CHARACTERIZATION OF THE SPECIFICITY OF DNA UPTAKE

AND TRANSFORMATION BY NATURALLY COMPETENT

Acinetobacter calcoaceticus

THESIS

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Mohsen Ahmadian-Tehrani, B.S.

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Although cultures of *A. calcoaceticus* BD413 are naturally
competent at all growth phases, genetic markers can only be
successfully transformed by DNA from the genus *Acinetobacter*.
Transformation frequencies of nearly 1.2% were obtained when
pcaA mutants were incubated in liquid culture with wild-type
DNA. Marker transformation was found to be competed for with
equal effectiveness by DNA derived from *A. calcoaceticus*,
Pseudomonas putida or *Escherichia coli*. Results further
suggest that DNA sequences derived from both *A. calcoaceticus*
and *E. coli* were successfully internalized by *Acinetobacter*
cells. These results suggest that *Acinetobacter* does not
utilize an "identity sequence" to identify *Acinetobacter* DNA
and that discrimination against non-*Acinetobacter* DNA
sequences occurs at the level of recombination, not DNA
uptake.

AKNOWLEDGEMENTS

TABLE OF CONTENTS

    I. INTRODUCTION.....................................1

        Evolution of Bacterial Genetics
        Mechanisms of Genetic Exchange or Transfer
        Natural Transformation Processes
        Properties of the Genus *Acinetobacter*
        Project Significance
        Project Objective

    II. MATERIALS AND METHODS...........................17

        Bacterial Strains and Plasmids
        Media and Growth Conditions
        Isotopes, Enzymes and Chemicals
        Preparation of Transforming Chromosomal DNA
        Cloning DNA Fragments Into pUC18
        E. coli Competent cell Preparation
        Transformation of Competent Cells with pUC18
        Rapid Plasmid Isolation by Alkaline
           Lysis-Analytical Scale
        Preparative Scale Recombinant Plasmid
           Isolation
        Final Purification of Plasmids From
           CsCl/EtBr Solution
        Ethanol Precipitation
        Acinetobacter Transformation Procedures
        End Labeling of DNA Fragments

    III. RESULTS......................................38

        Effect of Time of Incubation on
           Transformation Efficiency
        Optimal DNA Concentrations
        Effect of Size of Transforming DNA on
           Efficiency of Transformation
        Characterization of Cellular Competence

III. RESULTS (continued)

Recipient Cell Density
Transformation on Semisolid (Agar) Media
Effect of Incubation Temperature on the
   Efficiency of Transformation
Effect of Starvation of Cells Prior to
   Transformation
Effect of Incubation Medium on
   Transformation Efficiency
Comparison of Transformation of ADP6
   Cells by Wild-type and Ac138 (arg⁻)
   Chromosomal DNA
Effect of Restriction Digestion of the
   Transforming DNA on the Efficiency
   of Transformation
Transformation of ADP6 Cells by
   Heat-denatured DNA
Transformation of Other Strains of
   A. calcoaceticus With Chromosomal DNA
Effect of Presence of Competing DNA on the
   Frequency of Transformation in ADP6
Effect of Presence of Competing DNA on the
   Frequency of Transformation in Ac138
Effect of Presence of Restriction Digested
   Competing DNA on the Frequency of
   Transformation in ADP6
Uptake of Radioactive DNA by ADP6 Cells
Gel Analysis of DNA Fragments Taken Up by
   ADP6 Cells

v

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

A	absorbance
acetyl-CoA	acetyl coenzyme A
BAP	bacterial alkaline phosphatase
bp	base pair
°C	celsius
cAMP	adenosine-3',5'monophosphate
cm	centimeters
CsCl	cesium chloride
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
g	gram
IPTG	isopropyl-ß-D-thio-galactopyranoside
k	1,000
kbp	kilobase pair
l	liter
M	molar
MCS	multiple cloning site
ml	milliter
mM	millimolar
mm	millimeter
µl	microliter
µci	microcuri
nm	nanometer
PEG	polyethyleneglycol
POB	p-hydroxybenzoate
rpm	revolutions per minute
UV	ultraviolet
V	volts
Xgal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

CHAPTER I

INTRODUCTION

Bacterial transformation, the uptake of genetic material
from the surrounding medium and subsequent acquisition of a
heritable altered phenotype, is a major avenue of genetic
exchange for a number of scientifically and medically
important bacterial genera. Although it is likely that only
a minority of bacterial species are naturally transformable,
members of the *Bacillus*, *Streptococcus* and *Haemophilus* genera
utilize transformation as a primary mechanism of genetic
exchange, comparable in importance to either conjugation or
transduction. Within the Gram-negative genera, strains of
Haemophilus, *Neisseria*, *Acinetobacter* and *Moraxella* have been
observed to exhibit naturally high levels of transformation
competence (reviewed by Stewart and Carlson, 1985).
Haemophilus influenzae and *H. parainfluenzae* (and more
recently to some extent *Neisseria*) have been utilized
extensively as model Gram-negative organisms in
transformation studies. However, these genera include
significant human pathogens and generally require complex
expensive growth media and/or conditions. Collectively these
properties restrict the application of many recombinant DNA
techniques and significantly reduce their potential

1

usefulness as tools in the biotechnology industry for the production of recombinant DNA products.

Acinetobacter calcoaceticus is a common soil microorganism possessing a number of genetic and metabolic properties which strongly indicate the potential value of better understanding the transformation process in this Gram-negative organism. *A. calcoaceticus* strains such as BD413 are naturally competent for transformation (Juni, 1972).

Evolution of Bacterial Genetics

The development of gene transfer mechanisms in microorganisms is probably due to the competitive advantage of accumulating independently derived beneficial mutations within a single cell (Bodmer, 1972). Since most bacteria reproduce by binary fission, the mixing of genes requires genetic exchanges between cells followed by recombinational events. Such a process can lead to the rapid mixing and/or dispersion of genes through out a large population of cells (reviewed by Stewart and Carlson, 1985). In this way, cells may both acquire advantageous mutations and eliminate deleterious ones.

Mechanisms of Genetic Exchange or Transfer

Genetic exchange in microorganisms is mediated by one or more of three basic processes. These are conjugation,

transduction and transformation (reviewed by Smith et al., 1981). Conjugation involves the transfer of a copy of a portion or even all of a donor cell's chromosome to a recipient cell. This process is generally plasmid-mediated (e.g. the F plasmid in *E. coli*). Transduction is another common mechanism of gene transfer utilized by bacterial cells in which a small fraction of the donor cell's chromosome is transferred to an appropriate recipient cell via a "transducing" bacteriophage. The third mechanism is referred to as transformation. Transformation involves the uptake of naked DNA by cells from their surrounding environment. The ability or the inability to demonstrate transformation as a natural process in any given bacterial strain is dependent on the ability of the recipient cell to exhibit competence. Competence in this context is simply defined as the physiological state where cells exhibit the ability to bind and take up DNA (Venema, 1979). Competence development in some microorganisms occurs naturally (natural competence). However, in other cases the development of competence must be induced by chemical and/or other manupilations in the laboratory (Stewart and Carlson, 1985). This is referred to as artificial competence.

Natural Transformation Processes

Transformation can be divided into four different stages. Theses are: 1) competence development, 2) DNA

biding, 3) DNA uptake and 4) integration of the DNA into the recipient cell's chromosome with the subsequent exhibition of an altered trait (Stewart and Carlson, 1985). Natural transformation falls into two general categories as determined by the overall mechanism utilized in each case. These two different mechanisms are utilized by Gram-negative and Gram-positive organisms, respectively. The mechanism utilized by Gram-positive organisms has been put forth primarily based upon studies of *Streptococcus pneumonea* and *Bacillus subtilus*. The mechanism unique to Gram-negative organisms has been based upon studies of *Haemophilus influenzae*, *H. parainfluenzae*, and *Nisseria gonorroeae* (reviewed by Smith et al., 1981).

Most of the naturally competent bacteria studied exhibit competence development as a regulated phenomenom (Goodgal, 1982). In the case of Gram-positive organisms such as *Streptococcus*, the presence of a secreted extracellular protein called "competence factor" induces the development of competence (Goodgal, 1982). This protein is secreted continuously, but only attains critical levels in the medium as the cell density increases to a preset value. Once the protein concentration is attained, a series of genes are induced and competence results. Conditioned medium from high density cell cultures can therefore readily induce competence in new cells, even at low cell densities. In contrast to Gram-positive bacteria, transformation studies in *H.*

influenzae and *H. parainfluenzae* have failed to show the presence of any competence factor in Gram-negative bacteria (Smith, et al. 1981). Gram-negative competence development is induced during the stationary phase of the growth cycle (Ingraham, et al. 1983) as a result of the accumulation of growth-inhibitory metabolites such as cAMP (Stewart and Carlson, 1985).

Following the development of competence, the next step in the natural transformation process is the binding of donor DNA to the cell surface. Gram-negative and Gram-positive are both transformed only by double-stranded DNA molecules, single-stranded molecules being completely ineffective. Additionally, both Gram-negative and Gram-postive exhibit what is referred to as "loose" and "tight" DNA binding proteins. The initial interaction of donor DNA with the cell surface is through "loose binding" to relatively non-specific DNA binding proteins. Beyond this point the mechanisms of Gram-positive and Gram-negative organisms differ significantly. Gram-negative organisms require the presence of specific "identity sequences" in order for high affinity binding and subsequent DNA uptake to occur (Khan and Smith, 1984 and Dougherty, et al., 1979). These sequences identify DNA as being derived from the particular genus of which the individual cell is a member. On the other hand, Gram-positive organisms do not utilize specific DNA uptake sequences.

Gram-positive organisms have divalent cation-dependent nucleases which cleave the DNA and degrade one strand (only 1 strand is internalized). Lacks demonstrated that 50% of counts originally associated with radioactively-labeled transforming DNA were released into the medium by *S. pneumonea* cells that were undergoing transformation (Lacks, 1977). The internalized single-stranded DNA is protected against further nuclease digestion by specific single-stranded DNA binding proteins. The period when the DNA is single-stranded is referred to as the "eclipse phase" since no transforming activity is found in DNA recovered from the cells during this time (Morrison, 1977). This process is calcium-dependent and characteristically inhibited by EDTA in the medium (Seto, et al. 1976). Gram negative organisms, in contrast, do not cleave or degrade the DNA during the internalization process (reviewed by Stewart and Carlson, 1985). The presence of EDTA in the medium does not inhibit DNA uptake by Gram-negative organisms. For example, Ahlquist has reported that calcium treatment does not increase the frequence of transformation in *Acinetobacter calcoaceticus* (Ahlquist, et al. 1979). Not surprisingly, the eclipse period, if one occurs, in Gram-negative organisms is very short. Furthermore, the bound double-stranded DNA-receptor complex is contained within a surface-associated vesicle during uptake in *Haemophilus* and *Neisseria* (Stuy and van der Have, 1971).

As the final step in Gram-positive transformation, the internalized DNA molecule is integrated into the recipient cell's genome by a single-stranded displacement mechanism (Khan and Smith, 1984). Experiments carried out with *S. pnemoniae,* utilizing DNA preparations that were reisolated from recipient cells at various stages of the transformation process, indicated that single-stranded DNA in the eclipse phase is unable to transform other competent *S. pnemoniae* cells. However, the DNA reisolated from recipient cells at later stages of transformation, following integration into the recipient cell chromosome, was capable of transforming other *S. pnemoniae* cells (Lacks, 1964). In the case of the Gram-negative *Haemophilus* cells, where a double-stranded form of the transforming DNA is internalized, the integration may be made possible by a similar process involving the formation of a heteroduplex region with the recipient cell's chromosome by physically displacing the homologous strand (Stewart and Carlson, 1985). This strand displacement would involve either the degradation of one strand or simple strand displacement as catalyzed by a RecA type protein.

Properties of the Genus *Acinetobacter*

The name *Acinetobacter* was first used in 1954, but until recently there has been much confusion concerning the classification of these organisms (Brisou and Prevot, 1954). Acinetobacters are aerobic, Gram-negative, non-motile,

non-pigmented coccobacilli, which can be isolated from most environments including soil, water and sewage. Because they lack sufficient distinguishing characteristics, however, strains now known to be acinetobacters, were originally claimed to be members of 15 different genera, including *Moraxella*, *Achromobacter*, *Diplococus* and *Mima* (Juni, 1978).

The interstrain transformation assay, developed by Juni, has been used as a measure of genetic relatedness, and careful taxonomic studies have eliminated earlier confusion (Juni and Janik, 1969). For this assay, a stable auxotroph of *A. calcoaceticus* strain BD413, carrying mutation in the highly conserved *trpE* gene serves as a recipient for donor DNA samples. Those samples derived from bacteria of the same genus are able to transform the mutant to prototrophy, whereas samples from unrelated bacteria are not. This assay is still used quite frequently, often to identify hospital isolates. Although they are not primary pathogens, acinetobacters have been implicated in infections of debilitated or immunocompromised patients (Henriksen, 1973).

A. calcoaceticus strain BD413 is naturally competent for transformation by homologous DNA, with maximum competence developing at the beginning of the stationary phase (Juni, 1972). Strains used in the present study were derived from the BD413 stain of Juni. Although these are also naturally competent, but other strains of *A. calcoaceticus* are not (Juni, 1972). The strain ADP6 (a derivative of BD413) used

in this study is a mutant that lacks the ability to utilize
p-hydroxybenzoate (POB) due to a mutation in the pcaA gene in
the ß-ketoadipate pathway (Fig. 1). The ß-ketoadipate
pathway is used by acinetobacters and pseudomonads for the
biodegradation of aromatic and hydroaromatic compounds such
as benzoate, catechol or p-hydroxybenzoate (Ornston and Yeh,
1982). As opposed to the pseudomonads, the ß-ketoadipate
pathway of A. calcoaceticus is comprised of two totally
separate parallel metabolic branches. One branch, mediated
by six enzymes encoded by cat genes, converts catechol to
succinate and acetyl coenzyme A (acetyl-CoA). The other
branch, catalyzed by products of the pca genes, converts
protocatechuate to succinate and acetyl-CoA in six metabolic
reactions analogous or identical to those of the the catechol
branch (Doten et al., 1987a). The sequence of the reactions
in the biodegradative oxidation of POB by A. calcoaceticus is
illustrated in Fig. 1. Other auxotrophic derivatives of
BD413 have been isolated and the corresponding mutations have
been mapped. However, characterization of the chromosomal
arrangement of the corresponding genes in A. calcoaceticus is
far from complete (Sawula and Crawford, 1972; Twarog, 1972;
Ginther, 1978).

A conjugation system of plasmids capable of chromosome
mobilization, developed by Towner and Vivian (Towner and
Vivian, 1976), was used to demonstrate circularity of the A.
calcoaceticus chromosome. The exact size of the chromosome

^-OOC — [benzene ring] — OH

p-hydroxy benzoate
hydroxylase

pobA →

^-OOC — [benzene ring] with OH, OH

protocatechuate-
3,4-oxygenase

pcaA / O_2 →

^-OOC — C=C — COO^- ... C=O

ß-carboxymuconate
lactonizing enzyme | *pcaß*

O=C — CH_2 — COO^- ... COO^-

ß-ketoadipate
enol-lactone hydrolase

← *pcaD*

^-OOC — [ring with O] — COO^- ... C=O

← *pcaC* / CO_2

^-OOC — [ring with O] — COO^- ... C=O

Y-carboxymuconate
decarboxylase

pcaE | ß-ketoadipate
succinyl CoA transferase

ß-ketoadipyl CoA — *pcaF* → Succinyl CoA + Acetyl CoA

ß-ketoadipyl CoA
thiolase

Figure 1. The ß-ketoadipate pathway of *A. calcoaceticus* for biodegradation of *p*-hydroxybenzoate (POB). The enzyme encoded by *pcaA* gene, protocatechuate 3,4-dioxygenase, is not synthesized by strain ADP6. Hence, these mutants are unable to utilize POB as a sole carbon source.

has yet to be determined. The frequency of this plasmid-mediated chromosomal gene transfer was too low to use conventional interruption techniques, but it was possible to establish the approximate location of 23 markers on a circular linkage map (Towner, 1978).

Most of these loci represent auxotrophic markers. However, little is known about the organization of the genes which confer the great nutritional versatility characteristic of acinetobacters. *Acinetobacter* species are able to utilize many organic compounds as growth substrates, including alcohols, hydrocarbons, carbohydrates, aliphatic acids, amines, amides, amino acids and aromatic compounds (Baumann et al., 1968).

Project Significance

Genetic transformation, first discovered in *S. pneumoniae* by Griffith in 1928, is the process by which naked DNA is taken up from the surrounding medium by competent cells and incorporated so as to alter the genotype in a heritable fashion. Competence is a physiological state which may occur naturally in some bacterial species or artificially produced in other species (artificial competence).

Although it is likely that only a minority of bacterial species are naturally transformable, members of several genera can exhibit significant levels of natural transformation competence and the process does represent a

major avenue of genetic exchange for several biologically and medically important genera. For example, as discussed earlier, certain species of *Bacillus*, *Streptococcus* and *Haemophilus* genera utilize transformation as a primary mechanism of genetic exchange, comparable in importance to either conjugation or transduction. However, the actual mechanism utilized can vary substantially from species to species. For example, there is only limited similarity in the properties/mechanisms of transformation utilized by Gram-negative and Gram-positive organisms. Gram-positive transformation has been studied extensively in several *Streptococcus* and *Bacillus* species, while *Haemophilus* and *Neisseria* species have been utilized as the primary model organisms for the study of natural transformation in Gram-negative bacteria. However, these Gram-negative genera include a number of human pathogens (which can restrict the application of many recombinant DNA techniques) and generally require complex (and expensive) growth media. This has sometimes limited their usefulness for basic scientific studies as well as industrial applications which could benefit from the naturally high level of competence.

Acinetobacter calcoaceticus is a common soil micro-organism (Juni, 1972) possessing a number of genetic and metabolic properties which strongly indicate its superiority as a model system for the study of Gram-negative transforma-tion. These include:

1. It is not considered a human pathogen (although it can infect immunocompromised individuals as an opportunistic pathogen).

2. The genus *Acinetobacter* exhibits a high degree of metabolic diversity, being able to utilize a wide spectrum of simple inexpensive media (such as succinate or benzoate) as sole carbon sources.

3. *A. calcoaceticus* strain BD413 (in addition to a number of other strains) is naturally highly transformation competent strain (Juni, 1981), readily taking up large quantities of DNA from the surrounding medium. High marker transformation frequencies can be obtained under appropriate conditions, provided the donor gene is from the genus *Acinetobacter* (Juni, 1972).

4. Cloned pieces of *A. calcoaceticus* DNA isolated from plasmids maintained in *E. coli* or *Pseudomonas putida* also transform *A. calcoaceticus* BD413 efficiently (Neidle, et al. 1986; 1987). Thus, this strain does not exhibit a restriction barrier to transforming DNA and any unique properties of *Acinetobacter* DNA required for transformation are in the primary sequence.

Further properties of *A. calcoaceticus* which will facilitate transformation studies also make it a potentially attractive cloning and expression system for both research and applied biotechnology purposes. In addition to utilizing a wide spectrum of carbon sources not commonly used by many

microorganisms, *A. calcoaceticus* grows at substantially acidic pH (pH 5-6, see Juni, 1981). Together these traits could reduce sterilization costs of commercial scale production procedures involving *Acinetobacter* strains.

A further interesting property of *Acinetobacter* transformation is found at the level of incorporation of transforming DNA into the host chromosome. Attachment of non-*Acinetobacter* DNA to donor DNA is not inhibitory to the process of transformation. If the cloned donor DNA is linear (e.g. *Acinetobacter* DNA flanked by heterologous vector), only the *Acinetobacter* portion is incorporated into the recipient chromosome. However, if the donor is a circular plasmid, the presence of *Acinetobacter* sequences can direct the uptake and incorporation of the entire plasmid into the recipient cell chromosome (personal communication, Shanley, Neidle and Ornston). This raises the possibility of creating long term stable integrates of genetically engineered non-*Acinetobacter* genes into the host chromosome, eliminating problems related to vector size and incompatability issues. This could greatly facilitate the construction of large genetically engineered metabolic pathways not readily assembled on a single plasmid.

Project objectives

The overall objective of this thesis is to study the transformation process in *Acinetobacter calcoaceticus*. This

characterization involves further defining the requirements and the mechanism of high frequency transformation. The characterization of the mechanism of transformation is accomplished by meeting the following objectives:

1. Observe and report the optimal conditions and the frequency of natural transformation during incubation either in liquid or on solid media.

2. Estimation of the quantity of DNA taken up per competent cell.

3. Characterize the uptake of transforming DNA with respect to size, shape and species of origin.

4. Clarify the stage in the transformation process in which the discrimination occurs between *Acinetobacter* and non-*Acinetobacter* DNA.

CHAPTER II

MATERIALS AND METHODS

Bacterial Strains and plasmids

A. *calcoaceticus* strains were derived from strain BD413

(Juni, 1972; Juni and Janik, 1969) which is designated strain

ADP1 in our laboratory collection. Strains ADP1 and ADP6

(*pcaA*⁻) were kindly provided by Nicholas Ornston. ADP6 is a

spontaneous mutant and was selected for its inability to grow

on 5 mM *p*-hydroxybenzoate (POB). Unlike many other bacteria,

A. *calcoaceticus* is able to grow on POB as the sole carbon

source. Strains Ac47 (*trpA*), Ac52 (*ilv*), Ac138(*arg*),

Ac141(*arg*), Ac330 (*cys*), Ac411 (*trpE*) were kindly provided by

Elliot Juni. These strains were used in competition

experiments. The two *arg* mutants were obtained independently

and may not be linked (Juni, personal communication). The

trpA and *trpE* sites are not linked (Crawford, 1976). For

cloning, *E. coli* JM101 (*supE, thi,* Δ(*lac-proAB*), F' *traD36*

proAB⁺*lacI*�q*Z*ΔM15) was used as the recipient cell.

The pUC18 (Ap *lacp/o*) plasmid was used as a cloning

vector (Yanisch-Perron et al., 1985). It carries a region,

the multiple cloning site (MCS), in which there are

restriction enzyme recognition sites which occur only once in

the vector. This vector-host system allows direct screening

for recombinant plasmids. DNA inserts into the pUC18 MCS
destroy the vector's ability to complement a defective JM101
ß-galactosidase. Hence, transformants carrying recombinant
plasmids are not able to cleave Xgal (5-bromo-4-chloro-
3-indolyl-ß-D-galactopyranoside), a color-indicator substrate
of ß-galactosidase. These transformants appear as white
colonies on agar plates containing Xgal. On such plates,
transformants carrying intact pUC18, which are able cleave
the color indicator, appear as blue colonies. *A.
calcoaceticus* stains and plasmids carrying cloned *A.
calcoaceticus* fragments are listed in Table 1.

Media and Growth Conditions

Cultures of *A. calcoaceticus* were grown at 37°C on
minimal medium (Ornston and Stanier, 1966) supplemented with
Hunter's Metals 44 (Cohen-Bazire, 1957) and solidified with
1.5% Difco Agar Noble® as required. Either 10 mM succinate,
5 mM Na benzoate, or 5 mM POB were provided as the carbon
source. Cultures of *E. coli* were grown at 37°C on L broth
(LB) (10 g peptone, 5 g yeast extract, 10 g NaCl, in 1 liter
volume) or on L agar containing 1.5% Difco Bacto-Agar®.
Supplements were added as needed at the following
concentrations: ampicillin (Ap), 50 µg/ml; Xgal, 0.006%;
isopropyl-ß-D-thio-galactopyranoside (IPTG), 1mM. All liquid
cultures were incubated on a New Brunswick™ Environmental
Gyrotory shaker at 250 rpm.

Table 1. Strains and plasmids used in this study.

Strains and plasmids	Relevant description	Source or reference
A. calcoaceticus		
ADP1	Wild type (BD413)	Juni, 1969
ADP6	*pcaA3006*	Doten, 1987b
Ac47	*trpA*	Juni, 1969
Ac52	*ilv*	Juni, 1969
Ac138	*arg*	Juni, 1969
Ac141	*arg*	Juni, 1969
Ac330	*cys*	Juni, 1969
Ac411	*trpE27*	Juni, 1969
E.coli		
JM101	*supE thi* Δ(*lac-pro*AB) F' *tra*D36 *pro*AB *lac*I^{q}ZΔM15	Yanisch, 1985
Plasmids		
pUC18	Ap *lacp/o*	Yanish, 1985
pZR1	*pcaABCDFE* on 11-kbp *Eco*RI fragment in pUC18	Doten, 1987b
pZR2	*pcaAC* on 2.5-kbp *Hind*III fragment in pUC18	Doten, 1987b
pAN2	*catBCDE* on 5.0-kbp *Eco*RI fragment in pBR322	Shanley, 1985
pDK2-3087	*xylDLEGFH* on 17-kbp *Hind*III fragment in pBR322	Azadpour This lab
pMO8	*xylDEGFH* on 16-kbp *Hind*III fragment in pBR322	This study

Isotopes, Enzymes and Chemicals

Radiolabeled compounds were purchased from the Amersham Corporation. Bacterial alkaline phosphatase was obtained from Bethesda Research Laboratories, and DNA ligase was obtained from New England Biolabs Corporation. Restriction enzymes were purchased from Bethesda Research Laboratories. All other chemicals and enzymes were obtained commercially at the highest purity available.

Preparation of Transforming Chromosomal DNA

Transforming DNA from ADP1 was isolated according to Marmur (Marmur, 1961). The DNA was further purified by CsCl-ethidium bromide density gradient centrifugation. After removal of dye by water-saturated butanol extraction, the excess salts were removed by dialysis against 50 mM Tris-HCl, pH 8.0, containing 1 mM ethylene diamine tetraacetic acid (EDTA) and 15 mM NaCl. DNA was stored at -20°C throughout this study with no detectable loss of transforming activity.

Cloning DNA Fragments Into pUC18

The pUC18 DNA was first subjected to restriction endonuclease digestion. Recirculariztion of linearized vector was prevented by the use of bacterial alkaline phosphatase (BAP). It is customary to carry out the initial enzymatic digestion in 50 μl total volume. Upon completion of digestion, 70 to 100 μl of TE buffer and 1 μl of BAP was

added (100-200 units for 5 µg of DNA). The reaction mixture
was incubated at 65°C for 2 to 3 hours. The vector DNA was
then subjected to phenol extraction and ethanol
precipitation. The DNA was dissolved in a small volume of
distilled water or TE buffer. The DNA fragment which was to
be inserted into pUC18 was also subjected to endonuclease
enzyme digestion (30-50 µl total volume). Upon completion of
the digestion, both vector and insert were subjected to
phenol extraction, ethanol precipitation and dissolved in
small volumes of distilled water or TE buffer.

A ratio of 3 moles vector to 1 mole insert DNA was
routinely used. However, a ratio of up to 10 vector DNA can
be used without adverse effects. Vector and insert DNA were
added to 1.5 ml microfuge tube. An appropriate amount of 5X
ligation buffer (250 mM Tris-HCl, pH 7.6, 25% w/v PEG 8000,
50 mM $MgCl_2$, 5 mM dithiothreiotol) and 1 µl of T4 DNA ligase
(10 units) were added. The tube was manually mixed,
centrifuged briefly and then left at room temperature for 4
hours (22°C-25°C). The total volume of the ligation reaction
was usually 20 µl, but volumes of up to 30 µl may be used.

E. coli Competent Cell Preparation

Competent E. coli cells (strain JM101) were prepared by
inoculating 5 ml LB medium and incubating at 37°C overnight
in a New Brunswick™ shaker at 250 rpm. The following day,
0.5 to 1.0 ml of the starting culture was transferred

aseptically to 50 ml of LB medium in a 300 ml flask. This was incubated at 37°C in the same shaker and the absorbance (A) of the culture was monitored at 550 nm. At an A_{550} of 0.4 to 0.5, the culture was aseptically transferred to two sterilized, chilled 30 ml Corex™ or Oakridge™ style sterilized tubes. The culture was kept in an ice bath for 15 to 25 minutes and then centrifuged in a refrigerated centrifuge (4°C) at 3,000 xg for 8 minutes (Sorval™ SA600 rotor was used). The centrifuge was stopped with the brake off. The supernatant was discarded and the pellet gently resuspended in 10 ml of ice-cold 50 mM $CaCl_2$. Extra care should be used when resuspending the cells due to their fragile nature (a sterile Pasteur pipette may be used to resuspend the cells). The cells were left on ice for 30 minutes and collected at 3,000 xg for 5 minutes in the same centrifuge. The supernatant was discarded and the pellet was resuspended in 1 ml ice-cold calcium chloride solution per tube. To maximize competency of *E. coli* cells, they may be left in the ice bath for 12 to 24 hours. Afterwards, they may be stored at -70°C. Aseptic techniques were used throughout the procedure.

Transformation of Competent Cells With pUC18

Sterile 12 x 75 mm tubes were chilled in an ice bath, and 0.1 to 0.2 ml of competent cells was aseptically dispensed into these tubes. Approximately 0.1 to 1.0 µg of

the DNA ligation mixture was added and the tubes mixed by gently rolling between the palms. They were left in an ice bath for 30 minutes and then heat shocked in a 42°C water bath for 2 minutes. LB agar plates containing the proper antibiotic(s) were prepared by spreading on their surface 10 μl of 100 mM IPTG and 50 μl of 2% X-gal. About 30 minutes is needed for these liquids to be absorbed into the agar. The transformed cells were then transferred with a sterile Pasteur pipette to the petri plates and evenly spread over the surface with an alcohol sterilized bent-glass rod. These plates were then incubated at 37°C in an upright position to allow the liquid to soak in (1 hour is adequate). They were then inverted and left in the 37°C incubator overnight. After incubation, plates were evaluated and colonies carrying recombinant plasmids were identified. Blue colonies carry plasmids with no insert DNA. White colonies carry plasmids with inserted DNA, inactivating the *lacZ* complementation by the plasmid. Colonies were picked with a sterile toothpick and transferred to proper antibiotic plates (ampicillin), freshly prepared for this purpose. Following overnight incubation, the plates were again evaluated. Transformants were identified and subjected to further analysis.

It is advisable to set up controls when carrying out transformation procedures. This may be done by plating competent cells on non-selective medium at a proper concentration (10^{-2} or 10^{-3} dilution), which gives a viable

cell count. If many colonies are seen on control plates, but only few or none are seen on selective medium plates (plates that have antibiotic to which transformants are resistant), then the cells were not competent, or at a very low level of competence. A parallel transformation with 0.1-0.2 µg of uncut plasmid should produce numerous blue colonies on selective medium. This provides an estimate of the level of competence. In general, poor transformation may result from poor quality cells, failure of ligation, dilution errors, or incomplete enzyme digestion(s).

Rapid Plasmid Isolation by Alkaline Lysis-Analytical Scale

The alkaline lysis procedure (Maniatis, et al. 1982) was utilized for the isolation of analytical amounts of recombinant plasmids up to 15 kbp in size from *E. coli*. It is a very efficient procedure for screening up to 48 colonies at a time in 5-6 hours. Culture tubes that have 5 ml of LB medium and an appropriate antibiotic were inoculated with a single colony and incubated overnight at 37°C in a New Brunswick™ shaker at 250 rpm. On the following day, cells were collected in 1.5 ml microfuge tubes by centrifuging 1.5 ml of the culture at 10,000 xg for 2 minutes. The supernatant was discarded, another 1.5 ml of culture was added and the tubes centrifuged again. The pellet was resuspended by vortexing with 100 µl of an ice-cold solution

of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0, and 6

mg lysozyme per ml (added just before use). Tubes were left

at room temperature for 5 minutes. Afterwards, 200 μl of a

fresh solution of 0.2 N NaOH and 10% SDS were added and the

tubes were mixed by inversion several times. They were then

kept in an ice bath for 5 minutes. At this time 150 μl of an

ice-cold solution of 5 M potassium acetate, pH 4.8, was added

to neutralize the alkali. Tubes were mixed by inversion and

then kept on ice for an additional 5 minutes. They were

centrifuged at 10,000 xg for 5 minutes at 4°C and the

supernatant was transferred to a fresh 1.5 ml microfuge tube.

An equal volume of phenol-chloroform (about 450 μl) solution

was added. This solution was freshly made by mixing equal

volumes of phenol and chloroform. The tubes were mixed by

vortexing and centrifuged at 10,000 xg for 2 minutes. The

upper aqueous layer was transferred again to a fresh tube and

two volumes of 200 proof ethanol were added (about 750 μl).

The tubes were then vortex and left at room temperature for 2

minutes. They were then centrifuged at 10,000 xg for 5

minutes in a refrigerated centrifuge at 4°C and the

supernatant discarded. The pellets were washed with 1 ml of

70% ethanol, vortexed briefly and recollected by

centrifugation at 10,000 x g for 5 minutes. The supernatants

were removed and the pellets dried in a vacuum desiccator for

5 to 8 minutes. Afterwards, the pellets were dissolved in 30

μl of TE buffer (pH 8.0) containing 25 μg/ml heat-treated

RNase. The samples were kept at -20°C until further analyzed.

Preparative Scale Recombinant Plasmid Isolation

The method of Tanaka and Weisblum (Tanaka and Weisblum, 1975) was utilized for the large scale isolation of cloning vectors and recombinant plasmids. This method was highly efficient for the isolation of the 3-20 kilobasepair plasmids utilized in this project (e.g. pBR322, pUC18 and their recombinant forms). A 30-50 ml volume of L Broth (LB) containing appropriate antibiotics (such as ampicillin at a concentration of 10-50 µg/ml) was inoculated from a fresh selective plate and incubated overnight at 37°C in a New Brunswick™ shaker at 250 rpm. The following day, four 2,800 ml Fernbach flasks containing 1 liter of LB were inoculated with 10-15 ml each of the overnight culture. These were left to shake at 37°C in the New Brunswick™ shaker at 250-300 rpm.

The absorbance was monitored at 550 nanometers (A_{550}) until it reached a value of 0.6-0.8 OD units (mid exponential phase). Aseptic techniques were used in monitoring the absorbance of cultures. When the desired absorbance was attained, 2 ml of 60 mg/ml chloramphenicol dissolved in 200 proof ethanol was added to each flask. This allows plasmid amplification to occur by blocking host cell DNA replication, while plasmid replication is relatively unaffected. The flasks were then left overnight in the New Brunswick™ shaker at 37°C. The

cells were collected using a Sorvall™ GSA rotor at 10,000 xg
for 12 minutes at 4°C. Pellets were washed of residual media
by resuspending in 20 ml 0.15 M Nacl. The suspension was
then centrifuged in Oak Ridge™ style tubes (45-50 ml
capacity) at 10,000 xg for 10 minutes in a Sorvall™ SA 600
rotor. All further steps in the plasmid isolation procedure
were performed in a cold room (4°C) in order to reduce
nuclease activity and obtain maximum plasmid yields. The
pellets were partially resuspended with a spatula in 25%
sucrose, 50 mM Tris-HCl, pH 8, and then vortexed for short
intervals until a uniform suspension was obtained. Two ml of
5 mg/ml lysozyme was then added to each tube and these were
mixed by repeated inversion. The cell suspensions were
allowed to stand on ice for 5 minutes and then 4 ml of 0.25 M
ethylene diamine tetraacetic acid (EDTA), pH 8.0, was added
to each tube. The tubes were mixed thoroughly by inversion
and allowed to stand on ice for a further 5 minutes. The
following two steps were performed in quick succession. Five
milliliters of 5 M NaCl was added and mixed well by
inversion. This was immediately followed by the addition of
2 ml of 10% sodium dodecyl sulfate (SDS) and again each tube
was mixed thoroughly by inversion. They were then left to
stand on ice in the cold room for 2 hours. The tubes were
centrifuged at 37,000 xg in an SA600™ rotor for 1 hour and
the supernatants poured into a graduated cylinder. To this,
an equal volume of isopropanol was added. The mixture was

then allowed to remain at -20°C overnight. The resulting
suspension was transferred to 250 ml centrifuge bottles and
centrifuged in a GSA™ rotor for 15 minutes at 16,000 xg.
Pellets were resuspended in 6-8 ml of Tris-EDTA (TE) buffer
(10 mM Tris, 1 mM EDTA, pH 8.0) per liter of original
culture. A spatula may be used to gently dislodge the pellet
and a magnetic stirrer was generally used to aid in obtaining
a uniform suspension (excessive foaming should be avoided).
About 45-60 minutes stirring at 4°C was needed to achieve
adequate resuspension of the pellet. Heat-treated RNase was
then added to a final concentration of 20 µg/ml in order to
eliminate as much contaminating RNA as possible. The
resulting suspension was then centrifuged at 14,000 xg for 10
minutes in an SA600™ rotor. A large pellet of denatured
protein and other cellular debris was discarded. To the
supernatant, 1.04 g cesium chloride (CsCl) per 1 ml solution
was added and allowed to completely dissolve. The solution
was then dispensed into 10.5 ml Sorvall Ultracrimp™
ultracentrifuge tubes. Enough space was left in order to add
ethidium bromide (EtBr) to a final concentration of 0.4
mg/ml. The tubes were then centrifuged at 110,000 xg for
38-40 hours in a Sorvall™ TR70 rotor. The tubes may be
viewed briefly with long-wave UV light in a dark room to
observe the DNA bands (Fig. 2).

The lower band (supercoiled plasmid) was carefully
removed using a 20G hypodermic needle and a 3 ml disposable

syringe. The liquid was dispensed into a 15 ml conical polypropylene tubes and protected from direct light by aluminum foil.

Final Purification of Plasmid From CsCl/EtBr Solutions

Ethidium bromide (EtBr) was removed from the samples by successive extractions with water-saturated butanol. Water-saturated butanol was added to each tube in an amount approximately equal to the plasmid containing solution. The tubes were vortexed thoroughly and allowed to stand upright until the liquid separates into original liquid layers. The top layer is ethidium bromide and butanol. This layer was carefully removed and the process was repeated until the top layer turns colorless (no pink color). Care should be taken not to remove any of the bottom layer containing the plasmid. It is customary to perform this procedure in a room with low indirect light until most of the ethidium bromide is removed. CsCl was removed by adding 2 volumes of distilled water and 9 volumes of 100% cold ethanol. The sample was mixed well and the tubes were allowed to stand in a -70°C freezer for 15 minutes. The samples were then warmed to room temperature (to allow CsCl to redissolve) and centrifuged at 14,000 xg for 15 minutes (4°C). Siliconized glass tubes were generally used (15-30 ml capacity, depending upon the sample volume). The supernatant was decanted and the pellets dissolved in 1 ml of 0.3 M sodium acetate. Three volumes of 100% cold

Protein

Chromosomal
DNA

Plasmid
DNA

RNA
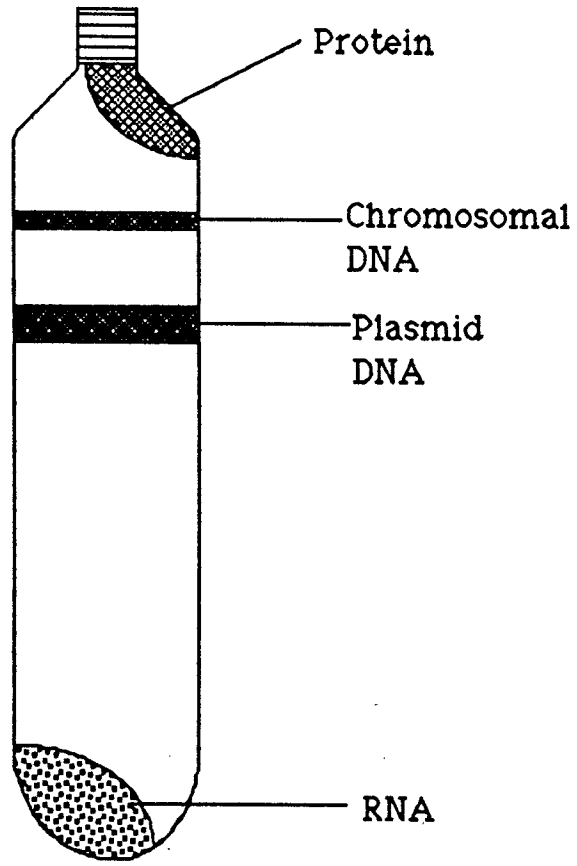
Figure 2. Ultracentrifuge tube containing plasmid and bacterial chromosomal DNA in CsCl/EtBr following ultracentrifugation as viewed under ultraviolet illumination.

ethanol were added. Samples were mixed well and then
centrifuged at 14,000 xg for 12 minutes at 4°C in a Sorvall™
SA600 rotor. The supernatant was decanted and 5 ml of 70%
ethanol was carefully added (to remove any residual
salts). The tubes were inverted to mix (instead of vortex-
ing) and then recentrifuged at 14,000 xg for 10 minutes. The
supernatant was removed, 3-5 ml of cold 100% denatured
ethanol was added and the tubes given a final centrifugation
at 14,000 xg for 5 minutes. The supernatant was discarded
and the pellet was allowed to stand for a few minutes
uncovered at room temperature in order to evaporate the
residual alcohol. An appropriate amount of sterile TE buffer
was added to dissolve the DNA pellet (generally 0.25-1.00
ml). An estimate of purity and yield was made by loading a
small sample on a 1% horizontal agarose gel. A more
quantitative estimate of yield was made by measuring the
absorbance at 260 nm of a 1:50 dilution of the dissolved
plasmid preparation (usually 20 µl of plasmid DNA is diluted
with 980 µl TE buffer). If the 1% agarose gel shows a large
amount of RNA present, the preparation can be further treated
with RNase. If this is done, the plasmid preparation should
be resubjected to ethanol precipitation in order to remove
the digestion products and redissolved in TE buffer.

Ethanol Precipitation

This protocol was performed primarily to concentrate a

DNA sample and/or to remove unwanted salts (Maxam and Gilbert 1980). An appropriate amount of 3 M sodium acetate was added to achieve a final concentration of 0.3 M. The sample was mixed on vortex and three volumes of 100% cold ethanol was added. The sample was vortex again and centrifuged at 10,000 xg for 10 minutes at 4°C. The supernatant was removed with a 9 inch pulled silconized Pasture pipette. The pellet was washed with 1 ml of 70% ethanol by inversion. The tube was then centrifuged at 10,000 xg for 5 minutes at 4°C. The supernatant was again removed with a pulled Pasteur pipette. The pellet was dried for a few minutes under vacuum and the DNA redissolved in distilled water or TE buffer.

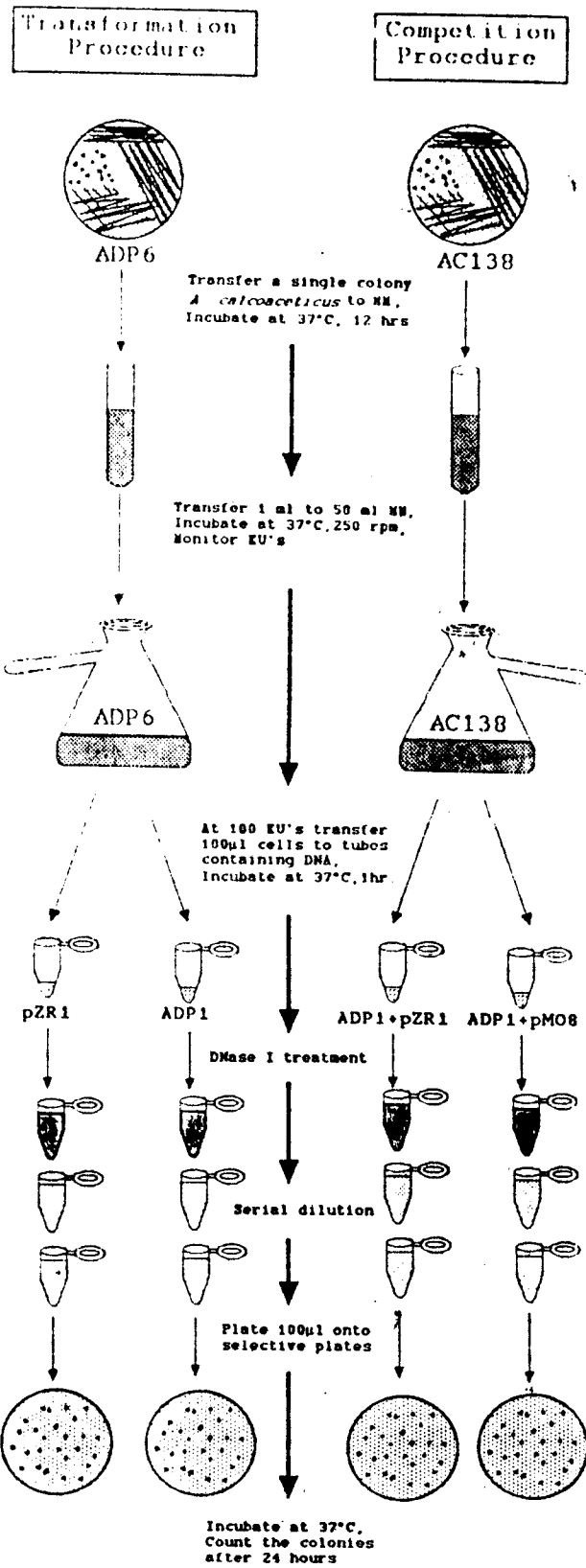## *Acinetobacter* Transformation Procedure

A. clcoaceticus auxotrophs were grown in 250 ml Klett flasks at 37°C to 100 Klett Sommerson Units (KU). At specific times, samples were withdrawn from the growth medium and diluted 1:100 into 1 ml of the sterile transformation medium. Unless otherwise specified, the transformation medium was identical to the growth medium. Purified DNA was added to 100 µl of the resulting cell suspension at a final concentration of 10 µg/ml. The transformation was carried out at 37°C for 60 minutes. Transformation was terminated by the addition of DNase I to a final concentration of 10 µg/ml. After an additional 10 minute incubation, cultures were diluted into minimal medium and 100 µl was plated onto

minimal media containing POB. The numbers of viable cells and prototrophic transformants were determined by dilution plating (see Fig. 3). Semisolid medium transformation assays were carried out utilizing an analogous procedure without the use of DNase I.

End Labeling of DNA Fragments

End labeling reactions were usually carried out using 5 to 10 μg of purified DNA fragments. Each aliquot was then digested with 20-40 units of the desired restriction enzyme. After the digestion had gone to completion, a sufficient amount of TE buffer was added to bring up the volume to 100 μl and 1 μl of bacterial alkaline phosphatase (BAP) was added (about 100 units). The samples were incubated at 65°C for 2 to 3 hours. Brief centrifugations at 10,000 xg for 15 seconds were carried out every 20-30 minutes to ensure that precipitation of the dephosphorylated DNA was carried out and the pellet was dissolved in 15 μl distilled water (the sample was vortexed well, placed at 65°C for 10 minutes, frozen at -20°C. This heat/freeze process was repeated as often as necessary to achieve complete dissolving of the sample).

^{32}P-labelling of the 5'-ends of dephosphorylated fragments was done using γ-labeled ^{32}P-ATP and T4 polynucleotide kinase. The dephosphorylated DNA was placed in a microfuge tube with 2 μl of 10X kinase buffer (500 mM Tris-HCl at pH 7.6, 100 mM magnesium chloride (MgCl$_2$), 50 mM

35

Figure 3. Typical transformation and competition procedures. MM represents minimal media with appropriate carbon and amino acid supplements.

dithiothreitol, 1 mM spermidine and 1 mM EDTA), 1-3 µl ^{32}P-ATP (about 200 µCi), and 1 µl of T4 polynucleotide kinase (about 10 units). The contents were mixed and the tube was given a brief centrifugation at 10,000 xg for 10 seconds. It was then placed in a 37°C water bath for 45 minutes to 1 hour.

Assay For Uptake Of DNA Fragments

Cells were taken directly from log phase cultures of ADP6 and incubated with radioactive DNA fragments at 37°C. Samples were removed at 15 minutes intervals for 60 minutes. At this time, mixtures were chilled, treated with DNase I, and the cells washed with 0.5M NaCl to eliminate residual extracellular DNA. The radioactivity associated with the cells was determined using a liquid scintillation counter. The cells were then lysed with SDS and the intracellular DNA was deproteinized by Proteinase K and successive extractions with phenol and phenol:chloroform. The DNA was precipitated with ethanol and digested with appropriate restriction endonuclease(s). This is done to regenerate any fragments which may have been ligated after entering the cells and to reduce viscosity of bacterial chromosomal DNA. The resulting fragments were separated by gel electrophoresis and compared to input (donor) fragments.

CHAPTER III

RESULTS

Effect of Time of Incubation on Transformation Efficiency

Experiments with *Acinetobacter calcoaceticus* strain ADP6 (POB⁻) were carried out to determine the effect of time of incubation of cells with transforming DNA on the transformation frequency (Fig. 4). Cells were grown in 5 ml of minimal medium containing 10 mM succinate at 37°C for 12 hours. Then, 1 ml of the culture was diluted into 50 ml of the same medium in a 250 ml klett flask. The culture was then incubated at 37°C in a New Brunswick™ shaker at 250 rpm. At 100 KU, approximately 9×10^8 cells/ml, samples were removed and diluted 1:10. 100 μl of the resulting diluted cell suspension was added to 10 μl minimal salt medium containing 1 μg of pZR1 DNA (10 μg/ml of cells). After incubation at 37°C for the indicated periods, the samples were DNase I treated for 10 minutes to insure the elimination of all of the pZR1 that had not been taken up by the ADP6 cells. The samples were then diluted 1:10 and 100 μl of the resulting cell suspension was plated on minimal medium containing 5 mM POB. An additional 100 μl aliquot was diluted (1:100) and 100 μl was plated on minimal media

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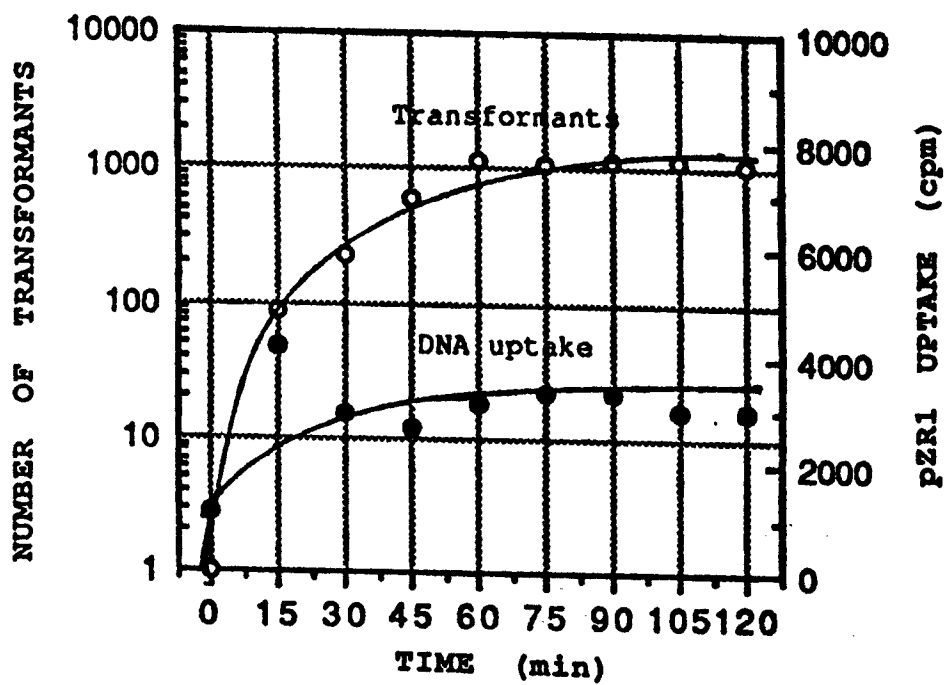
Figure 4. Effect of incubation time on DNA uptake and transformation efficiency of *A. calcoaceticus* ADP6 by pZR1 DNA. Open circles represent the number of transformants obtained after 9×10^6 cells were incubated with 10µg/ml of pZR1 at 37°C for the indicated times. Following incubation, the samples were treated with DNase I, diluted 1:10 and 100 µl of the diluted cell suspension was plated onto minimal POB plates. There were approximately 9×10^4 cells on each experimental plate. Solid circles represent α-^{32}P pZR1 uptake when 9×10^8 cells were incubated with 10 µg/ml (5 x 10^7 cpm) of pZR1. Following the indicated incubation times, the cells were DNase I treated and washed with 500 µl of LB and 500 µl of 0.5% NaCl. The pellets were then analyzed for the presence of radioactivity using a liquid scintillation counter.

containing 10 mM succinate as viable cell count control plates. Transformation frequency increased with the time of incubation to a maximum of 1.2% at 60 min.

Optimal DNA Concentration

Experiments were carried out to determine the minimal concentration of DNA necessary to yield maximum transformation efficiencies (Fig. 5). ADP6 was grown in minimal salt medium containing 10 mM succinate at 37°C to 100 KU. cells were diluted 1:10 and 100 μl of this resulting diluted cell suspension was added to 10 μl of minimal medium containing the indicated amounts of DNA for a period of 60 minutes. The samples were then DNase I treated for another 10 minutes at 37°C. The samples were then diluted once more and 100 μl of cells were plated onto minimal medium plates containing 5 mM POB. Transformation efficiency reached a maximum of 1.2% using 10 μg pZR1/ml (Fig. 5, upper curve). To test for the possible effects of the cell density on the transformation efficiency, a more concentrated recipient cell culture was also used (Fig. 5, lower curve). The recipient cells were obtained from the same culture as those used to obtain the results shown in the upper curve, but were not diluted prior to addition to the transformation medium. Transformation efficiency in the undiluted cell culture was about 0.1% with 1 μg/ml DNA and could be increased only
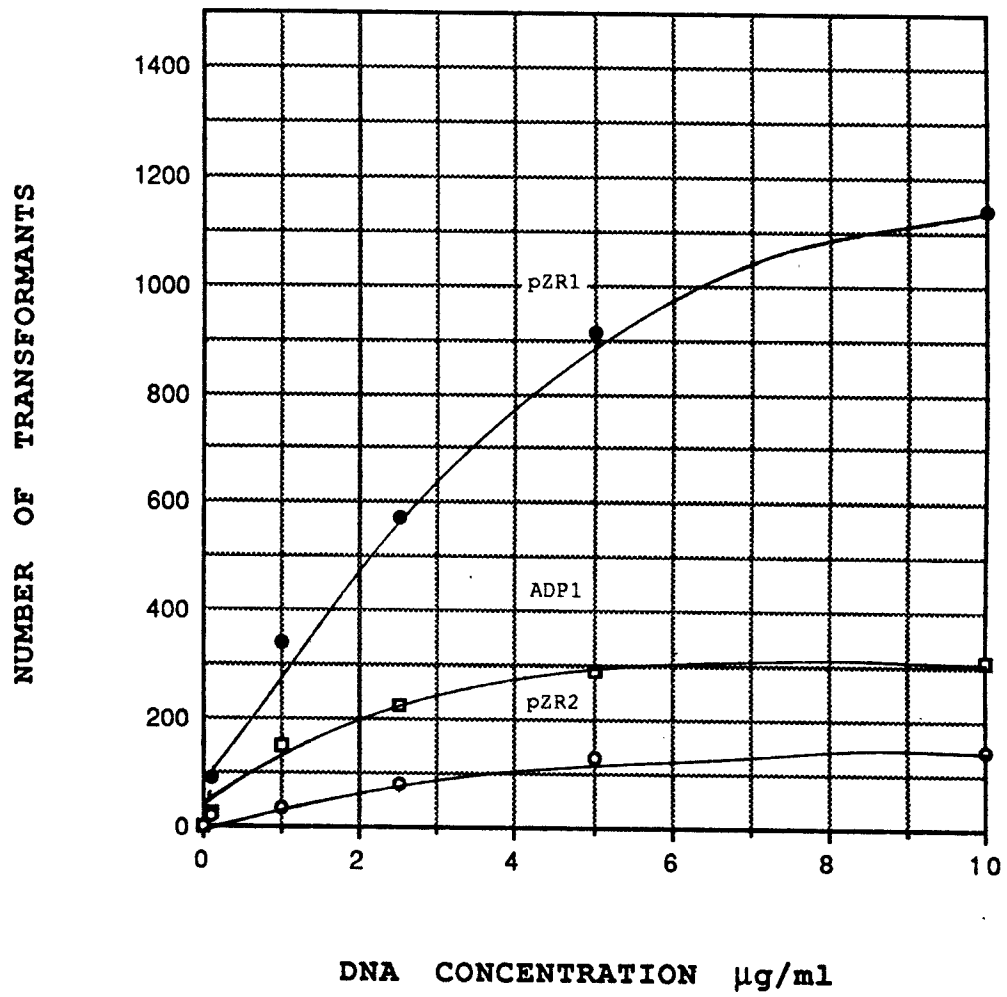
DNA CONCENTRATION (μg/ml)
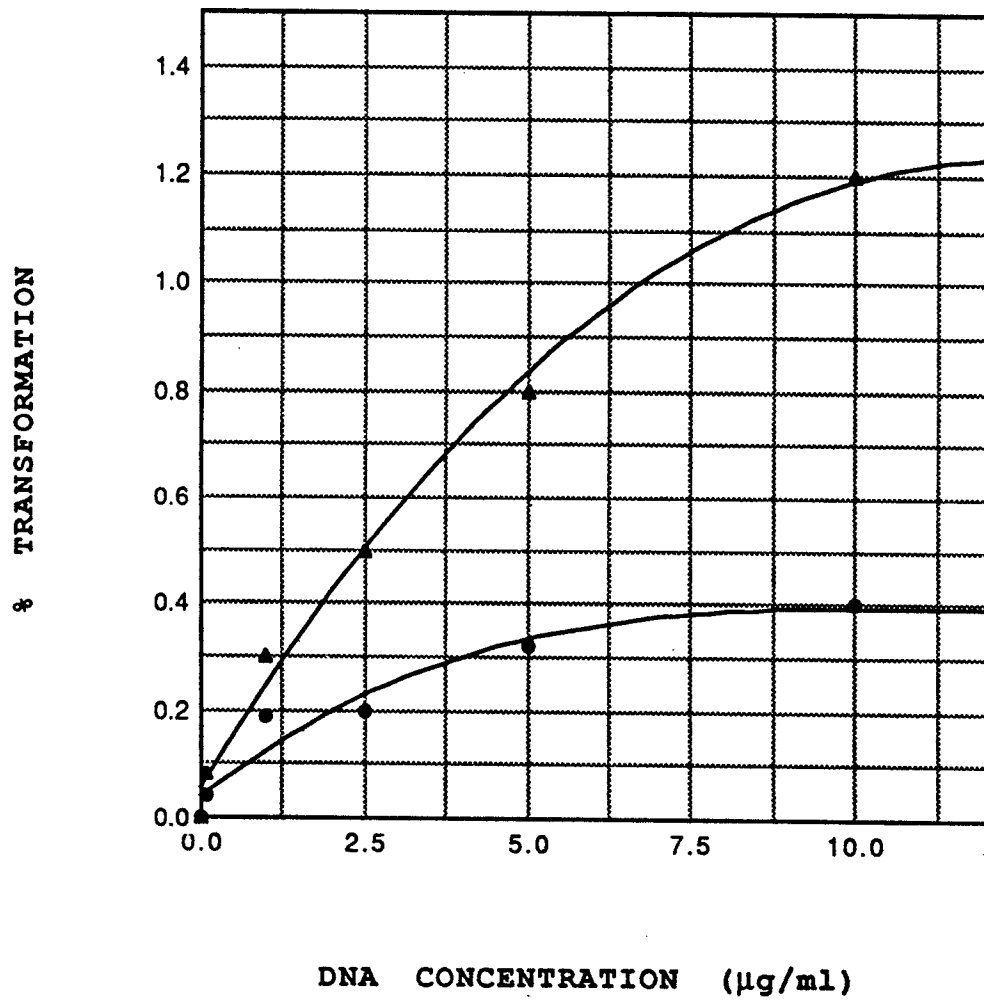
Figure 5. Transformation efficiency for *A. calcoaceticus* as a function of DNA concentration and initial cell concentration. Solid circles represent transformation efficiencies obtained using an initial cell concentration of 9.0×10^8 cells/ml. Triangles represent efficiencies obtained using 9.0×10^6 cells/ml.

4-fold by an additional 10-fold increase in the DNA concentration.

Effect of Size of Transforming DNA on Transformation Efficiency

The DNA dose response for transformation of ADP6 was also carried out using transforming DNA of various sizes. Three different transforming DNA's utilized in this experiment were pZR1 (14-kbp), pZR2 (5-kbp) and ADP1 chromosomal DNA with an average size of 20-30 kbp. The procedures used in this experiment were as indicated above. Fig. 6 illustrates the results of the experiment carried out using wild-type *A. calcoaceticus* (ADP1) chromosomal DNA as well as recombinant plasmids pZR2 and pZR1 containing *pcaA/C* (2.5 kbp insert in pUC18) and *pcaABCDFE* (11-kbp insert in pUC18) respectively in pUC18. As indicated in Fig. 6, the maximum transformation efficiency (1.2%) was obtained when pZR1 DNA was utilized as the transforming DNA. These results indicate a direct relationship between the size of the transforming DNA and the efficiency of transformation. Clearly, the use of larger fragments of transforming DNA significantly increase the efficiency of transformation. Since on average 1 out of 100 ADP1 chromosomal DNA fragments taken up by the cells has the potential of transforming ADP6 cells to prototrophy (about 1% would carry *pca* operon),

Figure 6. Transformation efficiency for *A. calcoaceticus* as a function of DNA concentration and size of the transforming DNA. Vertical axis represents the numbers of colony forming units (CFU) obtained in the presence of various transforming DNA's. The total number of cells plated in all cases was 9.0 X 10^5 cells.

it is reasonable that the transformation frequencies obtain
by ADP1 chromosomal DNA are lower than those obtained with
pZR1 despite the relative size differences between the two
transforming DNA.

Characterization of Cellular Competence

Results from experiments designed to measure competence
as a function of the growth phase of a culture are shown in
Fig. 7. Cells were grown in minimal media supplemented with
10 mM succinate. Samples were withdrawn and added to minimal
media containing pZR1 at a final concentration of 10 µg/ml.
Duplicate samples were withdrawn at several time points. *A.
calcoaceticus* ADP6 was found to be competent for
transformation over the entire growth cycle. The highest
level of competence was observed during late log phase and
decreased as the cells progressed into the stationary phase.
Transformation frequencies obtained during the peak of
competence were 3 to 5-fold higher than transformation
frequencies observed with recipient cells taken from late
stationary phase.

Recipient Cell Density

Possible effects of recipient cell density were examined
in relation to transformation efficiency (Table. 2). Samples
were drawn at the indicated times and diluted 1:10, 1:100;
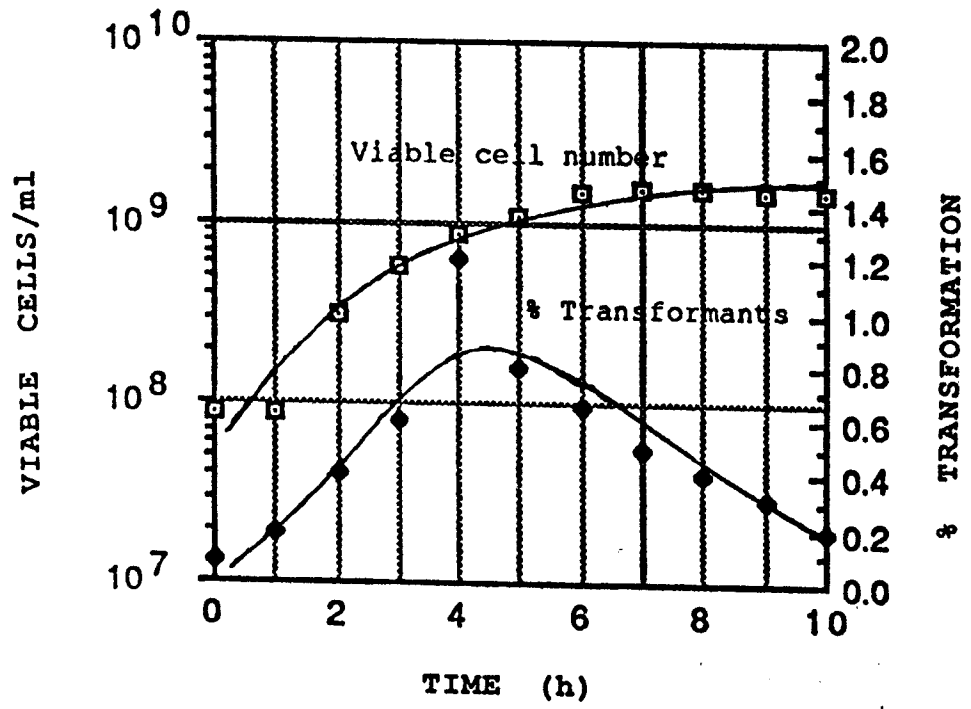
Figure 7. Competence of succinate-grown *A. calcoaceticus* cells as a function of growth phase. Samples were drawn at the times indicated for determination of viable cells and the number of transformants. The highest level of competence occurred during late log phase.

and 1:1000. 1 μg of transforming DNA (pZR1) was then added
to 100 μl of these diluted cell suspension. The highest
transformation frequencies were generally observed with a
cell concentration of 9 x 10^5 - 9 x 10^6 per ml in all growth
phases (Table 2). Similar results were obtained using the
semisolid transformation assay, where the highest
transformation frequencies were obtained with the lower cell
numbers per plate.

Transformation on Semisolid (Agar) Media

Transformation of ADP6 cells on semisolid medium was
carried out by allowing the cells to interact with the
transforming DNA on agar plates. ADP6 was grown to late log
phase (100 KU) in minimal media supplemented with succinate
and diluted to a final cell density of 2 X 10^3 cells/ml. 100
μl of the cells were then incubated with various amounts of
DNA for a period of 60 minutes at 37°C. These cultures were
then divided into two sets. For the first set, the contents
of the microfuge tubes were treated with DNase I and plated
directly onto the minimal POB plates. For the second set,
the DNase I treatment was omitted and both the cells and
transforming DNA were spread over the agar plates. The
results shown in Table 3 illustrate that very high levels of
transformation are obtainable with ADP6 cells if the cells
are plated on the selective media in the presence of

Table 2. Competence of succinate-grown *Acinetobacter calcoaceticus* cells as a function of recipient cell density. Dilutions of the cell culture are indicated. Initial recipient cell densities were: 9.0×10^8, 9.0×10^7, 9.0×10^6 cells/ml.

INITIAL CELL DENSITY/ml	CELL DENSITY IN TRANSFORMATION MIX	% TRANSFORMANTS
9×10^6	9×10^5	0.9
	9×10^4	0.9
	9×10^3	0.5
9×10^7	9×10^6	0.9
	9×10^5	0.9
	9×10^4	0.4
9×10^8	9×10^7	0.2
	9×10^6	1.2
	9×10^5	0.8

Table 3. Comparison of efficiencies of transformation obtained by liquid and solid transformation assay. Cells of a late log phase culture, diluted to a final concentration of 2×10^3 cells/ml, were incubated with various amounts of pZR1 for 60 min. The cells were then divided into two equal 100 μl sets. Set #2 was directly plated onto POB plates. Set #1 was treated with DNase I prior to plating. A denser (2×10^5) set of samples were also transformed to observe the effect of cell density on the effeicincy of solid transformation assay.

FINAL DNA CONC. (μg/ml)	# of TRANSFORMANTS	% TRANSFORMANTS
SET #1 W/ 2×10^2 cells		
50.00	3	1.5
10.00	3	1.5
5.00	2	1.0
1.00	1	0.5
0.10	ø	---
0.01	ø	---
SET #2 W/ 2×10^2 cells		
50.00	196	98
10.00	193	97
5.00	164	82
1.00	139	70
0.10	106	53
0.01	52	26
SET #2 W/ 2×10^5 cells		
50.00	1968	10
10.00	1854	9
5.00	1380	7
1.00	624	3
0.10	260	1
0.01	87	>1

transforming DNA. Transformation efficiencies of nearly 100%
were obtainable when cells were transformed using this
semisolid transformation assay. However, as was found with
the liquid transformation procedures, solid transformation
assays yielded lower transformation efficiencies (about 10%)
when 10^5 cells were spread per plate (Table 3). The high
number transformants obtained may be due to multiple
transformation events on a single recipient cell.

Effect of Incubation Temperature on the Efficiency of Transformation

The effect of incubation temperature on transformation
efficiency was examined using strain ADP6. Cells were grown
at 37°C in LB medium to 100 KU, and dilutions were made to
obtain a final cell concentration of 2 x 10^6 per ml.
Subsequently, 100 µl aliquots were withdrawn and added to 1.5
ml microfuge tubes containing either 1 µg (set A) or 0.1 µg
(set B) of pZR1 DNA. The transformation mixtures were
incubated at 15, 20, 25, 30 or 37°C for 60 minutes. The
samples were then treated with DNase I at a final
concentration of 10 µg/ml, each sample was diluted 1:10 and
100 µl samples of the resulting cell suspensions were plated
onto POB plates. As illustrated in Figure 8, the number of
colony forming units was significantly higher when the
samples were incubated at 37°C with the transforming DNA.
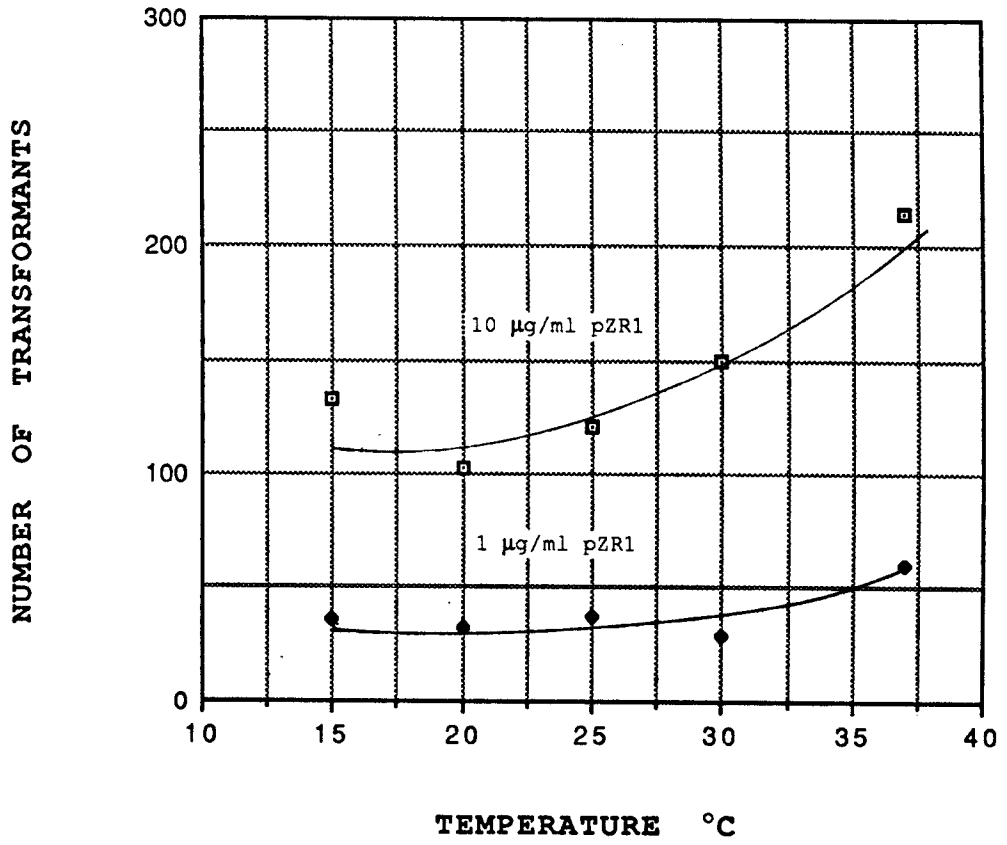
Figure 8. Effect of incubation temperature on the
efficiency of transformation. For each temperature, two
cultures were incubated with either 10 μg/ml (open circles)
or 1 μg/ml pZR1 (solid circles). Following incubation at the
noted temperatures, samples were treated with DNase I,
diluted and plated on selective plates.

Effect of Starvation of Cells Prior to Transformation

In order to find out whether starvation was instrumental in inducing competence in *A. calcoaceticus,* cultures were grown to 100 KU and diluted in minimal salt medium containing no carbon source to a final concentration of 2×10^3 cells/ml. Cultures were then divided into several 200 µl aliquots. One set of samples was allowed to incubate with pZR1 (10 µg/ml) at 37°C for various time intervals up to 70 hours (Fig. 9). The second set of samples, were starved for indicated times before pZR1 DNA was added to a final concentration of 10 µg/ml. Cells were then incubated for an additional 60 minutes and subjected to DNase I treatment (Fig. 10). In each case 100 µl samples were plated to determine transformation frequency and second sample was used to determine the viable cell number by serial dilution plating. Since the number of viable cells did not remain constant during this experiment, it was necessary to express the number of transformants as a fraction of total number of cells in each case (% transformation). As illustrated in Figure 9, the fraction of cells that were transformed to prototrophy increased up to a maximum of 20% when the cells were incubated in minimal salts with the transforming DNA for 25 hours. A qualitatively similar result was obtained when the cells were starved for various times prior to DNA addition (Fig. 10). The peak of transformation in each case
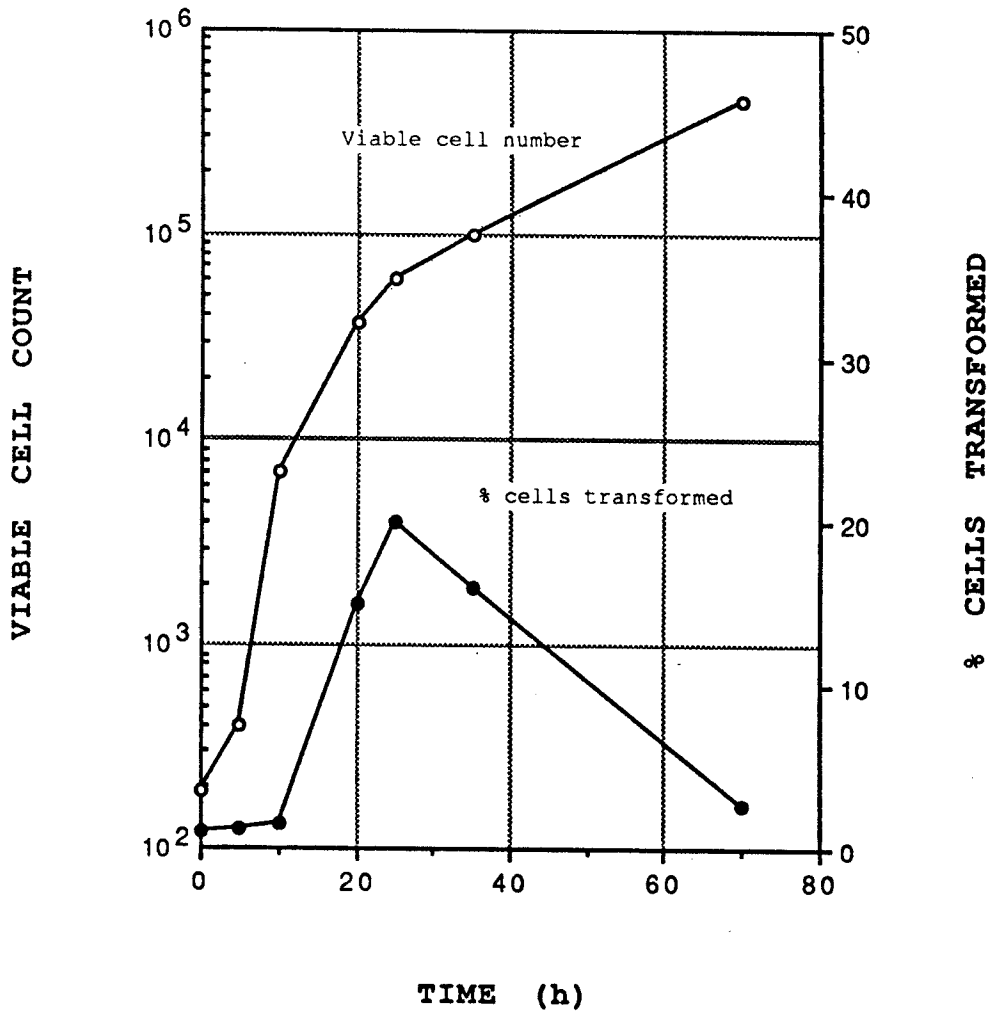
Figure 9. Effect of long term incubation of the ADP6 cells

with pZR1 DNA in the presence of no carbon source. ADP6

cells were diluted in minimal salt medium containing no

carbon source to a final concentration of 2×10^3 cells/ml.

The cells were then divided into several 200 μl aliquots.

Samples were allowed to incubate with pZR1 (10 μg/ml) at 37°C

for various time intervals up to 70 hours. After DNase I

treatment, the number of transformants and viable cell

numbers were determined by dilution plating onto selective

and non-selective plates, respectively.
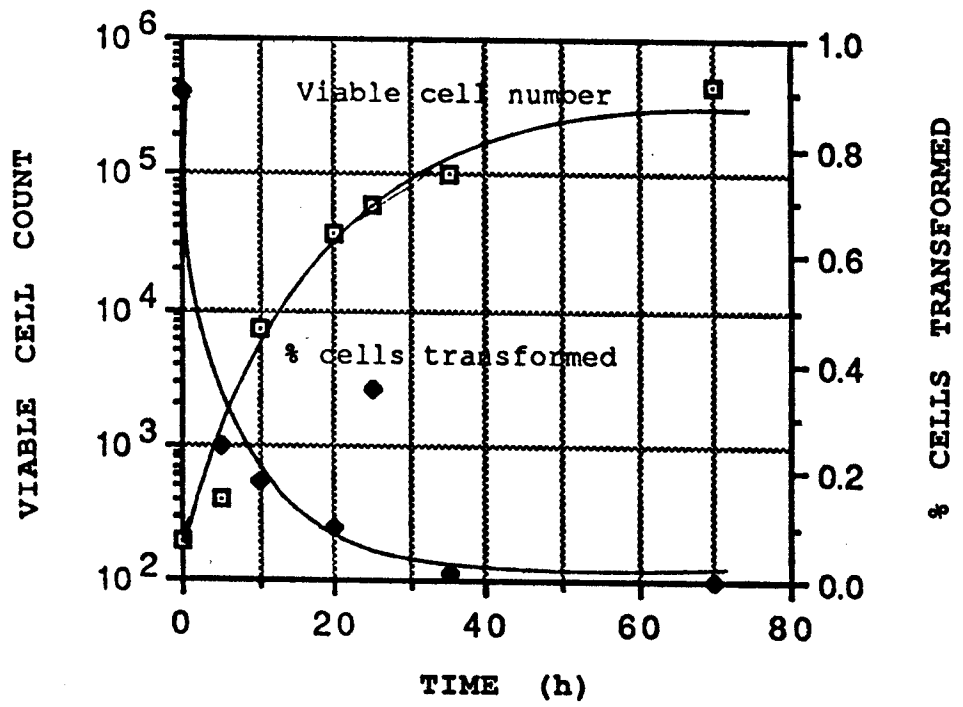
Figure 10. Effect of cell starvation on transformation efficiency. 200 µl of ADP6 cells were starved for indicated times before pZR1 was added to a final concentration of 10 µg/ml. Cells were then incubated for an additional 60 minutes followed by DNase I treatment (Fig. 10). In each case 100 µl of the samples were used to determine the viable cell numbers by dilution plating onto minimal salt medium containing 10 mM succinate.

occurred at about 25 hours. However, in this case the actual
transformation frequency was much lower. In addition the
highest level was obtained at zero time of starvation (Fig.
10). Thus although starvation may indeed have an effect on
the physiology of the natural transformation, the primary
factor here appears to be extended time of incubation with
transforming DNA.

**Effect of Incubation Medium on Transformation
Efficiency**

The impact of the incubation medium on the transform-
ation efficiency of ADP6 cells is depicted in Figure 11.
ADP6 cells were grown in 50 ml of LB medium at 37°C to 100
KU. Cells were then diluted to 2 x 10^7 cells/ml in either
LB, minimal salt medium, minimal salt medium containing 5 mM
POB or minimal salt medium containing 10 mM succinate. 100
μl aliquots were incubated with various amounts of pZR1 (0,
5, 10, 25 μg/ml) for 60 minutes at 37°C. After DNase I
treatment, the cells were diluted 1:10 and 100 μl of the
resulting cell suspension was plated on selective plates. As
shown in Figure 11, LB medium provided a much more suitable
environment for uptake of DNA by the cells. Experiments
where cells were initially grown with minimum salt medium
with 10 mM succinate gave virtually identical results,
indicating that the important factor here is the medium

DNA CONCENTRATION (µg/ml)
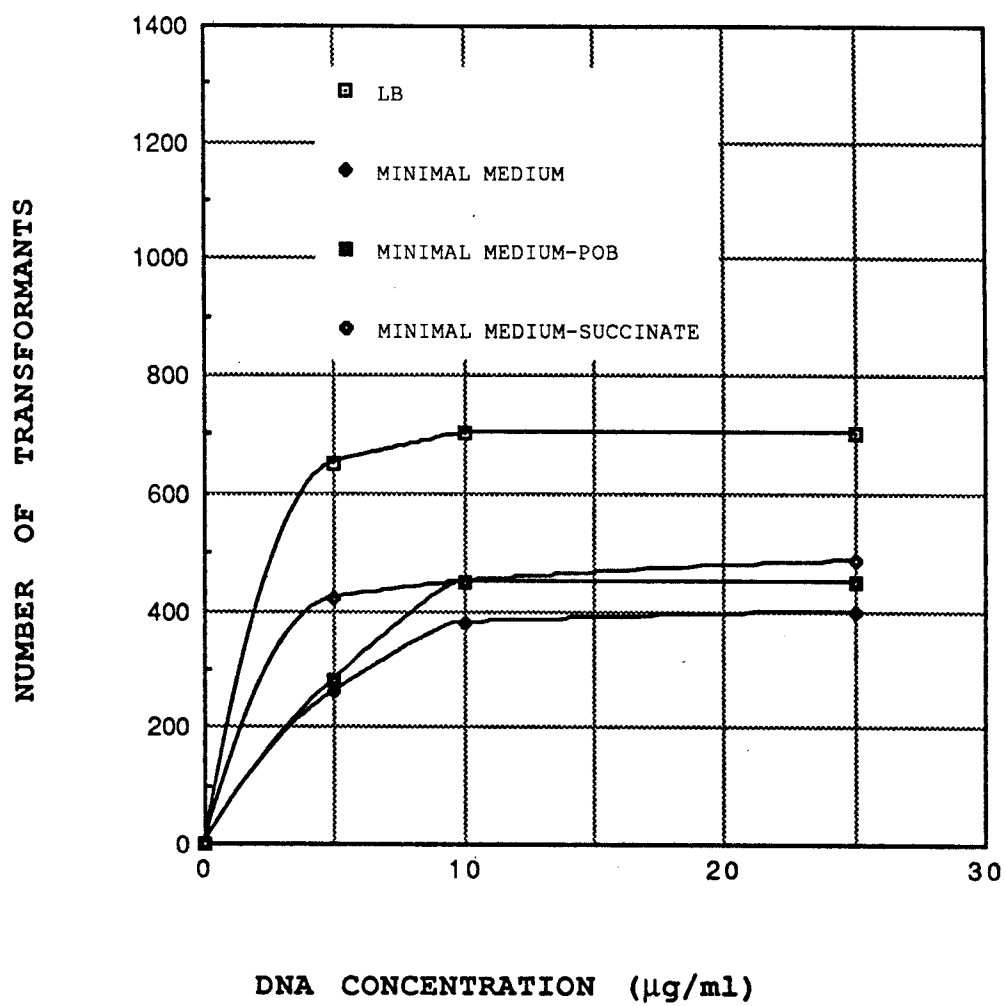
Figure 11. Effect of incubation medium on the ADP6

transformation efficiency. 2 x 10^6 cells were incubated with

various amounts of pZR1 (0, 5, 10, 25 µg/ml) in either LB,

minimal salts (no carbon source), minimal medium containing

POB or minimal medium containing succinate for 60 minutes at

37°C. After DNase I treatment, the cells were diluted 1:10

and 100 µl of the resulting cell suspensions were plated on

selective media.

present during transformation, not the medium on whichthe
cells were initially grown.

Comparison of Transformation of ADP6 Cells With
Wild-type and Ac138 (arg⁻) Chromosomal DNA

Results from experiments performed to compare the
abilities of wild-type (ADP1) and *arg⁻* (Ac138) chromosomal
DNA to transform ADP6 cells are shown in Figure 12. ADP6
cells were grown on minimal salt media supplemented with 10
mM succinate. Samples were withdrawn, diluted and added to
minimal media containing ADP1 or Ac138 DNA at a final
concentration of either 1 or 10 µg/ml. After 60 minutes of
incubation at 37°C, samples were DNase I treated, diluted and
plated onto selective media (minimal-POB plates). There are
several possible outcomes from presenting *pca⁺ arg⁻* DNA
(Ac138 DNA) to the cells that do not contain a functional *pca*
operon: 1) transformation of only one locus, in which case
only cells transformed by fragments containing *pcaA* gene
would appear on the minimal-POB plates, 2) cotransformation
of both *pca* and *arg* operon by the incoming DNA, which results
in formation of *pca⁺ agr⁻* transformants that are unable to
grow on the minimal-POB plates. As indicated in Figure 12,
the number of transformants obtained with ADP1 DNA were much
higher than those obtained with Ac138 DNA. Since the only
difference in the two DNA preparations used was that Ac138

CONCENTRATION OF DNA (μg/ml)
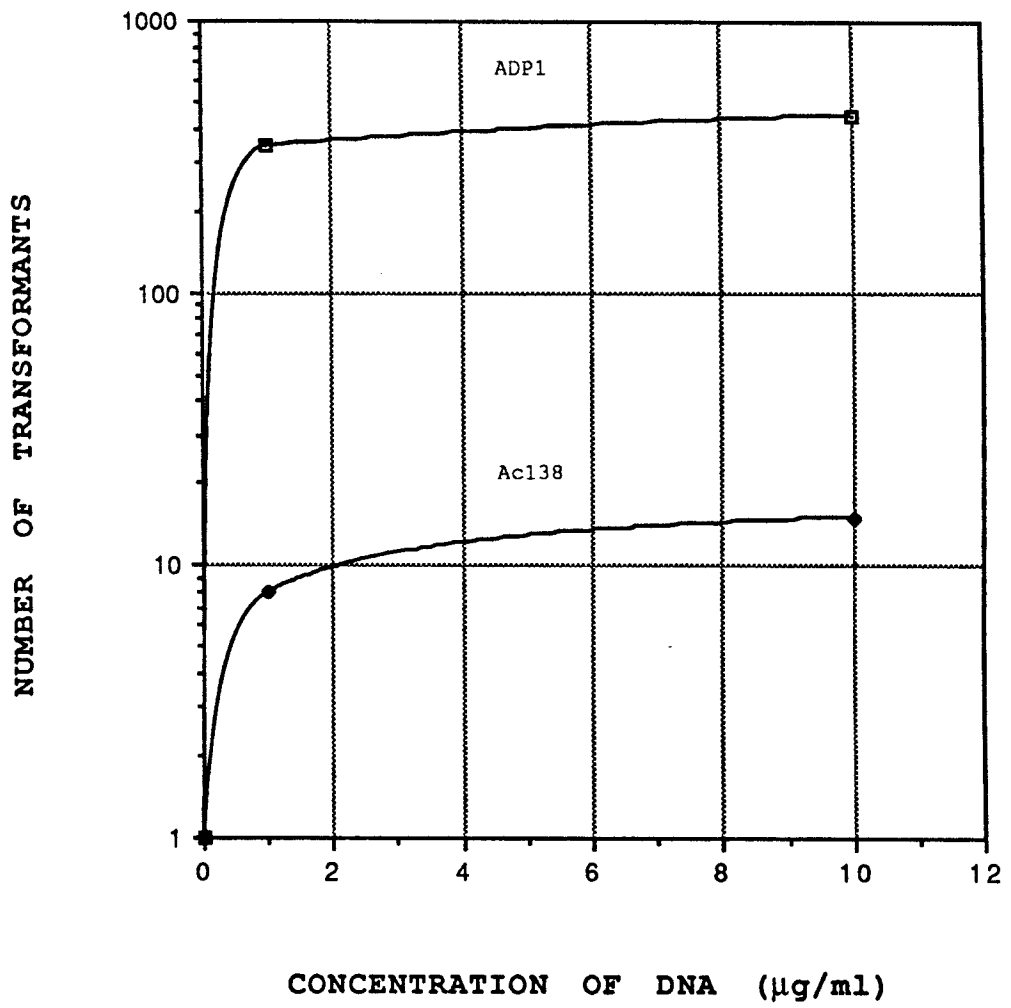
Figure 12. Comparison of the efficiency of transformation of ADP6 cells with wild-type and Ac138 (*arg⁻*) chromosomal DNA. ADP6 cells were grown in minimal salt media supplemented with 10 mM succinate. Samples were withdrawn, diluted and added to minimal media containing ADP1 or Ac138 DNA at a final concentration of either 0, 1 or 10 μg/ml. After 60 minutes of incubation at 37°C, samples were DNase I treated, diluted and plated onto selective media.

chromosomal DNA is *arg⁻*, it can be suggested that either the

pca operon is very closely linked to the *arg* operon or that

the levels of DNA uptake and subsequent recombination in *A.*

calcoaceticus are so high that both loci are almost always

transformed (Discussed in more detail below).

Effect of Restriction Digestion of the Transforming
DNA on the Efficiency of Transformation

The DNA dose response for transformation of ADP6 was

also carried out using transforming DNA that was cut with

restriction enzyme prior to transformation. Both pZR1 and

ADP1 DNA were cut with *Eco*RI. *Eco*RI was chosen because this

enzyme does not cut within the *pca* operon (Doten, et al.

1987). At 100 KU, ADP6 cells grown in LB medium were diluted

1:10 and 100 μl was transferred to tubes containing various

amounts of cut and uncut ADP1 or pZR1 DNA preparations.

After incubation at 37°C for 60 minutes, the transformation

mixtures were diluted to 2.5 x 10^5 cells/ml and 100 μl was

plated onto POB plates. The results of this experiment are

depicted in Figures 13 and 14. As illustrated, in each case

the number of transformants decreased when cut DNA was used

as the transforming DNA (as compared to uncut DNA). The

number of transformants obtained when an *Eco*RI digest of pZR1

was used as the transforming DNA was almost 10-fold higher

than that obtained with *Eco*RI digest of ADP1. There are

DNA CONCENTRATION (µg/ml)
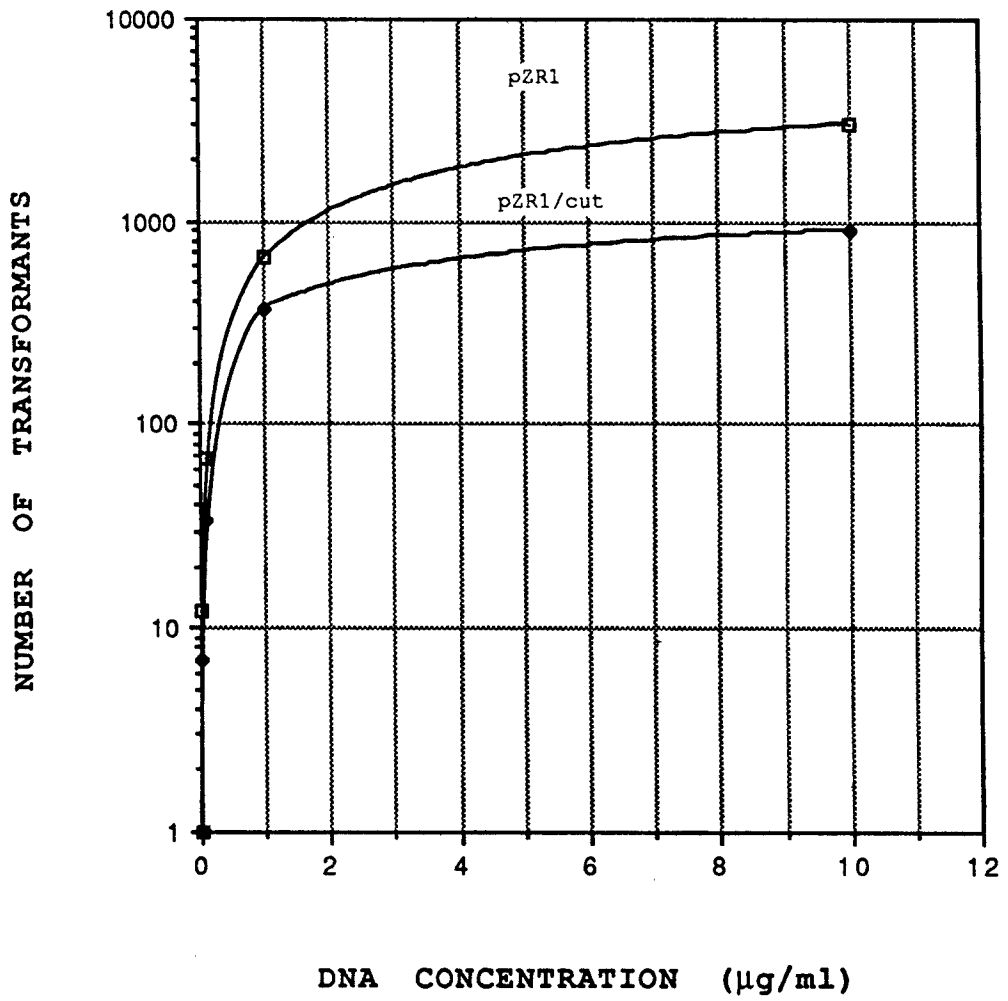
Figure 13. DNA dose response curves for the transformation of ADP6 by pZR1 cut with *Eco*RI and uncut pZR1. At 100 KU, ADP6 cells grown in LB medium were diluted 1:10 and transferred to tubes containing various amounts of cut and uncut pZR1 DNA. After incubation at 37°C for 60 minutes, the transformation mixtures were diluted and plated onto POB plates. The total number of cells on each plate was 2.5 x 10^4.
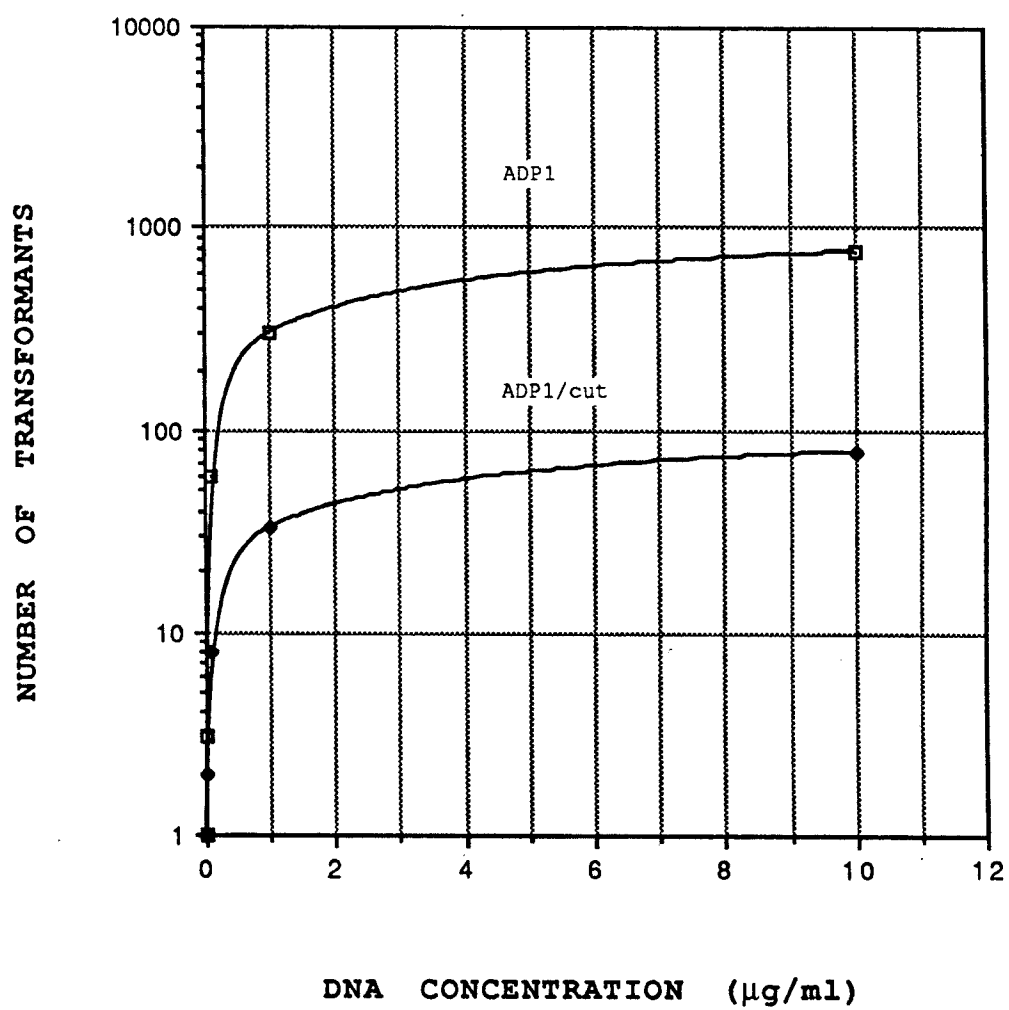
Figure 14. DNA dose response data for the transformation of ADP6 by ADP1 DNA cut with *Eco*RI and uncut ADP1 DNA. ADP6 cells were grown in LB medium to 100 KU, diluted 1:10 and transferred to tubes containing various amounts of cut and uncut ADP1 chromosomal DNA. After incubation at 37°C for 60 minutes, the transformation mixtures were diluted and spread onto POB plates. The total number of cells on each plate was 2.5×10^4.

several possible explanations for these results. Cutting the DNA produces additional fragments which do not carry the wild-type marker and which can thus act as competitive inhibitors of DNA uptake. Alternatively, cutting the DNA could separate the marker from some DNA sequence important for DNA uptake or recombination. This possibility seems less probable because it would be unlikely to find such sequence for *A. calcoaceticus* on pUC18 plasmid. Also, larger DNA could for an unknown reason simply transform better.

Transformation of ADP6 Cells by Heat-Denatured DNA

The possibility of transformation of *A. calcoaceticus* (ADP6) cells by single-stranded DNA was also examined (Table 4). ADP6 was grown to 100 KU in LB at 37°C. 100 μl of the culture was then diluted to a final cell density of 9 x 10^7 cells/ml. 100 μl cells were then transferred to minimal medium containing either heat-denatured or native DNA preparations of ADP1, pZR1, and pZR2 at final concentrations of 0, 1, or 10 μg/ml for a period of 60 minutes. After DNase I treatment, the cells were diluted 1:100 and 100 μl of the resulting cell suspensions were plated onto minimal salt medium containing 5 mM POB. As indicated in Table 4, no transformation was observed when single-stranded preparations of ADP1 and pZR2 DNA were utilized as the transforming DNA. A very small number of transformants (0.014%) were detected

when heat-denatured pZR1 DNA was used at a final
concentration of 10 μg/ml. However, renaturation of pZR1
molecules due to experimental error could easily explain the
the presence of a few colonies in this case.

Transformation of Other Strains of A. *calcoaceticus*
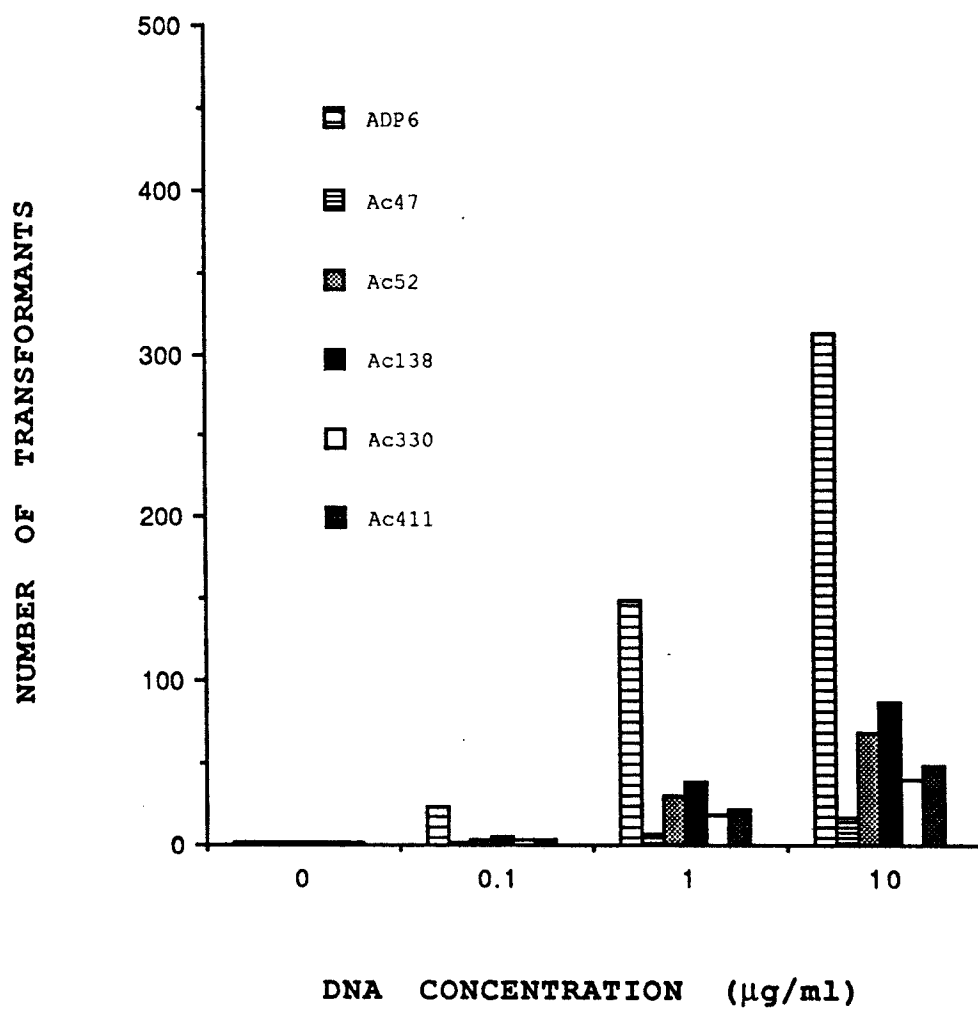**With ADP1 Chromosomal DNA**

Experiments were carried out to determine the minimal
concentration of DNA necessary to yield maximum transfor-
mation of strains Ac47 (*trpA*), Ac52 (*ilv*), Ac138 (*arg*), Ac330
(*cys*), Ac411 (*trpE27*). Each strain was grown to 100 KU in LB
at 37°C. Cells were then transformed in minimal medium
containing ADP1 chromosomal DNA at final concentrations of 0,
0.1, 1, or 10 μg/ml for a period of 60 minutes. The cells
were then diluted and plated onto minimal salt medium
containing 5 mM POB. ADP6 cells were also transformed at the
same time and the results were compared to those obtained
with the other *A. calcoaceticus* strains (Fig. 15). As shown
in Figure 15, the number of transformants was much higher for
ADP6 than the amino acid auxotrophs.

Effect of Presence of Competing DNA on the Frequency
of Transformation in ADP6

Results from experiments designed to determine the
effect of competing homologous ADP6 DNA on the transformation

Table 4. Comparison of transformation of ADP6 cells by native and single-stranded DNA. The number of cells plated in each case was 9×10^4.

Transforming DNA	DNA Conc. (μg/ml)	# of Transformants
Native DNA		
pZR1	0	< 1
	1	339
	10	1139
pZR2	0	< 1
	1	56
	10	168
ADP1	0	< 1
	1	153
	10	257
Single-stranded DNA		
pZR1	0	< 1
	1	13
	10	< 1
pZR2	0	< 1
	1	< 1
	10	< 1
ADP1	0	< 1
	1	< 1
	10	< 1

Figure 15. DNA dose response in *A. calcoaceticus* strains

ADP6, Ac47 (*trpA*), Ac52 (*ilv*), Ac138 (*arg*), Ac330 (*cys*),

Ac411 (*trpE27*). Strains were grown to 100 KU in LB at 37°C.

Cells were then transformed in minimal medium containing ADP1

chromosomal DNA at final concentrations indicated for 60

minutes. The cells were then diluted and plated onto minimal

salt medium containing 5 mM POB.

efficiencies of ADP6 cells are illustrated in Figures 16 and 17. ADP6 cells were grown at 37°C to 100 KU. Cells were then diluted to a final concentration of 2 x 10^7 cells/ml. Transformations were carried out using wild-type chromosomal DNA (ADP1) as well as pZR1 plasmid, which contains the entire *pca* operon, at final concentrations of 10 µg/ml. Competing ADP6 DNA was varied as indicated. 100 µl of the diluted cell suspension were used in the transformation mixtures. The total number of cells per plate was 2.0 x 10^5. As shown in each figure, the presence of homologous competing DNA resulted in an approximately 5-fold decrease in the number of transformants when the ratio of competing to transforming DNA was 10 to one. A decrease in the number of transformants in this case could be attributed to (1) ADP6 DNA inhibits the binding and uptake of transforming DNA (competitive inhibition of the transformation), or (2) there is a possibility of two successive recombination events by both transforming and competing DNA (ADP6 DNA preparation contains an identical sequence to the marker that was selected for transformation. Hence, ADP6 DNA is a poor choice as a competing DNA in transformation studies of the *pcaA* operon) (see Fig. 12). Further experiments were therefore done using the *arg⁻* strain Ac138 and cloned segments of *A. calcoaceticus* and non-*Acinetobater* DNA which do not encode enzymes for arginine biosynthesis.

NUMBER OF TRANSFORMANTS

pZR1 transformants

ADP1 transformants

COMPETING DNA (ADP6) μg/ml
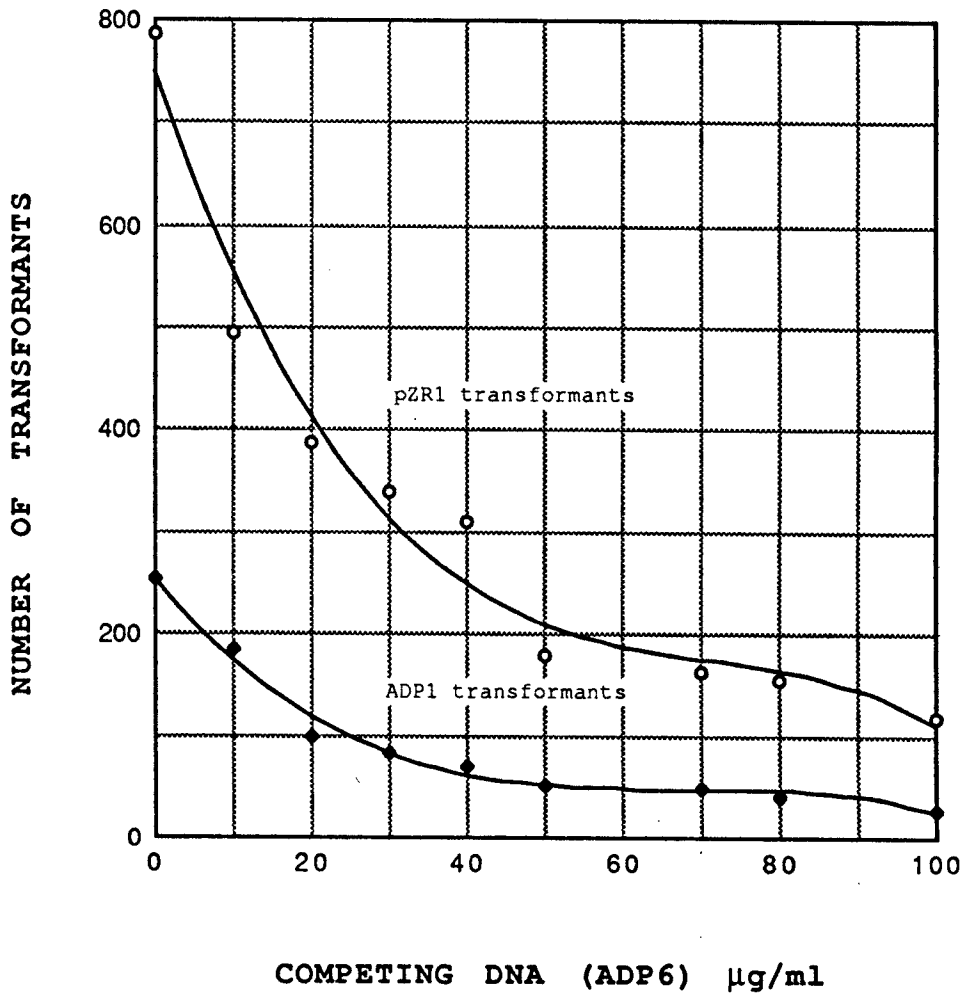
Figure 16. Transformation of *A. calcoaceticus* ADP6 (POB⁻)

to POB⁺ phenotype in the presence of competing homologous

ADP6 DNA. Transformations were carried out using wild-type

chromosomal DNA (ADP1) or pZR1 plasmid (which contains the

entire *pca* operon) at final concentrations of 10μg/ml.

Competing DNA was varied as indicated. The total number of

cells per plate was 2.0 x 10^5.

RATIO OF CFU WITHOUT COMPETING DNA
TO CFU WITH COMPETING DNA

RATIO OF COMPETING DNA TO
TRANSFORMING DNA
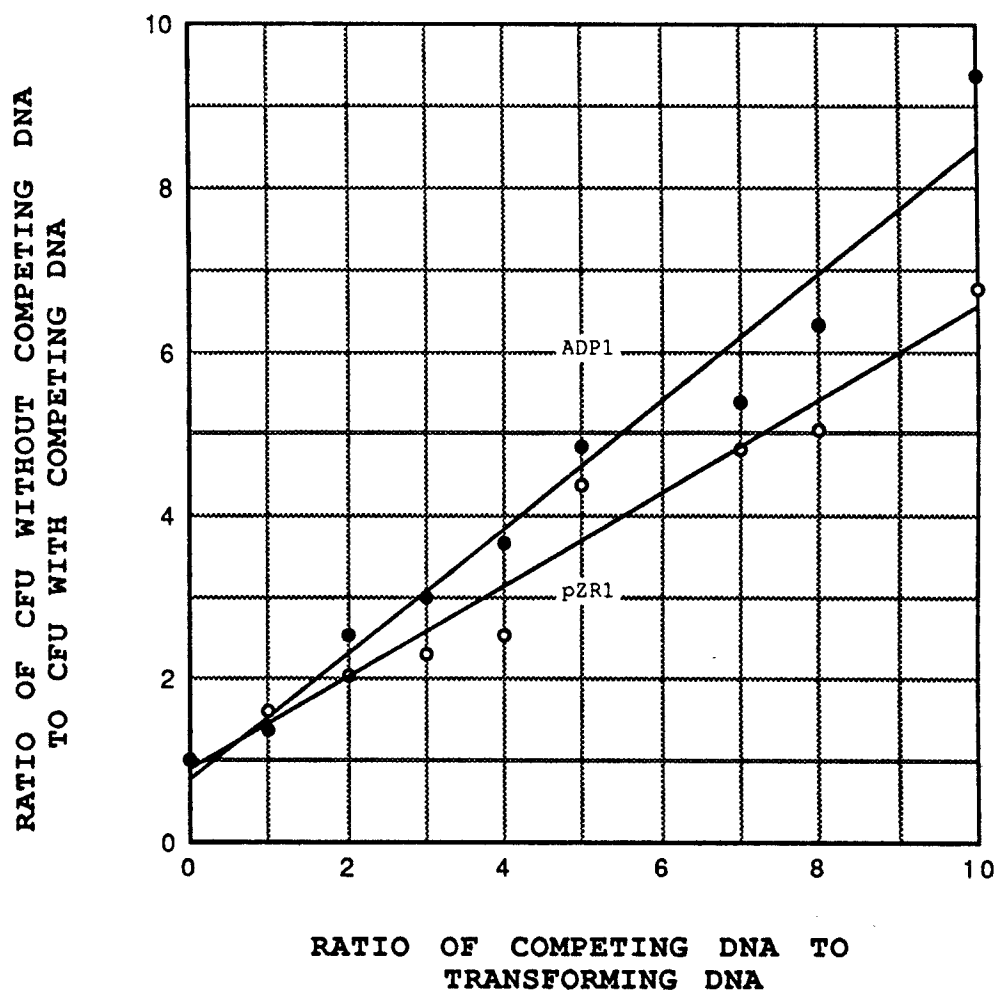
Figure 17. Competitive inhibition of *A. calcoaceticus* ADP6 (POB⁻) transformation to POB⁺ phenotype in the presence of competing homologous ADP6 DNA. This graph was plotted according to Sisco and Smith (Proc. Natl. Acad. Sci. USA 76, 972-976; 1979). Abscissa, the ratio of the concentration of competing DNA to transforming DNA; ordinate, the ratio of transformants without competing DNA to transformants in the presence of competing DNA.

Effect of Presence of Competing DNA on the Frequency of Transformation in Ac138

Results from the experiments designed to measure the effect of competing DNA in transformation of Ac138 (arg⁻) are shown in Figures 18 and 19. Transformations were carried out using wild-type chromosomal DNA (ADP1) as the transforming DNA at a final concentration of 10 µg/ml. pZR1 and pMO8 were selected as competing DNA's because the size of the cloned segments in both plasmids is about the same and while pZR1 carries the *A. calcoaceticus pcaA* operon, pMO8 carries the TOL lower pathway for *P. pudita*. Thus, pZR1 and pMO8 can serve as homologous (*A. calcoaceticus*) and heterologous (non-*Acinetobater*) competing DNAs, respectively. The concentration of competing DNA was varied as indicated. The total number of cells per plate was 2.0×10^5. As shown in Figures 18 and 19, the impact of competing DNA on the number of transformants obtained in both cases was virtually the same. This indicates once more that binding of DNA at the surface of the transforming cells occurs nonspecifically with respect to nucleotide sequence.

Effect of Restriction Digested Competing DNA on the Transformation Frequency of ADP6

Results from the experiments designed to reveal the effect of competing DNA size on the transformation efficiency
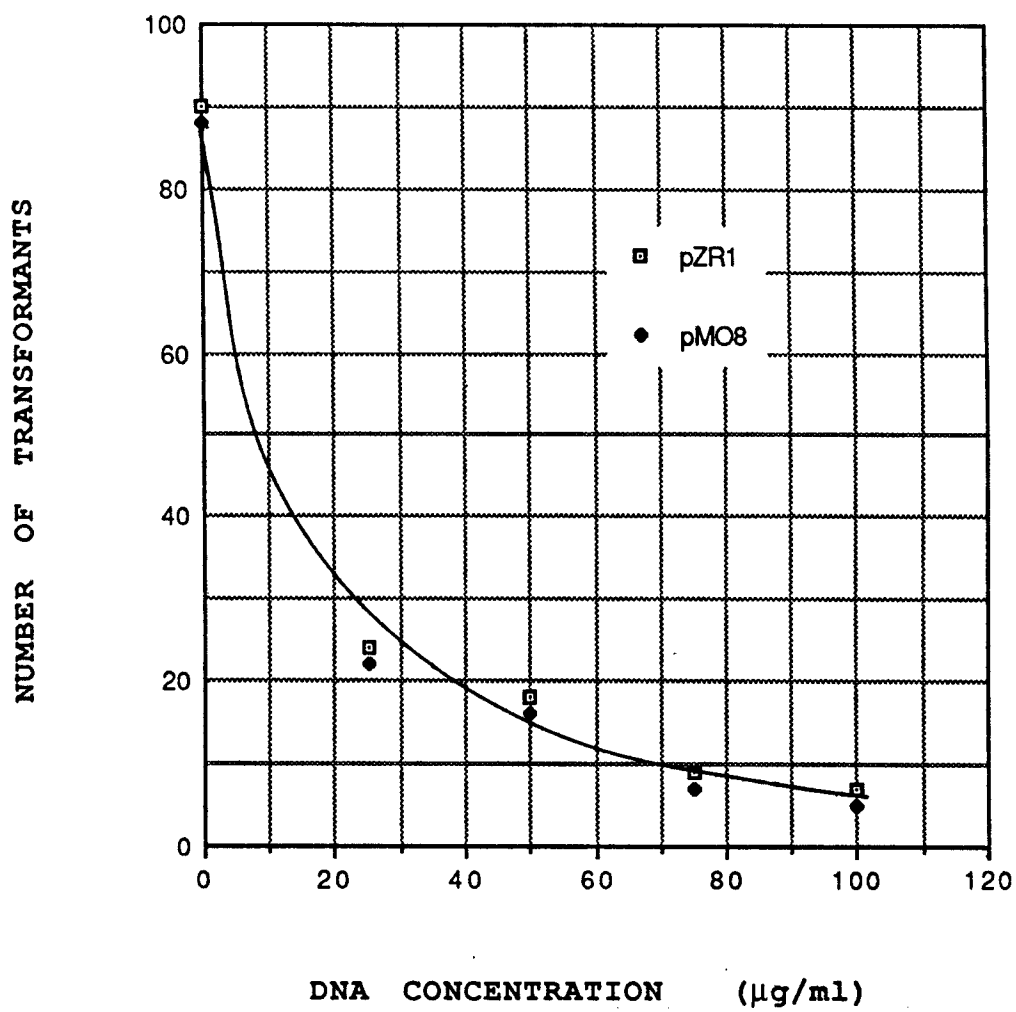
Figure 18. Transformation of *A. calcoaceticus* Ac138 (Arg⁻)

to Arg⁺ phenotype in the presence of competing pZR1

(homologous) and non-homologous pMO8 DNA. Transformations

were carried out using wild-type chromosomal DNA (ADP1) at

final concentration of 10 μg/ml. Competing DNA was varied as

indicated. The total number of cells per plate was 2.0 x

10^5.
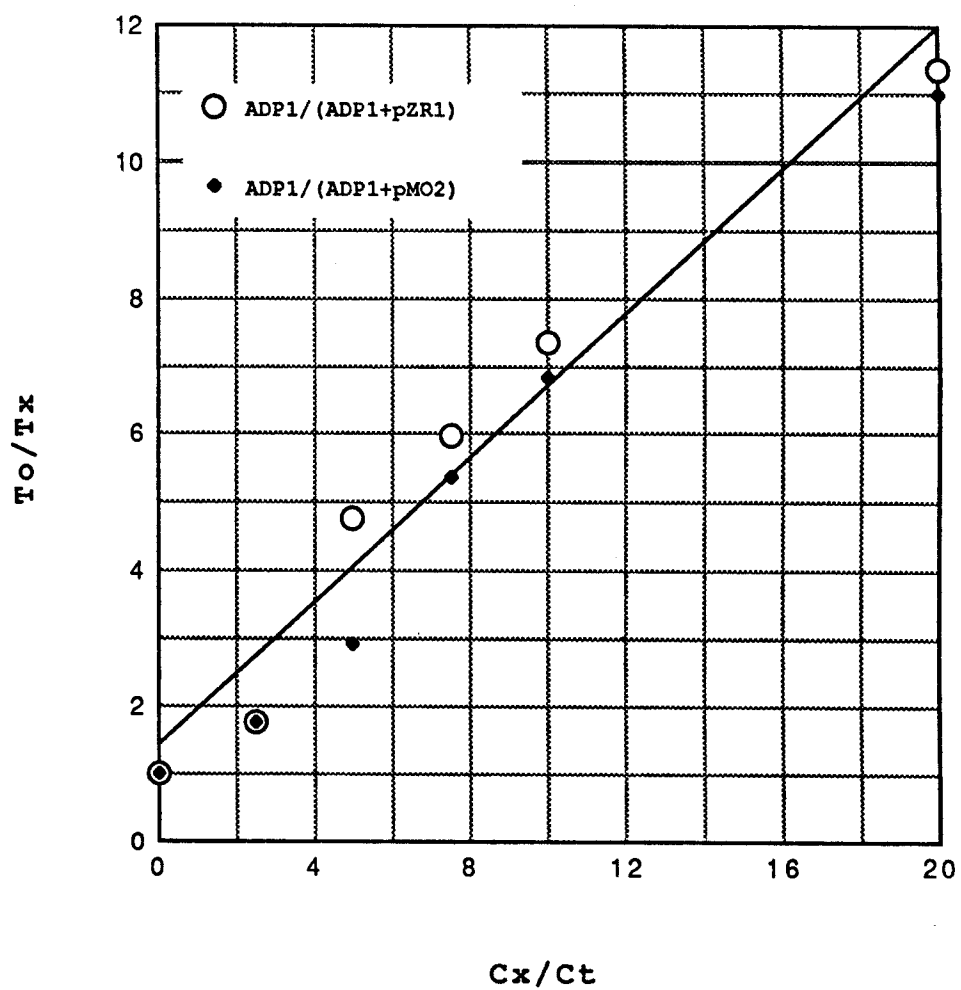
Figure 19. Competitive inhibition of *A. calcoaceticus* Ac138

(Arg⁻) transformation to Arg⁺ by homologous pZR1 and

non-homologous pMO8 DNA. This graph was plotted according to

Sisco and Smith (Proc. Natl. Acad. Sci. USA 76, 972-976;

1979). Abscissa, the ratio of the concentration of competing

DNA to transforming DNA; ordinate, the ratio of transformants

without competing DNA to transformants in the presence of

competing DNA.

of ADP6 are shown in Fig. 20. The DNA preparations from various bacteria, including *A.calcoaceticus* (ADP6), *P.pudita* (Paw 630), and *E.coli* (CSH12), were digested with *Hind*III. Undigested DNA preparations from each organism were also tested for their capability to effect the frequency of transformation. The chromosomal preparations from *E.coli* and *P.pudita* also served as an indicator for the possible effects of non-homologous competing DNA on ADP6 transformation. Procedures in this study were identical to all other competition studies described previously. As shown in Fig. 18, the number of transformants obtained using restricted chromosomal DNA preparations as competing DNA were always lower than those obtained with uncut competing DNA preparations. Thus, although reducing the size of donor DNA leads to a decrease in the efficiency of transformation (Fig. 14), reducing the average size of the competing DNA increases its effectiveness as a competitor in binding to receptores at the surface of the recipient cells (Fig. 20). A further interesting result illustrated in Fig. 20 is that regardless of the origin of the competing DNA, the number of transformants in all cases were very similar. Therefore, it can be concluded that homologous (*Acinetobacter*) and non-homologous (non-*Acinetobacter*) DNA compete for binding on the surface of transforming cells with a very similar effectiveness.
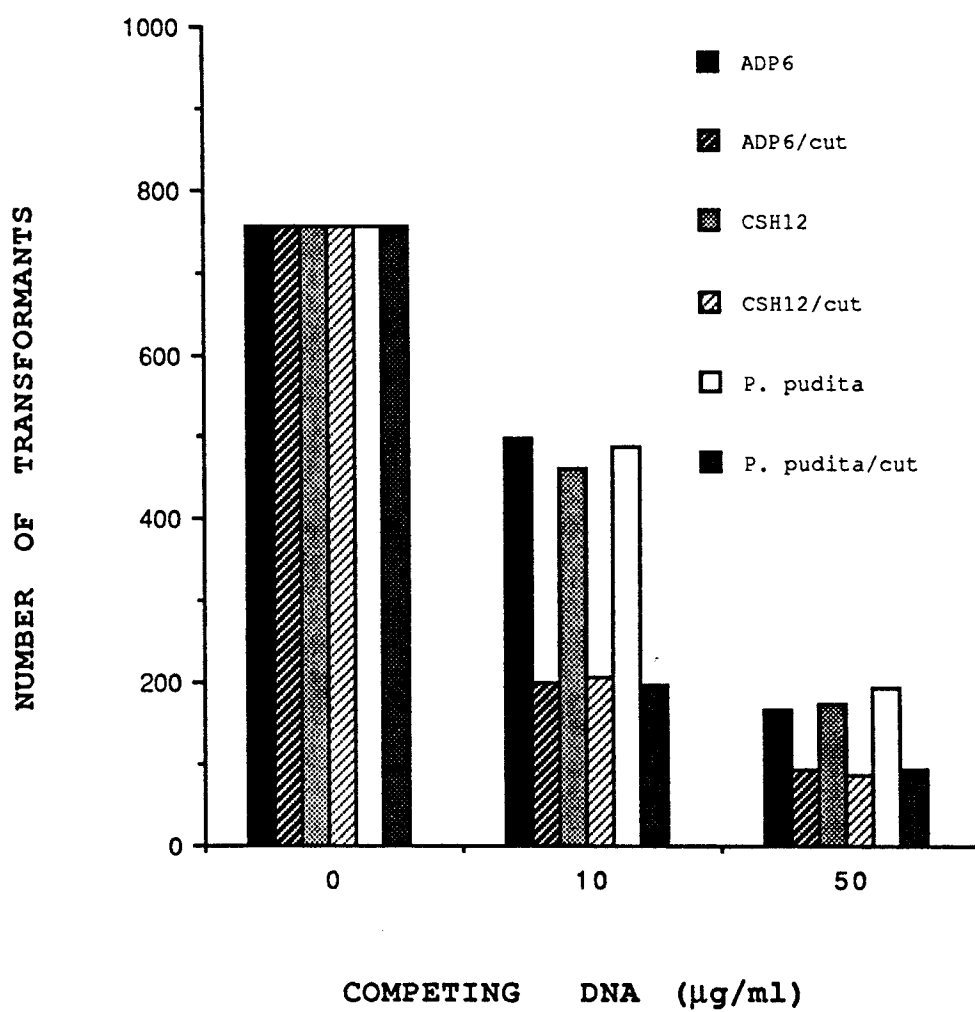
Figure 20. Effect of competing DNA on the frequency of

transformation of Ac138 from Arg⁻ to Arg⁺ phenotype. The DNA

preparations from various bacteria, including *A.calcoaceticus*

(ADP6), *P.pudita* (Paw 630), and *E.coli* (CSH12), were digested

with *Hind*III. Undigested DNA preparations from each organism

were also tested for their capability to effect the frequency

of transformation. Transformations were carried out using

wild-type chromosomal DNA (ADP1) at a final concentration of

10μg/ml. Competing DNA was varied as indicated. The total

number of cells per plate was 2.0 x 10⁵.

Uptake of Radioactive DNA by ADP6 Cells

Cells were taken from log phase cultures of ADP6 and
incubated with nick-translated pZR1 and pMO8 at 37°C.
Samples were removed at 15 minute intervals for 90
minutes. At this time mixtures were chilled, treated with
pancreatic DNase I, and washed with 0.5 M NaCl to eliminate
residual extracellular DNA. The radioactivity associated
with the cells was then determined using a liquid
scintillation counter. As indicated in Figure 21, a similar
pattern of DNA uptake was obtained in both cases with maximum
level of uptake occurring at about 15 minutes. Unexpectedly,
the cell-associated radioactivity declined significantly
after this maximum. At this time the results are
insufficient to reliably propose an explanation as to why the
associated radioactivity declined after the initial peak
value.

Gel Analysis of DNA Fragments Taken Up by ADP6 Cells

ADP6 cells were taken directly from late log phase
cultures and incubated with end-labeled *Eco*RI fragments of
pAN2 (*catBCDE* on 5.0-kbp *Eco*RI fragment in pBR322) at 37°C.
Samples were removed at 10 and 60 minutes, treated with DNase
I, and the cells were washed with 0.5 M NaCl to eliminate
residual extracellular DNA. The cells were then lysed with
SDS and the intracellular DNA was deproteinized by Proteinase
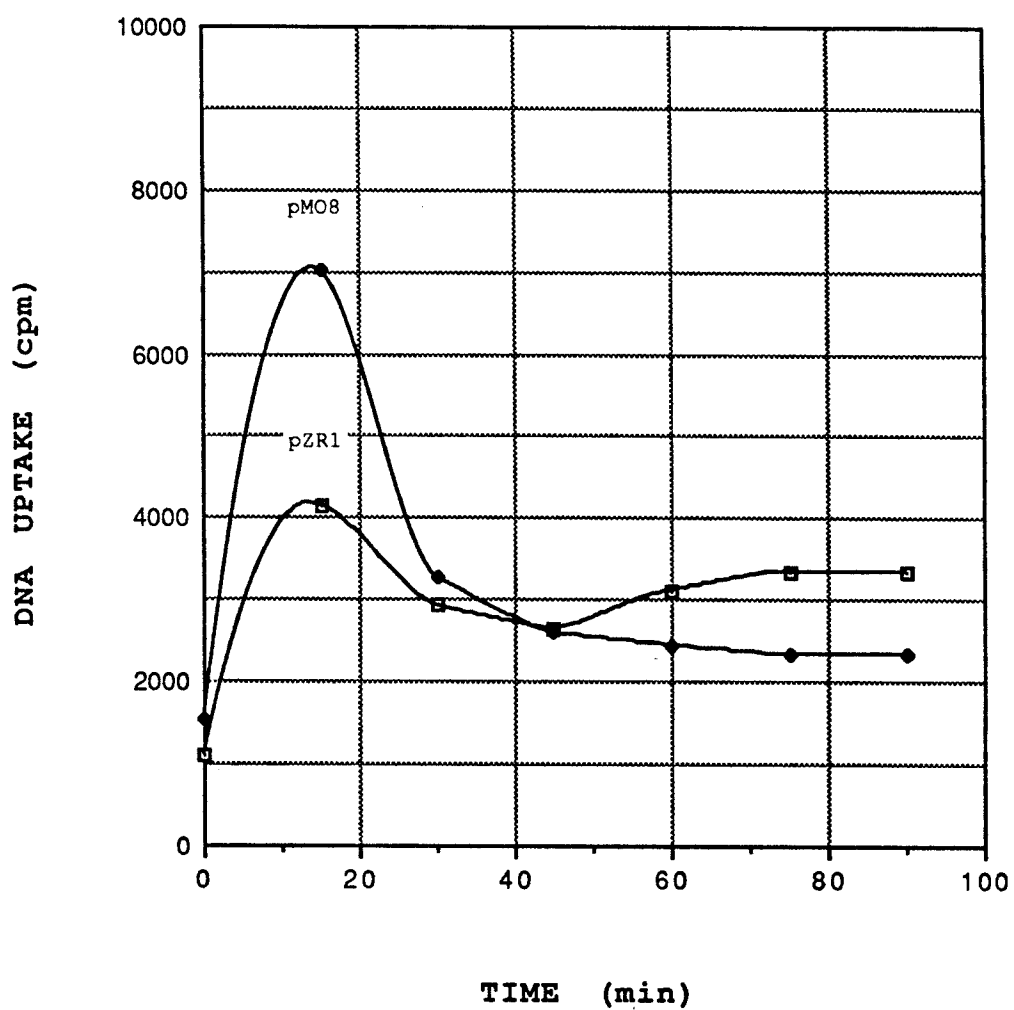
Figure 21. Uptake of radioactively-labelled DNA by ADP6

cells. Cells were taken directly from log phase cultures of

ADP6 and incubated with nick-translated pZR1 and pMO8 at

37°C. Samples were removed at 15 minutes intervals for the

next 90 minutes. At this time, mixtures were chilled,

treated with DNase I, and the cells were washed with 0.5 M

NaCl to eliminate residual extracellular DNA. The

radioactivity associated with the cells was determined using

a liquid scintillation counter.
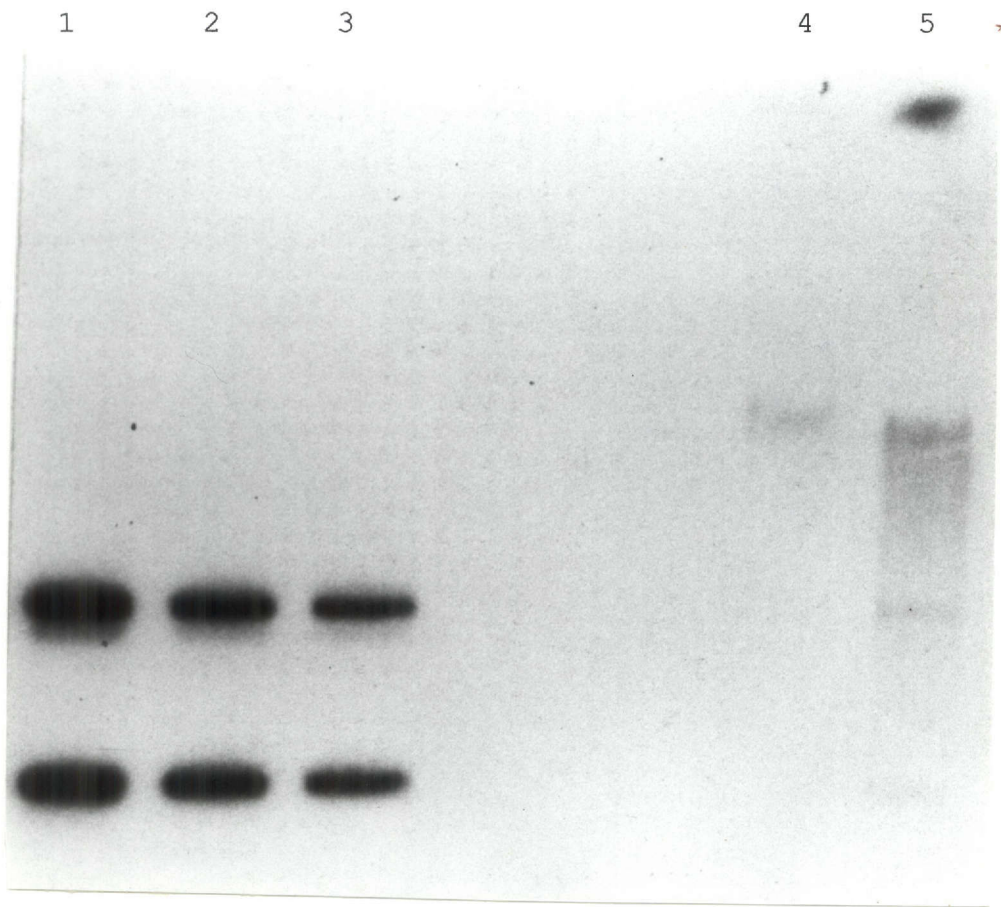
Figure 22. Autoradiogram of an agarose gel showing the uptake of an end-labeled *Eco*RI fragments of pAN2 (a 5.0-kb *Eco*RI fragment carrying *catBCDE* from ADP1 and linearized pBR322 vector) by *A. calcoaceticus* ADP6 cells. Lanes 1-3, end-labeled pAN2 digest before the uptake; lanes 4 and 5, DNA recovered from lysates of cells exposed to the DNA fragments for 10 and 60 minutes, respectively.

K and successive extractions with phenol and phenol:
chloroform. The DNA was precipitated with ethanol and
digested with *Eco*RI. This final digestion was done to
regenerate any fragments which may have been ligated after
entering the cells and to reduce the viscosity of bacterial
once more that *A. calcoaceticus* cells are capable of taking
up both homologous (insert) and heterologous (vector) DNA.
Hence, the discrimination against non-homologous DNA during
the transformation of *A. calcoaceticus* cells must occur at a
step of the transformation process occurring later than DNA
binding and uptake (*e.g.* recombination into the recipient
chromosome).

CHAPTER IV

Discussion

The appearance of competence for transformation is often

a fleeting phenomenon, its development is dependent upon the

species and strain of bacteria used, and on the conditions of

growth (see Notani, et al. 1974). The transformation system

of naturally competent *Acinetobacter calcoaceticus* cells was

investigated. A *pcaA* (catabolic mutant) and several amino

acid auxotrophic strains of BD413 were transformed in a

variety of liquid media with highly purified DNA's. Initial

efforts were directed at determining the conditions necessary

to obtain maximum levels of transformation competency. *A.

calcoaceticus* ADP6 (POB$^-$) was competent throughout the

entire growth cycle when grown and transformed in any of

several media. The highest transformation efficiencies,

approximately 1.2%, were repeatedly obtained with recipient

cells sampled during the late phase of exponential growth.

This peak of competence yielded transformation frequencies

4-fold higher than frequencies obtained using cells sampled

later in the growth cycle (Fig. 7). This result is, however,

in contrast with the earlier reports of others in which the

peak of transformation efficiencies occurred during the

early phase of exponential growth (Cruze, et al. 1979).

96

Competence was also higher in cells sampled from rapidly
growing cultures as compared to slowly growing cultures (Fig.
11). These results indicate that a requirement for the
development of maximum competence in *Acinetobacter* was the
use of actively growing recipient cells. This observation is
in contrast to the conditions for competence development by
Haemophilus. In *H. influenzae*, the requirement for
competence development is the transition of a batch culture
from exponential to stationary phase (Ingraham, et al. 1983).
On the other hand, competence development by Gram-positive
bacteria, such as *Streptococcus* and *Bacillus*, is induced by
the release of competence factors during the exponential
growth phase (Goodgal, 1982).

Transformation frequencies of A. calcoaceticus cultures
also increased as the length of time recipient cells were
incubated with transforming DNA was increased. The maximum
frequency of approximately 1.2% was obtained repeatedly when
the incubation time was increased to 60 minutes (Fig. 4).
During this time the cells continued to divide in growth
medium. For longer incubation times cells were placed in
non-growth media. Similar results were obtained when cells
were incubated with the transforming DNA in the absence of a
carbon source for extended periods of time. As illustrated
in Figure 9, the fraction of cells transformed to prototrophy
increased up to a maximum of 20% when the cells were
incubated in minimal salts with the transforming DNA for 25

hours. A qualitatively similar result was obtained when the cells were starved for various times prior to DNA addition (Fig. 10). The peak of transformation efficiency in each case occurred at about 25 hours. However, when DNA was not present during the initial incubation, the actual transformation frequency was much lower. In addition, in the latter case the highest efficiency was actually obtained at zero time of starvation (Fig. 10). The peak occurred only after an initial significant drop in the efficiency of transformation. Thus, although starvation may indeed have an effect on the physiology of natural transformation, the primary factor here appears to be the extended time of incubation with transforming DNA.

The incubation temperature also plays a significant role determining the number of transformants obtained. The maximum number of colonies always appeared when the cells were incubated at 37°C (Fig. 8).

As was reported earlier by others, recipient cell density plays a role in determining maximal transformation efficiencies (Cruze, et al. 1979). The highest transformation frequencies were obtained using recipient cell densities of about 9×10^5 to 9×10^6 cells in the transformation mixture (Table 2). This increase was not attributable to changes in the DNA/cell ratio, since transformation frequencies obtained with more concentrated cell suspensions could only be increased 1-fold by a

corresponding 10-fold increase in DNA concentration (Fig. 5).
The results obtained with semisolid transformations
procedures (cells and DNA on agar) further supported the
observation that a less concentrated cell suspension yields
higher transformation frequencies. Transformation
frequencies begin to fall off as the number of cells per
plate is raised to more than 2 x 10^5 (Table 3).

A comparison of liquid *versus* semisolid (agar)
transformation conditions was also carried out. When a
semisolid transformation assay was utilized (where donor DNA
was applied to the agar plate), approximately 100%
transformation efficiency was obtainable in the presence of
diluted cell suspensions (Table 3). This very interesting
phenomenon was previously utilized to produce deletions in
the recipient cells' chromosome with a very high frequency,
about 20% (Doten, et al., 1987). However, it should be noted
that this high frequency of colony transformation does not
represent a quantitative measure of transformation in *A.
calcoaceticus* cells, since in the absence of DNase I
treatment, multiple transformation events may occur. In
addition, it can not be determined whether the initial cell
transformed or whether one cell of a small "microcolony"
(often formed on agar plates by *A. calcoaceticus*) transformed
at a later time to produce a large "transformed" colony.

The optimal DNA concentration for achieving the highest
transformation efficiency was found to be 10 μg/ml. Although

higher concentrations were not inhibitory, no increase in transformation efficiency is seen by using more donor DNA. Furthermore, the size of the transforming DNA plays a substantial role in determining the transformation efficiencies obtained with *A. calcoaceticus* strain ADP6 (Fig. 6). Larger fragments of transforming DNA provide significantly increased numbers of transformants. However, transformation frequencies obtained by ADP1 chromosomal DNA were lower than those obtained with pZR1 despite the size difference between the two transforming DNA's. A possible explanation is that on average only 1 out of 100 ADP1 chromosomal DNA fragments taken up by the cells carries the *pca* operon and thus has the potential of transforming ADP6 cells to prototrophy. All pZR1 fragments, on the other hand, carry the *pca* operon. The number of transformants decreased when chromosomal donor DNA was cut with restriction endonuclease. The number of transformants obtained when an *Eco*RI digest of ADP1 was used as the transforming DNA was almost 10-fold lower than that obtained with undigested DNA (Fig. 14). Similar results were obtained when the transformation efficiencies of digested and undigested pZR1 were compared as the transforming DNA (Fig. 13). However, in the latter case the difference between the effectiveness of cut and uncut DNA was not as great as that obtained with ADP1 chromosomal DNA. There are several possible explanations for these results. Cutting the DNA produces additional fragments

which do not carry the wild-type marker and which can thus act as competitive inhibitors of DNA uptake. Alternatively, cutting the DNA could separate the marker from some DNA sequence important for DNA uptake or recombination. This possibility seems less probable because it would be unlikely to find such sequences for *A. calcoaceticus* on the pUC18 plasmid. Also, larger DNA could for an unknown reason simply transform better. In the case of ADP1, if the cells take up a limited number of DNA molecules, larger ones are more likely to carry the necessary markers.

Experiments were also initiated to determine how much DNA is taken up by competent *A. calcoaceticus* cells by measuring co-transformation of independent markers. As indicated in Figure 12 the number of ADP6 (*pcaA*) transformants obtained with ADP1 DNA was much higher than that obtained with Ac138 DNA. There are several possible outcomes from presenting *pca$^+$ arg$^-$* DNA (Ac138 DNA) to the cells that do not contain a functional *pca* operon: 1) transformation of only one locus, in which case only cells transformed by fragments containing *pcaA* gene would appear on the minimal-POB plates, 2) co-transformation of both the *pca* and *arg* operon, by the incoming DNA, which results in formation of *pca$^+$ agr$^-$* transformants that are unable to grow on the minimal-POB plates. As indicated in Figure 12, the number of transformants obtained with ADP1 DNA were much higher than those obtained with Ac138 DNA. Since the only

difference in the two DNA preparations used was that Ac138
chromosomal DNA is *arg⁻*, it can be suggested that the levels
of DNA uptake and subsequent recombination in *A.*
calcoaceticus are so high that both loci are co-transformed
about 80% of the time. Investigations are currently underway
using other auxotrophic strains to further catalog the exact
number of DNA molecules taken up by each cell.

The transformation machinery of the *A. calcoaceticus* is
unable to utilize single-stranded DNA as a suitable donor
DNA. This result is consistent with the earlier reports that
other naturally competent microorganism are not transformed
with the denatured DNA (Smith, et al. 1981).

Results from competitive inhibition experiments (Figures
16-19) suggest that the binding of DNA at the surface of the
transforming cells occurs nonspecifically with respect to the
nucleotide sequence. In addition, reducing the size of the
competing DNA by restriction digestion "increased the impact"
of the competing DNA on the transformation (Fig. 20). This
strongly suggests that the concentration of molecules (or
ends) is the critical parameter in binding. Furthermore,
results from experiments measuring the uptake of radioactive
DNA fragments indicates that *A. calcoaceticus* cells are
capable of taking up both homologous and non-*Acinetobacter*
DNA with equal efficiency (Fig. 21 and 22). This suggests
that the uptake of DNA by the transforming cells occurs
nonspecifically with respect to the nucleotide sequence.

Hence, the previously described discrimination against non-homologous DNA for transforming *A. calcoaceticus* cells (Juni, 1972) must occur at a step of the transformation process occurring later than DNA binding and uptake (e.g. recombination into the recipient chromosome).

In summary, the natural transformation of various strains of *A. calcoaceticus* was investigated. Unlike other systems characterized, *A. calcoaceticus* ADP6 was competent throughout the entire growth cycle when grown and transformed in several media. The highest frequencies were obtained when the cells were actively growing at 37°C. Results also suggest that raising the incubation time with the transforming DNA significantly increase the efficiency of transformation in ADP6 cells. The transformation mechanism in *A. calcoaceticus* was further characterized with respect to size, shape and the origin of the transforming DNA. Transformation frequencies of 1.2% were repeatedly obtained with a final DNA concentration of 10 μg/ml. The size of the transforming DNA appears to be an important factor in the transformation of *A. calcoaceticus* cells. Transformation efficiencies by larger DNA fragments were higher (even if attached to non-*Acinetobacter* DNA) than those obtained with smaller DNA fragments. In addition to size, the duplex nature of the DNA is important, almost no transformation was observed when heat-denatured DNA was utilized in transforming the ADP6 cells. Both competition and uptake assays suggest

that binding and uptake of the DNA molecules by the cells occur nonspecifically with respect to DNA sequence. Therefore, *A. calcoaceticus* does not follow the typical Gram-negative mechanism of transformation in which specific sequences are required for binding and subsequent uptake of the donor DNA.

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