. This strain was then examined for loss of drug resistance, metal ion resistance, growth substrate abilities, and luxury phosphate uptake.

A secondary goal of this study was to isolate growth substrate mutants generated by N-methyl-N-nitro-N-nitrosoguanidine (NTG) treatment. These mutants were then characterized by testing for growth on different substrates and by examining the metabolism of various radiolabeled compounds by these mutants.

CHAPTER 2

MATERIALS AND METHODS

Microorganisms

Acinetobacter phosphadevorus P-7 was obtained from Irving Yall, Ph.D. *Acinetobacter calcoaceticus*, *Acinetobacter anitritus* originally ATCC #12381, and *Herella vaginicola* derived from ATCC #12359 were obtained from the stock culture collection at the Microbiology Department at the University of Arizona. *Escherichia coli* strain V517 was obtained from Donald Zink, Ph.D. The mutant P-7 strains used in this study are described in Table 1.

Chemicals

The chemicals used were of reagent grade. All hydrocarbons employed were 99% pure n-alkanes. Texas crude oil was obtained from Phillips Petroleum Company. N-methyl-N-nitro-N-nitrosoguanidine was obtained from Sigma Chemical Company. The source of orthophosphate-^{32}P, glycine-1-^{14}C, glycine-2-^{14}C, acetate-1-^{14}C, octanoate-1-^{14}C, α-keto-glutarate-U-^{14}C, and citrate-1,5^{14}C was New England Nuclear Corporation. Acetate-2-^{14}C was obtained from the International Chemical and Nuclear Corporation. All isotopes had a radiometric purity of 97.5-99%.

Table 1. Bacterial Strains

Strain	Genotype	Source
P-7	wildtype (pYG1)[a]	I. Yall
EB-22	wildtype	Ethidium bromide curing of P-7
CP-1	cit^{-}[b] (pYG1)	NTG mutant of P-7
AP-2	ace^{-} (pYG1)	NTG mutant of P-7
HP-2	hex^{-} (pYG1)	NTG mutant of P-7
HE-5	hex^{-}	ethidium bromide curing of HP-2
HP-6	dod^{-}	NTG mutant of P-7 which spontaneously lost pYG1
V517	*E. coli* reference[c]	D. Zink

[a]The plasmid in P-7 has been designated pYG1.

[b]The abbreviations used are: cit = citrate; ace = acetate; hex = hexadecane; dod = dodecane; and NTG = N-methyl-N-nitro-N-nitrosoguanidine.

[c]Harbors reference plasmids with molecular weights of 35.8×10^6, 4.8×10^6, 3.7×10^6, 3.4×10^6, 2.6×10^6, 2.0×10^6, 1.8×10^6, and 1.2×10^6.

Media

Koser's citrate broth (Difco Laboratories) contained per liter of distilled water: 1.5 g sodium ammonium phosphate, 1.0 g monobasic potassium phosphate, 0.2 g magnesium sulfate-7-hydrate, and 3.0 g sodium citrate.

Carbon-free basal medium, used to determine which carbon sources would support growth, contained per liter of distilled water: 1.5 g sodium ammonium phosphate, 1.0 g monobasic potassium phosphate, and 0.2 g magnesium sulfate-7-hydrate.

Peptone broth and acetate broth contained per liter of basal medium: 20 g Bacto-peptone (Difco) and 4.0 g sodium acetate, respectively.

Hexadecane agar plates consisted of basal medium agar plates spread with 0.1 ml hexadecane.

Trypticase soy broth (BBL) plus yeast extract (TSB+YE) contained per liter of distilled water: 17.0 g trypticase peptone, 3.0 g phytone peptone, 5.0 g sodium chloride, 2.5 g dipotassium phosphate, and 6.0 g yeast extract (Difco).

The pH of all media was adjusted to 7.5 with 5.6% KOH, unless otherwise indicated. Solid media was prepared by adding 15 g of agar to one liter of the appropriate broth.

Penassay broth (Difco) contained per liter of distilled water: 1.5 g Bacto yeast extract, 5.0 g Bacto peptone, 1.0 g Bacto dextrose, 3.5 g sodium chloride, 3.68 g dipotassium phosphate, and 1.32 g monopotassium phosphate. The pH was adjusted to 7.60 with 4.0% sodium

hydroxide. The media was filter sterilized to prevent any pH changes due to autoclaving.

Tris-citrate-ammonium sulfate contained per liter of distilled water: 12.1 g Tris (hydroxymethyl) aminomethane, 3.0 g sodium citrate, and 0.6 g ammonium sulfate. The pH was adjusted with 5.0 M hydrochloric acid.

Growth Conditions

For mutagenesis and isotope studies, cells were grown in 100 ml of media per 500 ml Erlenmeyer flask. Broth cultures for mini-lysate preparations were grown in 20 ml of TSB+YE media in a 125 ml Erlenmeyer flask. Aeration for broth cultures was provided by continuous shaking on a New Brunswick rotary shaker (Model C.S.) at 200 RPM. Plate, slant, and broth cultures were incubated at 25 C, unless otherwise indicated.

Centrifugation Conditions

Cells were harvested in a Servall RC-2 or a Beckman Model J2-21 refrigerated centrifuge at 17,000 x g, 4 C, for 15 minutes, unless otherwise indicated.

Mutant Isolation

Growth substrate mutants were obtained by NTG treatment (1). Selection was accomplished by ampicillin treatment (17). Cells were grown in peptone broth to mid-logarithmic phase, harvested, and resuspended in 0.25 original volume of basal medium (pH 6.0). NTG was added to a final concentration of 50 µg/ml and the suspension incubated at 25 C for 30 minutes. Treated cells were harvested, washed once with

basal medium (pH 7.5), resuspended in 100 ml of peptone broth, and incubated at 25 C. After three hours, the cells were harvested, washed once, and resuspended in basal medium (0.1 original volume). The suspension was transferred to growth media containing 1.0 mg/ml ampicillin, incubated for two hours, the cells were harvested, washed once with basal medium, and resuspended in basal medium. A flask containing 100 ml of peptone broth was inoculated with the selected cells, incubated overnight, and the cells plated on peptone agar plates. The plates were incubated for two days, then isolated colonies were transferred to peptone agar plates on a grid allowing 40 colonies per plate. These master plates were incubated for two days, then replicated onto Koser citrate agar, acetate II agar, and hexadecane agar plates. The isolates unable to grow on a particular substrate but able to grow on the other two substrates were selected for further study.

Mutant Characterization

Basal medium agar plates containing 1,000 ppm total carbon of a particular substrate were used to screen for other growth substrate defects present in the mutants. The mutant strains were streaked onto the various substrate agar plates, the plates were incubated for three days, then scored for growth.

Determination of Radioactivity

A Packard Tri-Carb liquid scintillation counting system, Model 3320, was used to measure radioactivity. The scintillation fluid

contained per liter: 4 g 2,5-bis-2(5-tert-butylbenzoxazolyl)-thiophene (BBOT), 80 g naphthalene, 400 ml ethylene glycol monoethyl ether, and 600 ml toluene.

Determination of Dry Weight

Microporous filtration was used for dry weight of cell mass determinations. Cells were concentrated 10 fold by centrifuging in a Beckman Model J2-21 centrifuge (17,000 x g; 10 minutes) and resuspended in basal medium. A 1 ml aliquot was passed through a tared 0.22 μm (pore size) Millipore membrane filter pad (47 mm). The filters were dried to a constant weight at 70 C in a Blue Dot drying oven for 18 hours before weighing.

Radioisotope Studies

Warburg flasks were used as reaction vessels. The center well was filled with 0.3 ml of 20% KOH; the side arm contained 0.5 ml of 0.1 M H_2SO_4. Mid-exponential phase cells were harvested, washed once with basal medium, and resuspended in 10% of the original volume in basal medium. Two ml of cells were added into each flask along with 0.5 ml of the appropriate labeled substrate. The flasks were set on the Warburg water bath shaker at 25 C. After 30 minutes, the 0.1 M H_2SO_4 was dumped into the main chamber to stop the reaction, and the flasks incubated for 15 minutes.

A 0.1 ml sample from the KOH of each flask was removed for scintillation counting; the remaining KOH was removed from the well. The cell suspension was transferred to a centrifuge tube; the flask

was washed two times with 0.15 M NaCl and the washes added to the centrifuge tube. The cells were washed once with 0.15 M NaCl. The wash and supernatant fractions were pooled, and the cells were resuspended in 1.0 ml of basal medium. Samples of the cells and supernatant fraction were removed for scintillation counting.

Mini-Lysate Preparation

Cells were grown in TSB-YE media to late log phase (40). The cells (1.5 ml portions) were harvested in a Beckman microcentrifuge Model BG, (five minutes) at room temperature. The cells were washed once with 1.0 ml of TE (50 mM Tris-hydrochloride pH 8.0, 10 mM EDTA) buffer, recentrifuged, and resuspended in 40 µl of TE. Cells were lysed and the chromosomal DNA denatured by transferring the cell suspension to 0.6 ml of lysis buffer (TE + 4% SDS, pH 12.42), then incubating at 37 C for 20 minutes. After denaturation, the sample was neutralized by adding 50 µl of 2 M Tris-HCL (pH 7.0), mixed, 160 µl of 5 M NaCl was added, and the sample incubated at 0 C for at least 60 minutes. The precipitated chromosomal DNA was pelleted (five minutes, 25 C, in a microcentrifuge), the supernatant fraction transferred to another tube, and 550 µl of cold isopropanol (-20 C) was added to the supernatant. The supernatant fraction was incubated for 30 minutes at -20 C, and the precipitate pelleted by centrifuging for five minutes (in the microcentrifuge at 25 C). The pellet was dried in a vacuum and resuspended in 30 µl of TES (30 mM Tris-hydrochloride pH 8.0, 5 mM EDTA, 50 mM NaCl buffer. The samples were stored at -20 C if not used immediately.

Gel Electrophoresis of Mini-Lysates

Mini-lysates were electrophoresed in 0.7% agarose (Bethesda Research Laboratories), dissolved in Tris-borate buffer (89 mM Tris, 2.5 mM disodium EDTA, 89 mM boric acid) (33). A 15 µl aliquot of each sample was mixed with 5 µl of marker dye solution (0.07% bromophenol blue, 7% sodium dodecylsulfate, 33% glycerol) and loaded onto the gel. Electrophoresis was carried out on a BRL Model HO horizontal gel apparatus. The power source was an LKB Model 2103 power supply. Electrophoresis was carried out at 60 mA, 125 V. The gel was stained in Tris-borate buffer containing 0.5 µg/ml of ethidium bromide for 30 minutes. The gel was destained and the DNA bands visualized on a Chromato-Vue Transilluminator. The gel was photographed with a Minolta SRT 201 camera using Kodak Plus X pan film.

Plasmid Molecular Weight Determination

Plasmids of known molecular weight were obtained from *Escherichia coli* strain V517 (obtained from D. Zink). This strain contains eight plasmids and serves as a ready reference plasmid source (31). The weights for reference plasmids were 35.8×10^6, 4.8×10^6, 3.7×10^6, 3.4×10^6, 2.6×10^6, 2.0×10^6, 1.8×10^6, and 1.2×10^6. A plot of the log of relative mobility and the log of molecular weight (MW) yielded a linear curve. This relationship was used to determine the size of the *Acinetobacter* sp. plasmids.

Ethidium Bromide Curing of Plasmid DNA

The minimum growth inhibitory concentration of ethidium bromide was determined (3). A small inoculum (0.1 ml inoculum of a 10^{-3} dilution of log phase cells) was grown for three days at 37 C, without shaking, in TSB+YE containing a subinhibitory concentration of ethidium bromide. The culture was plated on TSB+YE agar plates. Isolated colonies were picked and grown on master plates with 40 colonies/plate. These isolates were screened for a loss of plasmid DNA by gel electrophoresis.

Growth Substrate Studies

Hydrocarbon growth substrates were tested by streaking carbon-free basal medium agar plates with the strains to be examined; several strains (up to six) were streaked on a single plate. A 2 cm x 2 cm square of Gelman saturation pad (Gelman Instrument Company) was taped to the top of the petri dish and saturated with 0.1 ml of hydrocarbon. The plates were sealed with tape or large wide rubber bands and incubated for five days at 25 C.

Screening tests for soluble growth substrates were carried out with 7 mm concentration discs. Discs were inoculated with 50 µl of a 1 M (or saturated) solution of an organic salt, dried, and placed on basal medium agar plates that had been inoculated with approximately 10^7 colony forming units (CFU) of the strain to be tested. Plates were incubated for 48 hours before determining the size of the growth zones.

Antibiotic Sensitivity Screening

Screening tests for antibiotic resistance were carried out with commercially-available antibiotic discs. The discs were placed on a TSB+YE agar plate (six per plate) that had been inoculated with approximately 10^7 CFU of the strain to be examined. Plates were incubated for 48 hours before zone of inhibition sizes were measured.

Metal Cation and Oxyanion Sensitivity Screening

Screening tests for ion resistance were carried out with blank 7 mm concentration discs (37). Discs were impregnated with 50 µl aliquots of a salt solution, dried, and then placed on a TSB+YE agar plate that had been inoculated with approximately 10^7 CFU of the strain to be examined.

Uptake of ^{32}P-Orthophosphate

Strains to be tested were grown to mid-log phase in Koser's citrate broth, harvested, washed once with Tris buffer (100 mM, pH 7.5), reharvested, and suspended in Tris buffer (total concentration factor - 40 fold). One ml samples of cells were added to 125 ml Erlenmeyer flasks containing 9.0 ml of Tris-citrate-ammonium sulfate growth media and 10 µCi ^{32}P-orthophosphate and incubated with shaking. Duplicate 100 µl samples were taken at the indicated time intervals. Each sample was added to 0.9 ml of basal medium (pH 5.5) at 0 C to stop the reaction. The cells were pelleted by centrifuging for three minutes in a Beckman microcentrifuge. A 100 µl aliquot of the supernatant fraction

was removed for scintillation counting. An uninoculated flask served as a control.

CHAPTER 3

RESULTS

Mutant Isolation and Characterization

Growth substrate mutants of A. phosphadevorus were generated by NTG mutagenesis, followed by ampicillin selection. Strains unable to grow on citrate (strain CP-1), acetate (strain AP-2), hexadecane (strain HP-2), and dodecane (strain HP-6) as sole carbon sources were isolated. These mutants were tested for growth on basal medium plates containing acetate, citrate, malate, fumarate, dodecane, dodecanoic acid, or hexadecane as sole carbon sources. It was found that the mutant strains grew on all the substrates other than the substrate characteristic of that strain.

Strains CP-1 and AP-2 were further characterized using radiolabeled compounds. The uptake and utilization of these substrates were measured and compared to that of the wildtype strain P-7 (Tables 2-6).

From the growth studies described above, it was shown that CP-1 was unable to grow on citrate media but was able to grow on TCA cycle intermediates (malate and fumarate) as sole carbon sources. An explanation for this could be that CP-1 was unable to take up citrate, but the TCA cycle was still functioning. To test this possibility, the uptake and utilization of citric acid (1,5-^{14}C) and α-ketoglutaric acid (U-^{14}C) by CP-1 was compared to P-7. Acetic acid (1-^{14}C), acetic acid

Table 2. Uptake of Various Labeled Substrates by Peptone-Grown Strains P-7 and CP-1

Isotope/Strain	% Uptake[a]	% Lost as CO_2[b]
citric acid (1,5-^{14}C)		
P-7	76	98
CP-1	10	73
α-ketoglutaric acid (U-^{14}C)		
P-7	28	95
CP-1	46	94
octanoic acid (1-^{14}C)		
P-7	8	79
CP-1	8	95
acetic acid (1-^{14}C)		
P-7	95	87
CP-1	94	89
acetic acid (2-^{14}C)		
P-7	82	73
CP-1	89	70

Cells were exposed to label for 30 minutes at 25 C with shaking.

All counts were obtained per milligram cell dry weight.

Initial radioactivity available: citric acid (1,5^{14}C), 1.1 x 10^6 CPM; α-ketoglutaric acid (U-^{14}C), 8.0 x 10^4 CPM; octanoic acid (1-^{14}C), 2.6 x 10^5 CPM; acetic acid (1-^{14}C), 1.4 x 10^5 CPM; acetic acid (2-^{14}C), 8.4 x 10^4 CPM.

[a]Expressed as the percent uptake of radioactivity available.

[b]Expressed as a percentage of ^{14}C taken up by the cells.

(2-^{14}C), and octanoic acid (1-^{14}C) were included in this experiment to determine if the uptake of other carboxylic acids was affected.

Citric acid (1,5-^{14}C) was readily taken up by P-7 (76%), but CP-1 only removed 10% of the available label from the media (Table 2). However, CP-1 was able to take up considerably more α-ketoglutaric acid (U-^{14}C) (46%) than was P-7 (28%). Ninety-four percent of the α-ketoglutaric acid (U-^{14}C) taken up by CP-1 was oxidized to CO_2, indicating that the TCA cycle was functioning in CP-1. The other labeled carboxylic acids tested were taken up at comparable levels by CP-1 and P-7.

To determine if the mutation in CP-1 was involved in the citrate permease system, cell-free preparations of CP-1 and P-7 were compared for the ability to degrade citric acid (1,5-^{14}C) (Table 3). CP-1 was able to oxidize citric acid (1,5-^{14}C) as was P-7, indicating that the enzymes for the oxidation of citrate are present in CP-1. Therefore, CP-1 may be mutated in the citrate transport system.

Cell-free preparations of CP-1 and P-7 were also tested for oxidation of octanoic acid (1-^{14}C). This carboxylic acid was not readily taken up by the intact cells (Table 2), and the cell-free preparations oxidized the labeled compound to only a negligible degree. Therefore, it was concluded that peptone-grown cells of A. phosphadevorus do not contain the enzymes necessary for octanoate oxidation.

An experiment designed to further characterize the citrate transport system is outlined in Table 4. Various metabolic inhibitors were used to determine their effects on the uptake of citric acid (1,5-^{14}C) by wildtype P-7 cells. Sodium arsenite, a potent inhibitor

Table 3. Oxidation of Labeled Citric Acid and Octanoic Acid by Cell-Free Preparations of P-7 and CP-1 (Peptone Grown)

Isotope/Strain	% Lost as CO_2[a]
citric acid (1,5-^{14}C)	
P-7	74
CP-1	97
octanoic acid (1-^{14}C)	
P-7	1.3
CP-1	4.9

Cells were exposed to label for 30 minutes at 25 C with shaking.

All counts were obtained per milligram cell dry weight.

Initial radioactivity available: citric acid (1,5-^{14}C), 7.2×10^4 CPM; octanoic acid (1-^{14}C), 2.3×10^5 CPM.

[a]Expressed as a percentage of ^{14}C initially available.

Table 4. Effects of Inhibitors on Labeled Citric Acid Oxidation in P-7 (Peptone Grown)

Inhibitors[a]	% Uptake[b]	% Lost as CO_2[c]
control	77	97
2,4-dinitrophenol	16	97
azide	56	99
arsenite	2	65

Cells were exposed to label for 30 minutes at 25 C with shaking.

All counts were obtained per milligram cell dry weight.

Initial radioactivity available: citric acid (1,5-^{14}C), 1.0×10^5 CPM.

Final inhibitor concentrations: 2,4-dinitrophenol, 10^{-2} M; sodium azide, 10^{-2} M; sodium arsenite, 5×10^{-3} M.

[a]Cells preincubated for 30 minutes in the presence of the inhibitor.

[b]Expressed as the percent uptake of radioactivity available.

[c]Expressed as a percentage of ^{14}C taken up by the cells.

of the TCA cycle, almost completely inhibited the uptake of labeled citrate (2% of the available label was removed). Sodium azide and 2,4-dinitrophenol are inhibitors of electron transport and oxidative phosphorylation, respectively, and have differing effects on the uptake of labeled citrate (16% versus 56%, respectively).

Experimentation involving utilization of acetic acid ($1-^{14}C$) and acetic acid ($2-^{14}C$) by AP-2 and P-7 was performed (Table 5). Although AP-2 was unable to grow on acetate agar plates, it was able to take up and oxidize acetate. AP-2 and P-7 took up the labeled acetate at comparable levels; there were, however, differences in the fate of the carbon atoms. Experiments with acetic acid ($1-^{14}C$) demonstrated that the carboxyl group is oxidized by AP-2. P-7 exhibits the normal preference for the methyl group of acetate when using acetic acid ($2-^{14}C$); i.e., only 34% of the label taken up is lost as CO_2, as opposed to 71% of the label when using acetic acid ($1-^{14}C$) (25). AP-2, however, does not exhibit a great preference for the methyl group of acetate, because 71% of the label taken up is lost as CO_2 when using acetic acid ($2-^{14}C$).

Previous work has indicated that unstarved mid-logarithmic phase A. phosphadevorus cells incorporate the methyl group via the serine pathway with a loss of the carboxyl group as CO_2 (25). Since AP-2 oxidizes the methyl group of acetate so readily, a mutation may be present in the serine pathway. An important intermediate in this pathway is glycine, which reacts with the methyl group of acetate (carried by tetrahydrofolic acid) to form serine (13, 25).

Table 5. Uptake of Labeled Acetic Acid by Citrate-Grown Strains P-7 and AP-2

Isotope/Strain	% Uptake[a]	% Lost as CO_2[b]
acetic acid (1-^{14}C)		
P-7	96	71
AP-2	94	84
acetic acid (2-^{14}C)		
P-7	87	34
AP-2	89	71

Cells were exposed to label for 30 minutes at 25 C with shaking.

All counts were obtained per milligram cell dry weight.

Initial radioactivity available: acetic acid (1-^{14}C), 1.4 x 10^5 CPM; acetic acid (2-^{14}C), 1.3 x 10^5 CPM.

[a]Expressed as the percent uptake of radioactivity available.

[b]Expressed as a percentage of ^{14}C taken up by the cells.

The uptake and utilization of glycine (1-^{14}C) and glycine (2-^{14}C) were examined for AP-2 and P-7 in the presence of acetate and without acetate (Table 6). The primary effect of acetate with either glycine label was to cut the percent CO_2 lost approximately in half; there were no apparent effects on the amount of glycine taken up by the cells. The only difference observed between AP-2 and P-7 was that the carboxyl group of glycine (1-^{14}C) was lost as CO_2 to a greater degree with AP-2 than with P-7 (81% and 59%, respectively). This effect was particularly apparent when acetate was present. The data is insufficient to determine the mutational lesion in AP-2.

Plasmid Detection and Curing

The plasmid content of three Acinetobacter-type species strains were compared with that of A. phosphadevorus. As seen in Figure 1, of the four strains examined, three contained extrachromosomal DNA. The results are compiled in Table 7. The sizes listed should only be considered approximations, not definitive values.

A. phosphadevorus was readily cured of the plasmid by growth in the presence of ethidium bromide. After treatment, 35 isolates were screened by gel electrophoresis for loss of plasmid DNA; 26 colonies lost the plasmid, for a curing rate of 74%. A single isolate from among the 26 cured colonies (EB-22) was selected for further study.

Determination of Plasmid Function

In order to determine if the hydrocarbon growth characteristics of A. phosphadevorus were plasmid mediated, strain EB-22 (pYG1$^-$) was

Table 6. Uptake of Labeled Glycine by Citrate-Grown Strains P-7 and AP-2

Isotope/Strain	% Uptake[a]	% Lost as CO_2[b]
glycine (1-^{14}C)		
P-7	48	59
AP-2	37	81
glycine (1-^{14}C) + acetate		
P-7	43	21
AP-2	49	46
glycine (2-^{14}C)		
P-7	43	27
AP-2	51	30
glycine (2-^{14}C) + acetate		
P-7	38	10
AP-2	42	13

Cells were exposed to label for 30 minutes at 25 C with shaking.

All counts were obtained per milligram cell dry weight.

Initial radioactivity available: glycine (1-^{14}C), 9.1 x 10^4 CPM; glycine (2-^{14}C), 9.2 x 10^4 CPM.

[a]Expressed as the percent uptake of radioactivity available.

[b]Expressed as a percentage of ^{14}C taken up by the cells.

Table 7. Plasmids of Various Acinetobacter-Type Species Microorganisms Resolved by Mini-Lysate Electrophoresis

Bacterium	No. of Plasmids Detected	Molecular Weight of Plasmids (Mdal)
A. calcoaceticus	0	--
A. phosphadevorus	1	29
A. anitritus	2	5.0, 7.4
H. vaginicola	6	1.4, 2.0, 3.0, 3.5, 4.1, 17

Figure 1. Gel Electrophoresis of Mini-Lysates of *Acinetobacter* sp.

Lane 1. *A. phosphadevorus*
Lane 2. *A. calcoaceticus*
Lane 3. *A. anitritus*
Lane 4. *E. coli* V517
Lane 5. *H. vaginicola*

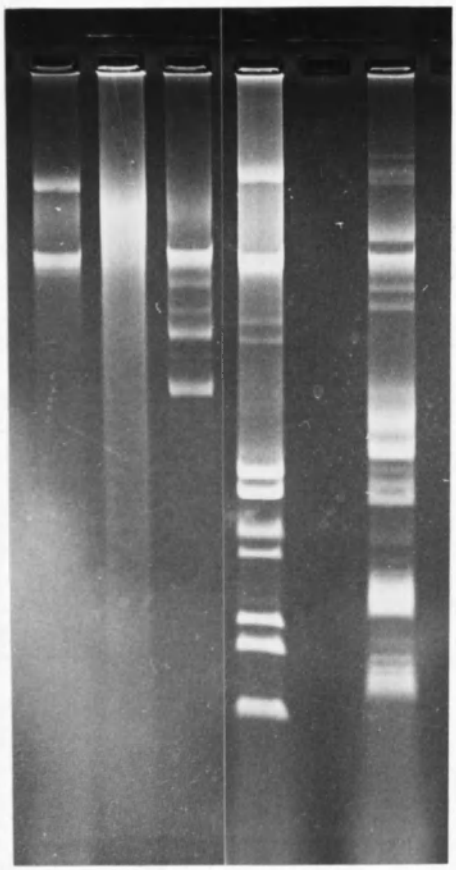

Figure 1. Gel Electrophoresis of Mini-Lysates of Acinetobacter sp.

compared to P-7 (pYG1$^+$) for differences in hydrocarbon-induced growth. A wide range of hydrocarbons were tested as growth substrates, including n-alkanes, crude oil, benzene, phenylethyl alcohol, tributyrin, camphor, and n-butanol. Other hydrocarbons screened but unable to support growth of either P-7 or EB-22 are listed in Table 8.

The results of the growth studies are included in Table 9. Strain P-7 grew on all the hydrocarbons listed except heptane (C_7). Strain EB-22 was unable to grow on the n-alkanes C_8, C_9, or C_{10}. Therefore, the plasmid pYG1 appeared to mediate growth of P-7 on the n-alkanes C_8, C_9, and C_{10}.

The verify this result, the 35 isolates previously screened for the loss of pYG1 were replicated onto basal medium plates containing C_9, C_{10}, C_{12}, or C_{16} n-alkanes as growth substrates. On the C_9 and C_{10} plates, the nine isolates containing the plasmid, pYG1, grew; the others did not. All 35 isolates grew on the C_{12} and C_{16} plates.

Since pYG1 appeared to mediate growth on n-alkanes, the two hydrocarbon growth mutants previously described were examined for the presence of plasmid pYG1. It was found that HP-2 still harbored pYG1, but HP-6 did not (Figure 2).

Curing of pYG1 from HP-2 was effected by ethidium bromide treatment (Figure 2). The growth of one of the cured HP-2 strains, HE-5, on n-alkanes and crude oil was compared to that of HP-2. The only difference found was the inability of HE-5 to grow on C_8, C_9, or C_{10} n-alkanes; all other hydrocarbon growth characteristics were the same.

Table 8. Hydrocarbons Unable to Support Growth of *A. phosphadevorus*

naphthalene
α-naphthol
tert-butanol
n-octanol
chloroform
carbon tetrachloride
n-hexane
n-heptane
terpinol
toluene

Table 9. Hydrocarbon Growth Characteristics of A. phosphadevorus Strains

Hydrocarbon	Strain P-7	EB-22	HP-2	HE-5	HP-6
n-heptane	-	-	-	-	-
n-octane	+	-	+	-	-
n-nonane	+	-	+	-	-
n-decane	+	-	+	-	-
n-undecane	+	+	+	+	-
n-dodecane	+	+	+	+	-
n-tridecane	+	+	M[a]	M	M
n-tetradecane	+	+	M	M	M
n-pentadecane	+	+	-	-	+
n-hexadecane	+	+	-	-	+
n-heptadecane	+	+	-	-	+
n-octadecane	+	+	M	M	+
n-eicosane	+	+	M	M	+
crude oil	+	+	-	-	+
benzene	+	+	ND[b]	ND	ND
camphor	+	+	ND	ND	ND
phenylethyl alc.	+	+	ND	ND	ND
tributyrin	+	+	ND	ND	ND
n-butanol	+	+	ND	ND	ND

[a]Microcolonies formed (i.e., slight growth observed).

[b]Not done.

Figure 2. Gel Electrophoresis of Various Strains of *A. phosphadevorus*

Lane 1. P-7
Lane 2. EB-22
Lane 3. HP-2
Lane 4. HE-5
Lane 5. HE-6
Lane 6. *E. coli* V517

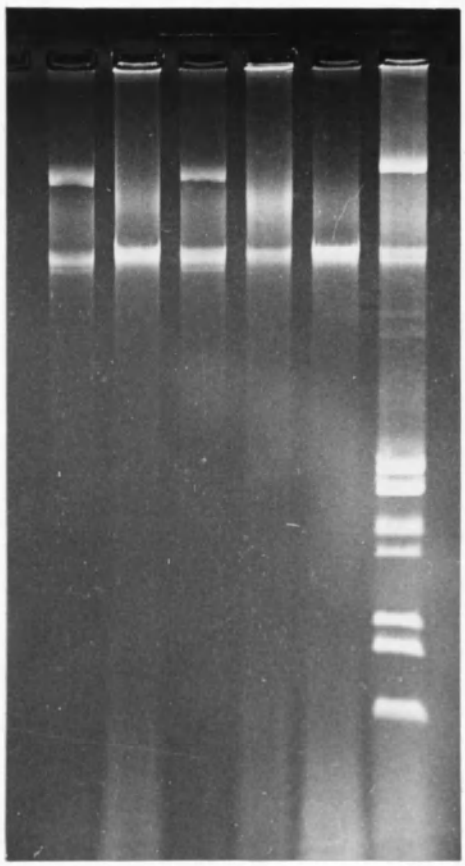

Figure 2. Gel Electrophoresis of Various Strains of A. phosphadevorus

The growth characteristics of strains P-7, EB-22, HP-2, HE-5, and HP-6 are summarized in Table 9.

Screening for Other Plasmid Functions

Strains P-7 ($pYG1^+$) and EB-22 ($pYG1^-$) were compared for further differences in growth substrate characteristics, antibiotic resistances, metal cation and oxyanion resistances, and luxury phosphate uptake abilities.

A wide range of organic salts were tested as growth substrates for P-7 and EB-22. There were no differences in growth of the two strains on any of the 32 substrates tested (Table 10).

Plasmids present in *Acinetobacter* sp. have been shown to code for antibiotic resistance (21). Antibiotic discs were used to test for antibiotic resistance variance between *A. phosphadevorus* strains P-7 and EB-22. There were no apparent differences in the antibiotic resistances of the two strains. The antibiotics tested are listed in Table 11.

Resistance to heavy metals and other oxyanions has been shown to be plasmid mediated in various species of bacteria and in *Acinetobacter* sp. (37, 21). Filter discs impregnated with the chemical to be tested were used to compare the two *A. phosphadevorus* strains. Some of the chemicals did not produce any zone of inhibition; inhibitory chemicals produced the same diameter zone with P-7 and EB-22. The chemicals are listed in Table 12.

The luxury uptake of phosphate during log phase is a unique characteristic of some strains of *Acinetobacter* sp. (10, 30). To

Table 10. Organic Compounds Screened as Growth Substrates for Strains P-7 and EB-22

Growth by Both Strains		No Growth by Both Strains	
Compound	Concentration	Compound	Concentration
alanine	0.1	valine	0.1
leucine	S[a]	phenylalanine	S
isoleucine	0.1	tryptophane	S
proline	0.1	methionine	0.1
tyrosine	S	glycine	0.1
asparagine	0.1	serine	0.1
glutamate	0.1	threonine	0.1
aspartic acid	S	cysteine	S
lysine	S	glutamine	0.1
arginine	0.1	hydroxyproline	0.1
histidine	0.1	tartrate	1.0
citrate	1.0	formate[b]	1.0
acetate	1.0	isocitrate[b]	1.0
propionate	1.0	fumarate[b]	1.0
pyruvate	1.0	succinate[b]	1.0
malate	1.0		
α-ketoglutarate	1.0		

[a]Saturated distilled water solution.

[b]These substrates had been previously reported to support growth (14).

Table 11. Antibiotic Discs Used to Compare the Antibiotic Sensitivities of Strains P-7 and EB-22

Antibiotic	Concentration (µg/disc)	Zone Size (mm) P-7	EB-22
ampicillin	5	0	0
carbenicillin	50	9	9
methecillin	5	0	0
penicillin G.	10 (units/disc)	0	0
ticarcillin	75	13	14
albamycin	30	15	15
aureomycin	30	18	17
clindamycin	2	0	0
erythromycin	15	15	15
gentamycin	10	17	18
kanamycin	10	17	17
lincomycin	5	0	0
neomycin	5	10	9
streptomycin	10	15	14
tobramycin	10	13	12
vancomycin	30	7	7
vibramycin	30	20	20
sulfadiazine	0.25	20	19
sulfisoxazole	2	26	25
nalidixic acid	30	22	23
rifampin	15	19	18
tetracycline	30	15	15

Table 12. Chemicals Screened for Resistance Differences Between Strains P-7 and EB-22

Inhibitory Chemicals		Non-Inhibitory Chemicals	
Compound	Concentration (M)	Compound	Concentration (M)
$CuCl_2$	1.0	$BaCl_2$	0.1
$Pb(NO_3)_2$	0.1	$NaMoO_4$	0.1
H_2PtCl_2	10%	KI	0.1
$CdCl_2$	0.1	KIO_3	0.1
$CeSO_4$	0.1	$MnCl_2$	0.1
$CoCl_2$	0.1	$NaWO_4$	0.1
$AgNO_3$	0.1	$K_3Fe(CN)_6$	S
$SnCl_2$	0.1	cacodylic acid	1.0
PHMB[a]	0.01	phenol	1.0
mersalyl acid	0.01	$Na_2B_4O_7$	1.0
$HgCl_2$	0.1	$NaHAsO_4$	1.0
$SrCl_2$	0.1		
$ZnSO_4$	0.1		
Na_2CrO_4	0.1		
$NaAsO_2$	0.1		
NaN_3	0.1		
K_2TeO_3	S		
NaCN	1.0		

[a]p-hydroxymercuribenzoic acid

determine if this is plasmid mediated in A. phosphadevorus, ^{33}P-ortho-phosphate removal from the growth media, during log phase growth, was measured for P-7 and EB-22. There were no apparent differences in the phosphate removal capabilities over time of these two strains (Figure 3).

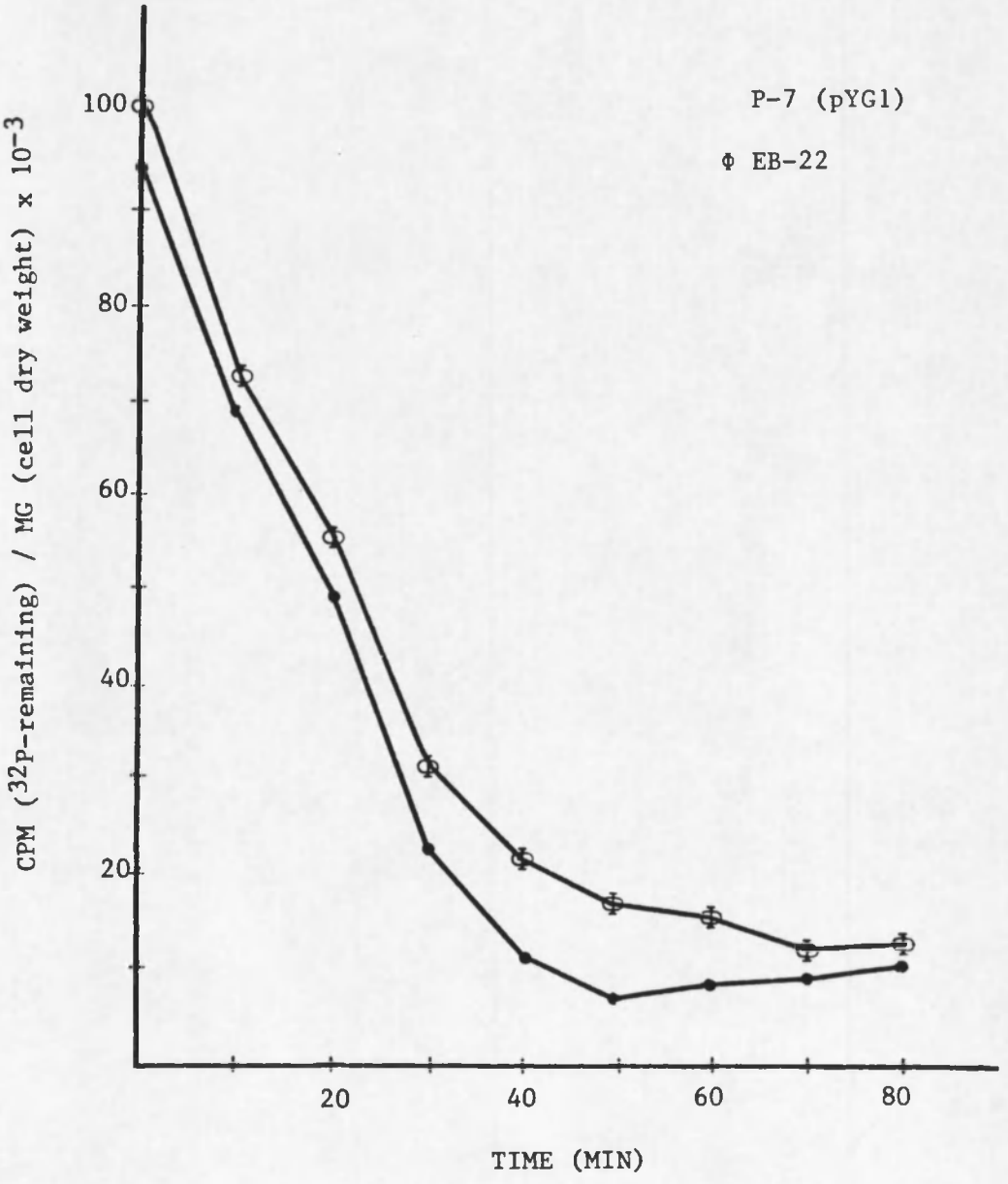

Figure 3. Removal of ^{32}P-Orthophosphate by Strains P-7 and EB-22

CHAPTER 4

DISCUSSION

The n-alkane degradative capacities of A. phosphadevorus have been well established (34). Previous work by Ferguson (13) demonstrated that the organism was unable to grow on most aromatic hydrocarbons, in broth culture. This study indicates that A. phosphadevorus can grow on camphor, benzene, and phenylethyl alcohol using agar plates. These differences may be explained by the fact that with the agar plate technique, the organism is exposed to hydrocarbon vapors and does not come in direct contact with high concentrations of hydrocarbon. This contention is supported by the observation that when agar plates are flooded with hydrocarbon before streaking, no growth is observed with benzene or phenylethyl alcohol. N-butanol and tributyrin also supported growth, although a previous study reported the opposite result (13).

Hydrocarbon degradation is not restricted to only a few genera of bacteria. This characteristic has been observed in a wide variety of bacteria genera, yeast and other fungi, and algae, including cyanobacteria and eukaryotic algae (39). These organisms are found in a wide variety of environments, including marine, fresh water, and soil habitats (2). In a few bacteria, the hydrocarbon degradation has been shown to be plasmid mediated (5, 15, 35, 47).

This is the first report of plasmid mediated hydrocarbon utilization in Acinetobacter sp. Although A. phosphadevorus is able to degrade n-alkanes as small as octane and as large as tetracontane (C_{40}) (13), the plasmid (pYG1) only codes for utilization of octane, nonane, and decane. There was no indication that plasmid functions were involved in the utilization of aromatic hydrocarbons or any of the other n-alkanes tested.

The enzymes of the decane utilization pathway encoded by pYG1 are not known. A similar plasmid (OCT) found in Pseudomonas oleorovans only codes for the alkane hydroxylation and alcohol dehydrogenation steps of the terminal oxidation pathway used by this organism (36).

Chromosomally-mediated alkane catabolism can occur via a number of different pathways. The most prevalent mechanism in Acinetobacter sp. is the terminal oxidation pathway; i.e., $R\text{-}CH_3 \rightarrow R\text{-}CH_2OH \rightarrow R\text{-}COOH \rightarrow$ beta-oxidation pathway of fatty acids (26). Less ubiquitous mechanisms, in other genera, are omega (diterminal) oxidation and subterminal oxidation (27, 32). An unusual mechanism found by Finnerty (14) in an Acinetobacter sp. involves terminal oxidation followed by splitting the hydrocarbon at position ten with subsequent formation of a hydroxy acid and an alcohol.

The pathway(s) by which A. phosphadevorus catabolizes n-alkanes has not been elucidated. Preliminary work has shown that the fate of labeled hexadecane and hexadecanoic acid are significantly different (34). This indicates that hexadecanoic acid is not an intermediate in

the degradation of hexadecane and mitigates against the existence of a terminal oxidation mechanism for hexadecane catabolism.

Results from the growth studies on hydrocarbon mutants HP-2 and HP-6 indicate that alkane utilization in A. phosphadevorus may be quite complex (Table 8). HP-2 does not grow on C_{15}, C_{16}, or C_{17}, but does grow on the other n-alkanes (on C_{13}, C_{14}, C_{18}, and C_{20}, slight growth was observed). Strain HP-6 (pYG1$^-$), however, cannot grow on C_8 through C_{12}, grows slightly on C_{13} and C_{14}, but does grow well on C_{15} through C_{18} and C_{20}. Therefore, it appears that there are at least two chromosomally-mediated n-alkane catabolic pathways, differing by one or more enzymes. One pathway may mediate degradation of C_{11}, C_{12}, C_{18}, and C_{20} n-alkanes, the other may mediate catabolism of C_{15}, C_{16}, and C_{17}. The pathways may overlap in function; this would explain the slight growth observed by either mutant on C_{13}, C_{14}, C_{18}, and C_{20}. However, any proposals at this point are highly speculative.

Other growth substrate mutants isolated in this study were an acetate mutant (AP-2) and a citrate mutant (CP-1). These were characterized using growth substrate studies and labeled substrate uptake experiments.

The citrate mutant was found to be unable to take up labeled citrate, although cell-free preparations could oxidize the compound. Therefore, it was concluded that CP-1 represented a citrate transport mutant. Furthermore, citrate transport was found to be energy dependent. Inhibitors of the TCA cycle ($NaAsO_2$) also inhibit uptake of citrate. This mutation in the citrate transport system did not prevent

growth on TCA cycle intermediates nor did it prevent uptake and oxidation of α-ketoglutarate. Previous work has indicated that transport is inducible and does not require citrate lyase for uptake (13, 25).

Jones (25) has demonstrated that the methyl carbon of acetate is incorporated via the serine pathway by unstarved logarithmic phase cells. As a consequence of this, the carboxyl group of acetate is released as CO_2 (70%), whereas the methyl group of acetate is incorporated into cellular carbon (25).

The acetate mutant AP-2 is unable to grow on acetate media, yet is able to oxidize acetate. Furthermore, it was found that very little of the methyl group of acetate (29%) is incorporated into cellular carbon as compared to the wildtype (66%). Also, glycine was decarboxylated at a very high rate (AP-2, 81%; P-7, 59%), but the number two carbon of both strains had similar fates (AP-2, 30%; P-7, 27%). These results do not allow firm conclusions, but it can be proposed that the inability of AP-2 to grow on acetate is due to a failure to incorporate sufficient carbon from the methyl group of acetate via the serine pathway.

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