

A NOVEL MECHANISM FOR SITE-DIRECTED MUTAGENESIS OF LARGE
CATABOLIC PLASMIDS USING NATURAL TRANSFORMATION

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Natural transformation is the process by which cells take up DNA from the surrounding medium under physiological conditions, altering the genotype in a heritable fashion. This occurs without chemical or physical treatment of the cells. Certain *Acinetobacter* strains exhibit a strong tendency to incorporate homologous DNA into their chromosomes by natural transformation. Transformation in *Acinetobacter* exhibits several unique properties that indicate this system's superiority as a model for transformation studies or studies which benefit from the use of transformation as an experimental method of gene manipulation. *Pseudomonas putida* is the natural host of TOL plasmids, ranging between 50 kbp and 300 kbp in size and encoding genes for the catabolism of toluene, *meta*-toluate, and xylene. These very large, single-copy plasmids are difficult to isolate, manipulate, or modify *in vitro*. In this study, the TOL plasmid pDKR1 was introduced into *Acinetobacter calcoaceticus* strains and genetically engineered utilizing natural transformation as part of the process. Following engineering by transformation, the recombinant DNA molecule was returned to the native genetic background of the original host *P. putida* strain. Specific parameters for the successful manipulation of large plasmids by natural transformation in *Acinetobacter* were identified and are outlined. The effects of growth phase, total transforming DNA concentration, transforming DNA conformation, and gene dosage on transformation efficiency are

presented. Addition of *Acinetobacter* plasmid DNA sequences to the manipulated constructs did not have an effect on transformation rates. Results suggest that a broadly applicable and efficient method to carry out site-directed genetic manipulations of large plasmids has been identified. The ability to easily reintroduce the recombinant DNA molecules back into the original host organism was maintained.

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TABLE OF CONTENTS

Page	
LIST OF TABLES.....	vii
LIST OF ILLUSTRATIONS.....	viii
LIST OF ABBREVIATIONS.....	x
CHAPTER	
I. INTRODUCTION.....	1
Genetic transformation and the processes of natural transformation	
Molecular biotechnology and applications of transformation	
Natural transformation systems	
Properties of the genus <i>Acinetobacter</i> which make it uniquely suited for use as a molecular tool	
Catabolic plasmids and the genus <i>Pseudomonas</i> – a model system	
Research objectives	
II. MATERIALS AND METHODS.....	27
Bacterial strains and plasmids	
Media and growth conditions	
Long-term storage of cultures	
Enzymes and chemicals	
Gram stains	
Oxidase test	
Selective plating	
Complete digestion of DNA with restriction endonucleases	
Partial DNA digestion by restriction endonucleases	
Recovery of duplex DNA from low-melting temperature agarose gel	
Quantitative and qualitative identification of DNA	
Horizontal agarose gel electrophoresis	
Calf intestinal phosphatase treatment of vector DNA	
DNA ligation	
Screening of recombinant plasmids	
Rapid analytical scale isolation of plasmid DNA by alkaline lysis	
Preparative scale plasmid isolation	

II. MATERIALS AND METHODS (continued)

- Rapid total DNA isolation
- Southern transfer of DNA to nylon membranes
- Generation of ^{32}P labeled DNA probes by polymerase chain reaction
- Hybridization of membranes with radiolabeled DNA probes
- Preparation of crude chromosomal transforming DNA
- Preparation of transforming plasmid DNA
- Triparental mating protocol
- Conjugation protocol
 - A. calcoaceticus* competent cell production
 - E. coli* competent cell production
 - A. calcoaceticus* transformation protocol
 - E. coli* transformation protocol
- DNA template preparation by the colony PCR method
- DNA amplification by polymerase chain reaction
- PCR product preparation for DNA sequence analysis
- Sanger dideoxyribonucleotide sequence analysis of PCR-generated DNA products
- Denaturing polyacrylamide gels for DNA sequence determination
- Autoradiography of sequence analysis gels

III. RESULTS AND DISCUSSION.....79

- Verification of bacterial strains
- Determination of the ability to transfer plasmids between *Acinetobacter* and *Pseudomonas* by conjugation
 - Conjugation of pDKR1 into ADP1
 - Verification of pDKR1 TOL genes in *Acinetobacter* strains
 - Verification of pDKR1 conjugal transfer from *A. calcoaceticus* to *P. putida*
- Optimization of *Acinetobacter* transformation protocol
 - Determination of optimal growth phase for *Acinetobacter* transformation
 - Dependence of transformation efficiency on donor DNA concentration
 - Dependence of transformation efficiency on donor DNA conformation
 - Dependence of transformation efficiency on donor DNA size
 - Transformation efficiency as a function of gene dosage
 - Increased transformation efficiencies as a result of dilute lysis solution with detergent
 - Summary of transformation protocol optimization
- Engineering of TOL genes for the generation of donor and target constructs
 - Generation of pPW595 containing $\Delta xyL'T159$
 - Subcloning of $\Delta xyL'T159$ into pUCP30T

III. RESULTS AND DISCUSSION (Continued)

Subcloning of pRAY into pUCP30T	
Subcloning $\Delta xyLL'T159$ into pWS1001	
Generation of strains for transformation experiments	
Generation of A125B1 by natural transformation	
Generation of A6R1, A125B1R1 and A141R1 by conjugation	
Generation of A6P1, A6P2, A141P1, A141P2, A141R1P1, by triparental mating	
Transformations of the pDKR1 TOL plasmid with the standard <i>Acinetobacter</i> transformation protocol	
Transformation of A1R1 with pPW596	
Cotransformation of A6R1 with pPW596 and pZR1 DNAs	
Cotransformation of A125B1R1 with pPW596 and ADP125 genomic DNA	
Cotransformation of A141R1 with pPW596 and ADP1 genomic DNA	
Cotransformation of A125B1R1 with pBK692mut3 and ADP125 genomic DNA	
Modification of the standard <i>Acinetobacter</i> transformation protocol	
Cotransformation studies using model constructs	
Cotransformation of A6P2 with pBK391 and pZR1 DNAs	
Cotransformation of A141P1 with pBK391 and ADP1 genomic DNA	
Cotransformation of A141P2 with pBK391 and ADP1 genomic DNA	
Summary of cotransformation studies using model constructs	
Cotransformation of A6R1 with pPW596 and pZR1 DNAs	
Summary of pDKR1 transformation studies	
Conjugation of modified pDKR1 plasmids in <i>P. putida</i>	
Effect of $\Delta xyLL'T159$ incorporation into pDKR1 on <i>m</i> -toluate metabolism	
IV. CONCLUSIONS.....	152
V. APPENDIX.....	164
REFERENCES.....	166

LIST OF ILLUSTRATIONS

	Page
1. Physical map of the arrangement of the genes encoded by the TOL.....8 pathway in the TOL plasmid pWWO	8
2. Generalized scheme for the development of competence in <i>Streptococcus</i>11	11
3. Mechanism for transformation of the <i>Streptococcus</i> genome.....14	14
4. Model of the DNA -translocation apparatus in <i>Acinetobacter</i>19 <i>calcoaceticus</i> sp. BD413 as proposed by Averhoff's laboratory	19
5. Screening grid used for creation of a master plates.....43	43
6. Diagram of a CsCl gradient T1270 ultracentrifuge tube containing.....49 bands after 40 hours of centrifugation at 118,700 x g	49
7. Nucleotide sequence of oligonucleotide primers used for PCR.....63-64 amplification	63-64
8. Summary of timed DNA sequence determination protocol.....71	71
9. Representative autoradiogram showing DNA fragments hybridized82 by ³² P labeled <i>xylLT</i> PCR product	82
10. Verification of TOL gene sequences in ADP1 recipient strains by.....85 PCR amplification	85
11. Competence development in <i>A. calcoaceticus</i> ADP6 as a function90 of growth phase	90
12. Transformation efficiency for competent <i>A. calcoaceticus</i> cells as93 a function of donor DNA concentration	93
13. Transformation efficiency for competent <i>A. calcoaceticus</i> cells as.....96 a function of donor DNA conformation	96
14. Dependence of transformation efficiency on donor DNA size.....100	100
15. Transformation efficiency as a function of gene dosage.....104	104

16. Replica plates demonstrating the effect of dilute lysis solution in110 the transformation mixture on transformation of ADP6 cells	110
17. Agarose gel demonstrating the presence of the $\Delta xyL'T159$114 fragment in pPW596	114
18. Agarose gel demonstrating the presence of the $\Delta xyL'T159$118 fragment in pPW1000	118
19. Agarose gel demonstrating the presence of pRAY in pWS1001.....121	121
20. Agarose gel demonstrating the presence of the $\Delta xyL'T159$124 fragment in pWS1001	124
21. Agarose gel demonstrating the absence of $\Delta xyL'T159$ in pDKR1.....131	131
22. Nucleotide sequence of the <i>xyIT</i> region mutated in pBK692mut3.....136	136
23. Agarose gel analysis demonstrating the incorporation of146 $\Delta xyL'T159$ into pDKR1	146
24. Plot of PR1 Δ 1 and wild-type pDKR1 growth curves using <i>m-tol</i> as.....150 the sole carbon and energy source.	150

LIST OF ABBREVIATIONS

A	adenine
aa	amino acid
Ap	ampicillin
Ap ^r	ampicillin resistance
Ben	benzoate
bp	base pair
C	cytosine
°C	degrees centigrade
cc	cubic centimeter
CFU	colony forming unit(s)
CIP	calf intestinal phosphatase
Ci	Curie
cm	centimeter
cpm	counts per minute
CsCl	cesium chloride
Da	Dalton
dNTP	deoxyribonucleotide
ddNTP	dideoxyribonucleotide
ddH ₂ O	distilled deionized water
DNA	deoxyribonucleic acid
dsDNA	double-strand DNA
EDTA	ethylenediamine tetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol
x g	times the force of gravity
g	gram
G	guanine

Gm	gentamicin
Gm ^r	gentamicin resistance
IPTG	isopropyl-β-D-thio-galactopyranoside
kbp	kilobase pair
kDa	kilodalton
Km	kanamycin
Km ^r	kanamycin resistance
LB	Luria-Bertani
LD-50	lethal dose - fifty
l	liter
M	molar
Mbp	megabase pair
MCi	millicurie
MCS	multiple cloning site
ml	milliliter
mM	millimolar
mol	mole
<i>m</i> -tol	<i>meta</i> -toluate
μg	microgram
μl	microliter
μm	micrometer
NaCl	sodium chloride
ng	nanogram
NaOH	sodium hydroxide
nm	nanometer
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol

pg	picogram
pmol	picomole
POB	<i>para</i> -hydroxybenzoate
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
Sm	streptomycin
Sm ^r	streptomycin resistance
SSC	standard saline citrate
ssDNA	single-strand DNA
SSPE	sodium chloride, sodium phosphate, ethylenediamine tetraacetic acid
T	thymine
TAE	(hydroxymethyl) aminomethane, acetic acid, ethylenediamine tetraacetic acid
TBE	(hydroxymethyl) aminomethane, boric acid, ethylenediamine tetraacetic acid
Tc	tetracycline
Tc ^r	tetracycline resistance
TE	(hydroxymethyl) aminomethane, ethylenediamine tetraacetic acid
TEMED	N, N',N'' -tetarmethylenediamine
Tris	(hydroxymethyl) aminomethane
Trp	tryptophan
UV	ultraviolet
U	uracil
V	volts
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER I

INTRODUCTION

Genetic exchange in microorganisms is a major avenue by which bacterial cells acquire a competitive advantage through the accumulation of independently- derived beneficial genotypes in a single cell. Bacteria reproduce by binary fission, with no meiosis and gamete fusion. The primary method of acquiring new genetic information is genetic exchanges between cells followed by recombinational events. These are necessary to increase the diversity of the genetic pool. Transfer of genetic material between cells may be accomplished by three major processes. These are conjugation, transduction, and transformation (Smith *et al.*, 1981) Of these three, bacterial transformation is a major mechanism for genetic exchange in a number of (scientifically and medically) important bacterial genera.

Bacterial transformation is the process by which competent cells take up naked DNA from the surrounding medium and incorporate it, thereby altering their genotype in a heritable fashion. Natural transformation allows the cellular uptake of DNA from the environment and the subsequent use of the DNA's encoded genes for the organism's benefit under physiological conditions. The uptake of DNA occurs without the use of chemical or physical cellular treatments. The transformation process requires that the

recipient cell be in a state of competence. Competence, is defined as the physiological state that occurs naturally, or an artificial condition that can be produced experimentally (Palmen & Hellingwerf, 1997), during which naked DNA is taken up from the environment.

Many bacterial genera have been observed to undergo natural transformation (Lorenz & Wagnackel, 1994), and the molecular details of the processes are variable among them. Genera that contain naturally transformable organisms include, but are not limited to, members of *Acinetobacter*, *Bacillus*, *Campylobacter*, *Haemophilus*, *Moraxella*, *Neisseria*, *Pseudomonas*, and *Streptococcus*. Many of these genera include pathogens and require complex and expensive growth media and conditions. Some members of the genera *Escherichia*, *Pseudomonas*, and *Salmonella* have also been made transformable by artificial means (Neidhardt *et al.*, 1990).

Distinct differences exist in the processes by which many of the bacterial species undergo natural transformation. Currently, the best understood natural transformation systems fall into two groups. One is typical of Gram-positive organisms, and the other found in some Gram-negatives. Mechanisms for both the Gram-positive *Bacillus subtilis* (Dudnau, 1991) and *Streptococcus pneumoniae* (Avery *et al.*, 1944), as well as the Gram-negative *Haemophilus influenzae* (Goodgal, 1982) and *Neisseria gonorrhoeae* (Goodman & Scocca, 1991) have been elucidated. The characterized mechanisms indicate a significant difference in the process of DNA binding and transportation across the cell's plasma membrane between the two groups.

Table 1. Representative bacterial species reported to be capable of natural transformation (adapted from Neidhardt *et al.*, 1990; Lorenz & Wagnackel, 1994)

Gram-negative bacteria

Acinetobacter calcoaceticus

Azotobacter agilis

Azotobacter vinelandii

Campylobacter jejuni

Haemophilus influenzae

Haemophilus parainfluenzae

Moraxella osloensis

Moraxella urethralis

Neisseria gonorrhoeae

Pseudomonas alcaligenes

Pseudomonas mendocina

Pseudomonas pseudoalcaligenes

Pseudomonas stutzeri

Psychrobacter sp.

Gram-positive bacteria

Bacillus cereus

Bacillus stearothermophilus

Bacillus subtilis

Streptococcus pneumoniae

Streptococcus sanguis

The precise details of many natural transformation systems remain relatively unknown. Recent discoveries related to the transformation system of *A. calcoaceticus* and properties of the organism itself, have set it apart as a prime candidate for further study. *Acinetobacter calcoaceticus* BD413 exhibits high levels of natural competence in specific strains (Juni & Janik, 1969) and has been the subject of many investigations in the past 40 years because of its metabolic resourcefulness. It presents itself as the best model system by which the processes of natural transformation might be studied. *Acinetobacter calcoaceticus* also possesses many unique characteristics that allow previously gained and newly discovered knowledge to be utilized in the development of its natural transformation system as a molecular tool. The establishment of a set of procedures for natural transformation in *Acinetobacter* should allow the genetic manipulation and production of recombinant DNA molecules beyond the capability of current technology.

Molecular biotechnology and applications of transformation

Molecular biotechnology is a scientific discipline that exploits recombinant DNA technology to modify and transfer genetic information from one organism to another. For example, recombinant organisms may be designed to produce useful products, carry out novel commercial processes, or be model systems for the investigation of biochemical pathways and their regulatory mechanisms.

Most current molecular protocols for producing recombinant DNA molecules (and organisms) utilize artificial genetic exchange to introduce and/or engineer DNA

constructs. Many cloning protocols require that the source DNA, containing the target sequence, and the cloning vector be consistently cut into discrete, reproducible fragments. Such manipulations must be carried out *in vitro* and then the recombinant molecule reintroduced into the host organism.

It is important that recombinant DNA techniques and systems for genetically engineering large DNA constructs be developed. *In vivo* manipulation of large DNA constructs is advantageous over conventional *in vitro* approaches. Development of *in vivo* techniques for the manipulation of large catabolic plasmids should alleviate many of the major barriers which preclude or obstruct certain types of experimentation. It is logical to assume, from the rapidity by which bacterial populations can adapt to a changing environment, that a similar system must be utilized as a mechanism of horizontal gene transfer in nature and that this system may be exploited to our advantage. Natural transformation in *Acinetobacter* may provide just such a system.

The assembly of new recombinant DNA constructs containing complete metabolic pathways for the catabolism of toxic waste or the production of beneficial molecules offers exciting possibilities for biotechnology and biological research. The ability to manipulate large DNA constructs *in vivo* allows the development of innovative industrial applications (e.g. the production of regulated gene constructs) which should provide increased catalytic activity under conditions that would normally repress catabolic genes. It should also allow increased investigation of the metabolic potential and evolutionary source by which these pathways were originally formed.

The characteristics of typical degradative plasmids (Table 2) and their associated operons frequently do not allow the ready application of many traditional molecular cloning procedures. Such plasmids are usually single copy and are very large, making them difficult to isolate and generally challenging to work with. Large plasmids are very susceptible to damage while being manipulated *in vitro*. Plasmid size and the number of unique restriction sites within the very large operons make it difficult to use traditional restriction endonuclease digestion techniques to modify the plasmids. Very rarely are there any unique sites for which there are readily available restriction enzymes that can be utilized for modification and engineering. Thus, it is hard to specifically alter or otherwise manipulate any desired intact segment or section of coding sequence.

The organization of the superoperonic clusters encoded on these plasmids makes successful manipulations even more difficult. The functionality of a catabolic pathway may require the expression of several different operons in a concerted fashion and can include several *cis* regulatory elements as well as regulatory proteins with their own expression units (Fig. 1). This presents significant problems for the investigator when trying to carry out many types of recombinant DNA procedures.

For example, almost every recombinant DNA technique requires the introduction of altered DNA back into the original (or another) organism for analysis. The very large size of catabolic operon constructs and the inability of many organisms to be readily transformed even with smaller plasmids, combine to make the task of manipulating these pathways in their native genetic backgrounds virtually impossible.

Table 2. Examples of degradative plasmids found in *Pseudomonas putida* (adapted from Haas, 1993).

Plasmid	Size	Growth Substrate	Reference
TOL pWWO	176 kbp	Toluene, <i>m</i> -xylene, <i>p</i> -xylene	Reineke <i>et al.</i> , 1982
NAH7	69 kbp	Naphthalene	Yen & Gunsalus, 1982
pWW60	80 kbp	Naphthalene	Cane & Williams, 1982
SAL	60-83 kbp	Salicylate	Franklin <i>et al.</i> , 1981
CAM	225 kbp	Camphor	Rheinwald <i>et al.</i> , 1973
OCT	150 kbp	<i>n</i> -Octane	Fennewald <i>et al.</i> , 1979
pWW17	280 kbp	Phenylacetate	Pickup <i>et al.</i> , 1983

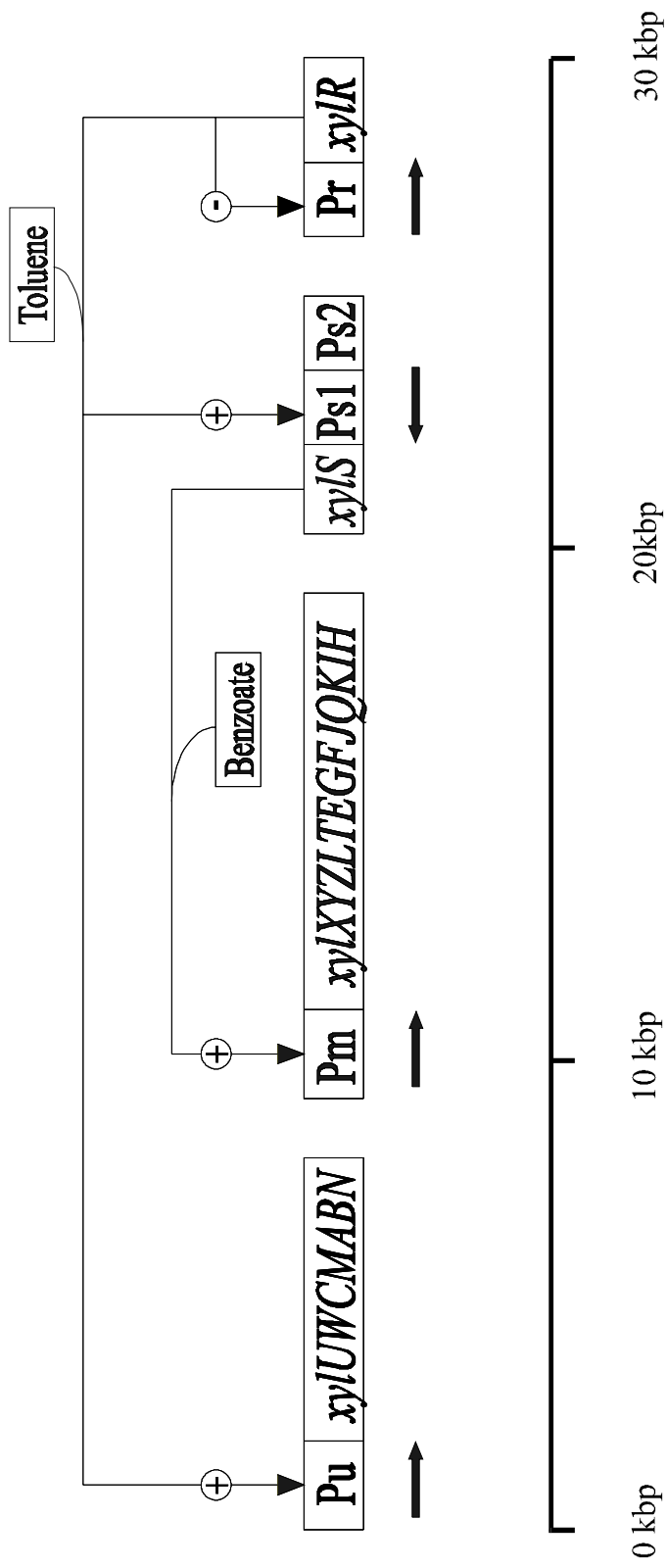


Fig. 1 Physical map of the arrangement of the genes encoded by the TOL pathway in the TOL plasmid pWWO. Arrows indicate the direction of transcription for each expression unit. Plus and minus symbols designate the regulatory impact on the respective promoter. This figure was adapted from Houghton & Shanley (1994).

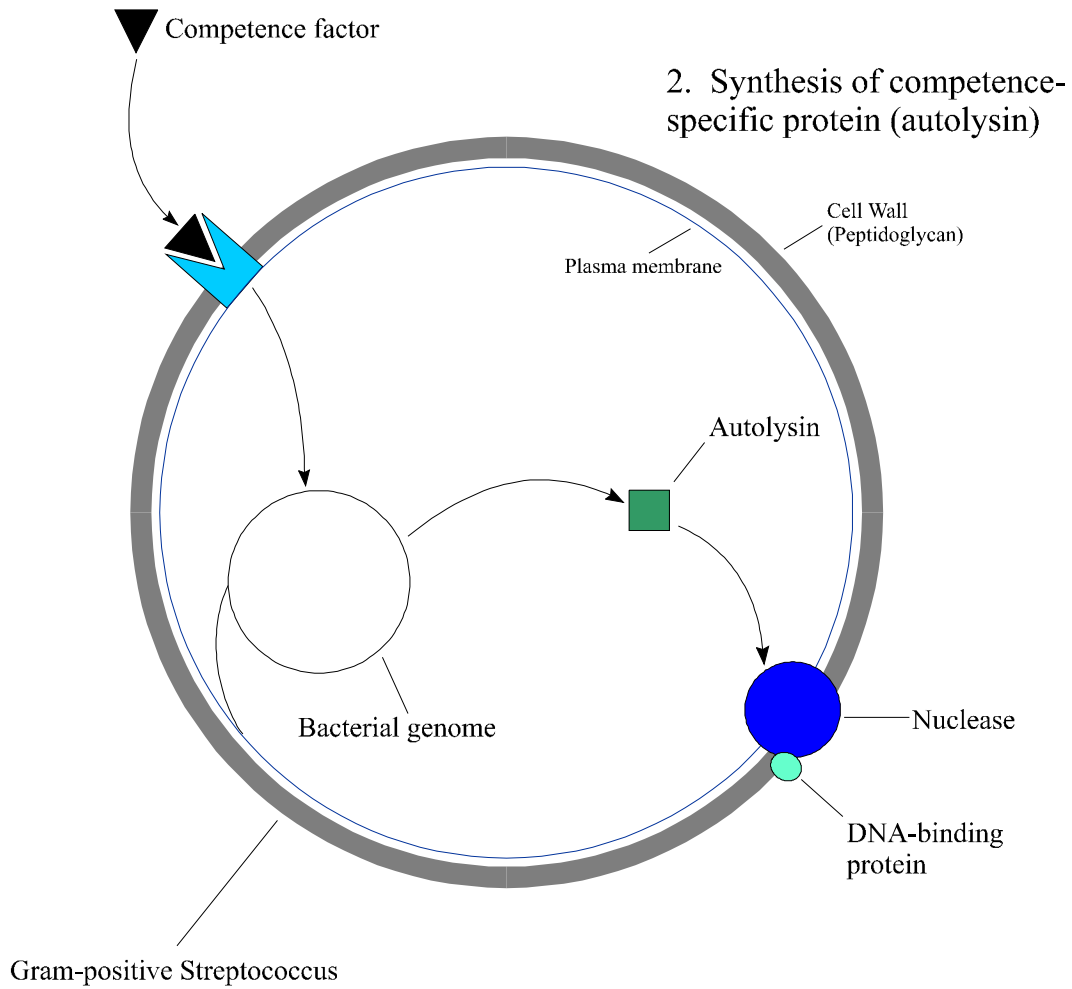
Natural transformation systems

Distinct differences and unique features in the natural transformation process have been observed across a range of organisms, particularly between Gram-negative and Gram-positive representatives (Stewart & Carlson, 1986). There is a significant difference in the process of DNA binding and transportation across the cell's plasma membrane between the two (Smith *et al.*, 1999). These and other differences in the natural transformation process suggest that its function varies between the species as well (Palmen, 1993). Currently understood natural transformation systems fall into two groups. Each group is defined by a model system (that seems to be) representative of each group. One is typical of Gram-positive organisms, and the other is found in Gram-negative representatives.

A. Gram-positive model (originally reviewed by Avery *et al.*, 1944)

The mechanism of natural transformation in Gram-positive organisms was first characterized in *Streptococcus pneumoniae*, and *Streptococcus* is still the model organism for many studies of transformation. *Streptococcus* cultures achieve the highest level of competence during the latter part of exponential growth (Avery *et al.*, 1944). Each cell produces a small amount of a low molecular mass protein (approximately 10,000 kDa) called competence factor (Neidhardt *et al.*, 1990). This competence factor induces the cells in the population to synthesize 8 to 10 different proteins that establish the competent state (Fig. 2). In *Bacillus subtilis*, *S. pneumoniae*, and *S. sanguis*, a single competent cell can bind and take up large numbers of DNA molecules regardless of the source(s) of the donor DNAs. During the uptake process, one strand of the duplex

1. Interaction of competence specific factor with cell surface receptor



3. Autolysin exposes DNA-binding protein and nuclease

Fig. 2 Generalized scheme for the development of competence in *Streptococcus*.

Interaction with cell-surface receptors cause the synthesis of the competence-specific protein, autolysin. Autolysin exposes a DNA-binding protein and nuclease on the cell's surface. (Adapted from Neidhardt *et al.*, 1990)

is degraded while the complementary strand is transported into the cell. In the case of *S. pneumoniae*, there are numerous membrane-associated protein multimers, composed of an exonuclease and a DNA-binding protein, that bind double-stranded DNA to the cell surface. At the same time, single strand breaks are introduced at 6 to 8 kbp intervals. Before the DNA enters the cell, double strand breaks occur in the DNA, presumably by a second cleavage opposite the single-strand nick introduced previously. A single strand of the DNA (duplex) is completely hydrolyzed by the envelope-bound exonuclease. The remaining internalized strand becomes associated with a specific binding protein that appears upon competence development (Fig. 3). A piece of DNA in this coated, single-stranded form is called an eclipse complex because if isolated it can no longer transform cells (only dsDNA can be taken up by cells). Still in single-stranded form, it becomes incorporated into the endogenote (recipient genome). If the transforming DNA sequence varies somewhat from the endogenote, a heteroduplex is formed. The heteroduplex is resolved by replication or the process of mismatch correction. The “correction” phenomenon is brought about by DNA repair mechanisms but the observed selectivity for donor genotype is not well understood, that is, the recipient cell preferentially uses the donor strand as the template for repair synthesis. Differentiation between the recipient and the donor is thought to be by methylation pattern but this is not yet proven.

B. Gram-negative model (Goodgal, 1982)

The best understood natural transformation mechanism of the Gram-negative group is in the genus *Haemophilus*. *Haemophilus influenzae* and *H. parainfluenzae*

1. A dsDNA fragment is bound to the cell at hundreds of sites

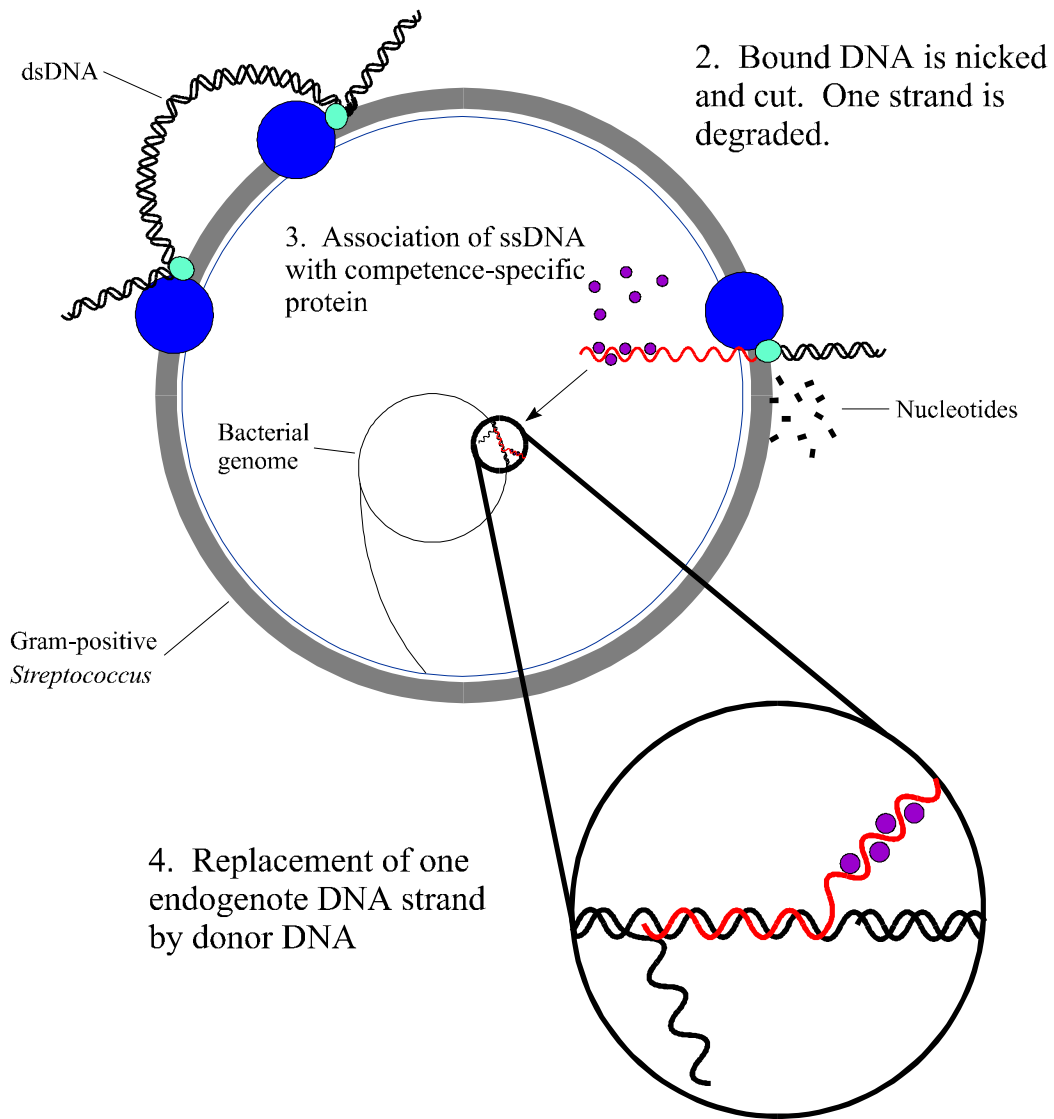


Fig. 3 Mechanism for transformation in *Streptococcus*. A fragment of dsDNA is bound to the cell's surface and the DNA is cut by the membrane-bound nuclease. After conversion to a single-stranded form, the ssDNA associates with a competence-specific protein. The DNA-protein complex replaces one endogenous strand with donor DNA. (Adapted from Neidhardt *et al.*, 1990)

have been the model organisms for the study of the transformable Gram-negative bacteria. Soluble competence factors such as those found in Gram-positives have never been demonstrated in the Gram-negative group. Cultures are induced to competence by a non-growth supporting medium that allows continued protein synthesis. Of the Gram-negative bacteria that are naturally transformable, the best understood mechanism of DNA uptake involves the binding of DNA molecules which contain specific “uptake” or recognition sequences. Only a few molecules of homologous DNA can be taken up by the competent cell, and heterologous DNAs lacking uptake sequences are taken up at a much lower frequency. Competent *Haemophilus* takes up only double-stranded DNA from closely related strains and this strain specificity has been shown to be mediated by specific nucleotide sequences called recognition sequences. In *Haemophilus*, the 11 bp sequence, 5'-AAGTGCGGTCA-3', is essential for DNA uptake. Similarly, *Neisseria gonorrhoeae* uses the 10 bp sequence 5'-GCCGTCTGAA-3'. A total of 1465 uptake recognition sequences were found in the 1.83 Mbp of *H. influenzae* genome, so even small fragments can be taken up by binding to receptors on the cell surface. The importance of transformation is clearly borne out by the frequency of these 11 base pair sequences.

The DNA is not degraded to a single-strand form as part of the process of entry into the cell. Therefore, transforming DNA does not go through an eclipse period as in Gram-positive bacteria. It is thought that the DNA then enters membrane bound vesicles called transformosomes from which the transforming DNA is able to “scan” the recipient’s genome until a homologous region is found. Only one strand of DNA is then

incorporated into the endogenote and the displaced strand plus the complementary donor strand are degraded.

C. *Acinetobacter calcoaceticus* BD413

Natural transformation was first observed in *A. calcoaceticus* by Juni and Janik in 1969. Juni & Janik (1969) described a highly efficient transformation system in the wild-type *A. calcoaceticus* strain BD413 (also known as ADP1) that has provided a useful model for the study of this mode of genetic transfer. Over 100 strains of *Acinetobacter* have since been studied and only two other weakly competent strains have been identified (Palmen, 1993). Transformation as demonstrated in *Acinetobacter* is highly strain-specific and does not follow the typical model that has been elucidated for other Gram-negative organisms such as *Haemophilus*.

Acinetobacter calcoaceticus strain BD413 is naturally competent at a very high level (Juni, 1972). It has also been shown that competent *Acinetobacter* cells demonstrate a very strong tendency to incorporate homologous transforming DNA into their chromosome (Palmen, 1993). Palmen (1993) found that transformational frequencies strongly depend on the kind of transforming DNA and the length of incubation. This was demonstrated by transformation experiments using multiple selectable markers and different types of transforming DNA. Palmen (1993) also found that DNA uptake by *Acinetobacter* did not show any sequence specificity, with DNA from other genera being taken up as efficiently as that from *Acinetobacter*. Transforming DNA enters the cells in a single-stranded form and the uptake appears to be magnesium

or calcium-dependent. These unique properties, not observed in other Gram-negative bacteria, make it ideal for the study of DNA translocation .

Acinetobacter is no exception when it comes to uniqueness of the process.

Much of the current literature is conflicting with respect to the most effective donor DNA molecule type and conformation, the growth phase at which *Acinetobacter* achieves the highest level of natural competence, and the frequency at which recombination leads to genotypic/phenotypic conversion of the bacterial cell.

Recently, novel genes said to be essential for natural transformation have been identified in *A. calcoaceticus* (Porstendorfer *et al.*, 1997; Busch *et al.*, 1999; Herzberg *et al.*, 2000). These newly identified genes and their respective proteins have been designated *comB* (Herzberg *et al.*, 2000), *comC* (Link *et al.*, 1998), *comE* (Busch *et al.*, 1999), *comF* (Busch *et al.*, 1999) and *comP* (Porstendorfer *et al.*, 1997). The gene products have been proposed to function as subunits of a cell surface structure that acts in binding and possibly forming channels for the translocation of DNA. The DNA sequence of these *com* genes shows a great deal of similarity to genes for factors necessary in assembly of Type IV pili. However, they have been shown not to play a role in the pilin formed by some *Acinetobacter* spp. The DNA translocation structures are predicted to be oligomeric, presumably comprised of pilin or pilin-like subunits. Many of the genes mentioned above have been demonstrated to be similar to pre-pilins of Type IV pili, pilin-like components of protein translocation machinery, and various types of Type IV pilus biogenesis factors (Link *et al.*, 1998). Averhoff (2000) has proposed a model by which the proteins interact at the cell membrane during DNA uptake (Fig. 4).

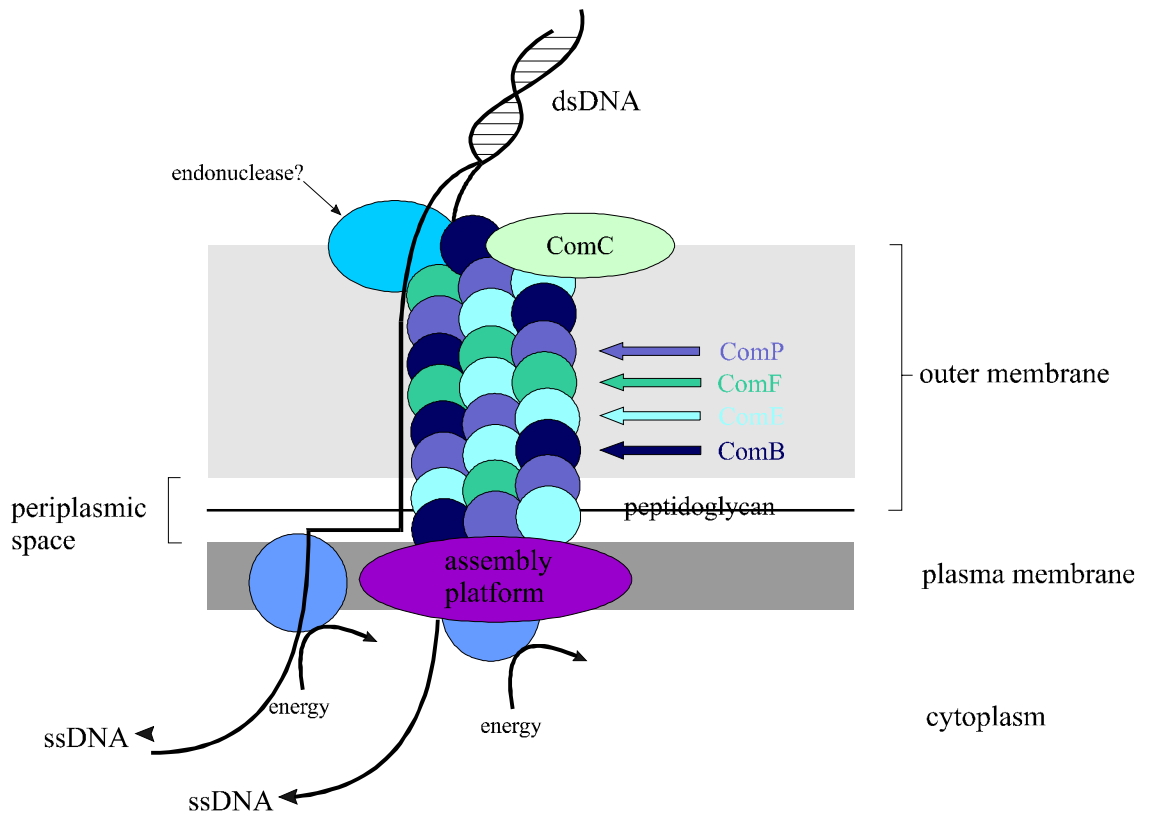


Fig. 4 Model of the DNA-transformation apparatus in *Acinetobacter calcoaceticus* BD413 as proposed by the laboratory of Averhoff. This figure was adapted from the website of Averhoff (<http://www.gwdg.de/~genmibio/lab255.html#1>)

Properties of the genus *Acinetobacter* which make it uniquely suited for use as a transformation vehicle

Acinetobacter calcoaceticus is a common non-pathogenic soil microorganism that is very interesting from a biotechnological standpoint. It exhibits several unique properties that indicate its superiority as a model system for transformation experiments and the expression of altered phenotypes for commercial application. These properties are:

1. *A. calcoaceticus* has the ability to grow on a wide range of substrates. This demonstrates a high degree metabolic diversity and makes the organism very nutritionally versatile. Its metabolic diversity allows it to grow on such substrates as simple sugars, aromatic hydrocarbons, and aliphatic hydrocarbons (Juni, 1978).
2. *A. calcoaceticus* has the ability to grow at an acid pH (Juni, 1978). Batch cultures, grown at an acid pH (around pH 5.0), dramatically reduce the need for expensive sterilization when bulk production is required.
3. *A. calcoaceticus* is usually non-pathogenic.
4. *A. calcoaceticus*' natural transformability and its high level of natural competence allows for rapid, inexpensive procedures which would be difficult or expensive with current recombinant DNA technologies. Transformation rates have been demonstrated to be high enough to allow screening of transformants by genotypic rather than phenotypic properties (Doten *et al.*, 1987a). These high transformation rates make screening by polymerase chain reaction (PCR) or DNA sequence analysis cost effective and time efficient approaches.

5. Broad host range plasmids such as RP1 can replicate in *Acinetobacter* strains. This affords the use of well-characterized vectors from other genera to transfer cloned DNA into *Acinetobacter* and subsequently back to the original donor (Benjamin & Shanley, unpublished data).
6. The ability to conjugate *A. calcoaceticus* with a broad range of donor and recipient species easily allows the introduction, engineering, and return of vector and plasmid constructs to the donor or host. This affords the easy construction of large genetically engineered metabolic pathways that can not be readily assembled on a single plasmid *in vivo*.
7. *A. calcoaceticus* takes up DNA in a non-discriminatory manner with respect to donor species (Palmen, 1993).
8. The attachment of non-*Acinetobacter* DNA to donor DNA is not inhibitory to the process of transformation (Palmen & Hellingwerf, 1997).
9. *A. calcoaceticus* incorporates only homologous DNA sequences into its chromosome (Benjamin, personal communication).
10. Cloned pieces of DNA in plasmids maintained in *E. coli* or *P. putida* can also transform *A. calcoaceticus* effectively (Shanley *et al.*, 1986; Doten *et al.*, 1987a).
11. DNA generated by polymerase chain reaction can transform *A. calcoaceticus* effectively (Kok *et al.*, 1999).

These properties constitute a unique combination not observed in other bacteria and indicate the possible existence of a specific mechanism that could be exploited to our benefit when dealing with large recombinant DNA constructs. It appears that

Acinetobacter is a prime candidate to allow rapid and economically feasible experimentation, which would be difficult and expensive to attempt using other organisms. This makes *Acinetobacter* ideally suited for detailed studies utilizing transformation.

Catabolic plasmids and the genus *Pseudomonas* – a model system

The genus *Pseudomonas* contains a group of organisms that is of particular interest to our laboratory. Pseudomonads are organisms that occur commonly in the soil, water, and other natural environments. The Pseudomonads are Gram-negative rods with polar flagella that are present singly and in tufts. They do not usually pose a threat as pathogens to healthy individuals, but may infect immunocompromised individuals as opportunistic pathogens. Pseudomonads are metabolically diverse, and are known for their ability to utilize a wide variety of compounds as carbon and energy sources. The genes used to produce enzymes for catabolism of these compounds may be located on the bacterial chromosome or on extra-chromosomal genetic elements such as transmissible plasmids (Williams & Worsey, 1976).

Catabolic plasmids carried by the Pseudomonads have been shown to encode enzymes that degrade many compounds such as pesticides, petroleum products, and a variety of other industrial and environmentally harmful substances to metabolically useful products (Haas, 1983). These plasmids are the center of many investigations and have proven to be industrially and biotechnologically relevant. *Pseudomonas putida*, which was first isolated from decaying material in 1886 by Flugge (Stanier *et al.*, 1966),

is a natural host of the TOL plasmids as well as many other plasmids encoding catabolic pathways.

TOL plasmids are usually between 50 kbp and 300 kbp in size and encode the genes which allow the catabolism of toluene, *meta*-toluate, and xylene. A TOL plasmid typically has four expression units (two encoding enzymes and two regulatory proteins) that encompass nearly 30 kbp of sequence and encode 22 proteins (reviewed by Hares, 1998). Some TOL plasmids can form cointegrates with the broad host range resistance plasmid RP1, which increases the conjugative nature of the TOL operons and provides significant antibiotic resistance (Shaw & Williams, 1988).

Pseudomonas putida is not naturally transformable, nor is it readily transformed by artificial means. Therefore, the TOL cointegrate plasmid pDKR1 in *P. putida* presents an ideal model target for genetically engineering an otherwise unmanageable construct by conjugation and transformation in *A. calcoaceticus*. Plasmid pDKR1 is 100 kbp, has the ability to be transferred by conjugation, and its RP1 cointegrate should allow for selection and stable maintenance of the plasmid in *Acinetobacter*. Utilizing the superior transformation properties of *Acinetobacter* for the manipulation and engineering of a large *P. putida* plasmid encoding a degradative pathway may provide a mechanism which would be generally applicable for the generation and manipulation of otherwise unmanageable large constructs.

Research objectives

The overall objective of this research is to test the feasibility for the genetic engineering of very large plasmid-encoded pathways utilizing transformation in *Acinetobacter* and define a molecular protocol. Although all types of DNA may be taken up by *A. calcoaceticus*, it is possible that *Acinetobacter* has the ability to distinguish exogenous DNA molecules and these may transform at a reduced rate. For example, a mechanism that requires a unique DNA sequence element for incorporation might exist. It is also possible that a significant difference exists for transformation efficiencies between chromosomal and plasmid targets and donor DNAs. In this case, super-coiling, methylation, associated proteins, or even guanine and cytosine mole percentage might be of significance. Characterization of large plasmid transformation in *Acinetobacter* will involve further defining the limits of transformation in general and for plasmids specifically. This will be accomplished by meeting the following objectives:

1. Reproduce previously published transformation experiments that utilize similar donor DNAs and verify natural competence of all new strains produced and utilized as part of this study.
2. Verify that a large, broad host range plasmid can be stably maintained in *A. calcoaceticus* using pDKR1 as a model system and show that pDKR1 is not inhibitory to the transformation process.
3. Create a range of additional plasmid constructs to use in the characterization of the plasmid transformation process.
4. Assess the success of manipulation.

5. Outline an improved transformation protocol for experimental use.

Fulfillment of these objectives should provide a better understanding of both the transformation process and how well it can be adapted for use as a general tool for genetic engineering of large plasmids.

CHAPTER II

MATERIALS AND METHODS

Bacterial strains and plasmids

Many of the bacterial strains utilized were included in our current laboratory collection, were created specifically to further this investigation, or acquired from outside sources. All *Acinetobacter calcoaceticus* strains are derived from strain BD413 (Juni & Janik, 1969; Juni, 1972), also known as ADP1. Strains of ADP125 were provided by Nicholas Ornston. Amino acid auxotrophs had been previously acquired from Elliot Juni (Ahmadian-Tehrani, 1990).

Media and growth conditions

Cultures of *A. calcoaceticus* were grown at 37 °C in minimal medium (Ornston & Stanier, 1966) supplemented with Hutner's Metals 44 (Cohen-Bazire *et al.*, 1957) and solidified with 1.6% Difco Bacto-Agar®. Either 10 mM succinate, 5 mM sodium benzoate, or 5 mM *para*-hydroxybenzoic acid (POB) was provided as the carbon and energy source. Cultures of *E. coli* were grown at 37 °C on LB broth containing 10 g Bacto Tryptone™, 5 g Yeast Extract (Difco™), and 10 g sodium chloride (NaCl), in one

Table 3. Strains and plasmids

Strain or plasmid	Relevant phenotype/genotype	Source or reference
<i>A. calcoaceticus</i>		
ADP1	Wild-type (BD413)	Juni & Janik, 1969
A1R1	ADP1 (BD413) with pDKR1	This study
ADP6	<i>PcaHG3006</i>	This laboratory
A6R1	ADP6 (<i>pcaHG3006</i>) with pDKR1	This study
A6P2	ADP6 (<i>pcaHG3006</i>) with pWS1100	This study
ADP125	<i>PcaIJ3125</i> (POB ⁻ {NTG ^d } of ADP1)	Doten <i>et al.</i> , 1987a
A125B1	ADP125 (<i>pcaIJ3125</i>) Δ <i>catM'BJ'FD30</i>	This study
A125B1R1	ADP125 (<i>pcaIJ3125</i>) Δ <i>catM'BJ'FD30</i> with pDKR1	This study
Ac141	<i>Arg</i>	Juni & Janik, 1969
A141R1	Ac141 with pDKR1	This study
A141R1P1	Ac141 with pDKR1 and pPW1000	This study
A141P1	Ac141 with pPW1000	This study
A141P2	Ac141 with pWS1100	This study
<i>P. putida</i>		
PRS2000		Kunz, DA
PaW630	Trp ⁻ Sm ^r	Kunz, DA
PR1 Δ 1	wild-type with Δ <i>xylL'T159</i> incorporated into pDKR1	This study
<i>E. coli</i>		
DH5 α	<i>supE44 hsdR17 recA1 gyrA96 thi-1 relA1</i>	Sambrook <i>et al.</i> , 1989
DH5 α F'	F' <i>supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Sambrook <i>et al.</i> , 1989
HB101	F Δ (<i>gpt-proA</i>)62 <i>leuB6 supE44 ara-14 galK2 lacY1</i> Δ (<i>mcrC-mrr</i>) <i>rpsL20</i> (Str ^r) <i>xyl-5 mtl-1 recA13</i>	Sambrook <i>et al.</i> , 1989

Table 3. (Cont.)

Strain or plasmid	Relevant phenotype/genotype	Source or reference
<i>E. coli</i> (Cont.)		
JM101	F' { <i>traD36 proAB⁺ lacI^q lacZΔM15</i> } <i>supE thi Δ(lac-proAB)</i>	Invitrogen®, 1998
JM109	F' { <i>traD36 proAB⁺ lacI^q lacZΔM15</i> } <i>endAI recAI hsdR17 (r_k⁻, m_k⁺) supE44 thi -1 gyrA96 relAI λ⁻ Δ(lac-proAB)</i>	Invitrogen®, 1998
Plasmids		
pUC18	<i>Ap lacp/o</i>	Yanisch-Perron <i>et al.</i> , 1985
pUC19	<i>Ap lacp/o</i>	Yanisch-Perron <i>et al.</i> , 1985
pUCP20	<i>Ap lacp/o</i>	West <i>et al.</i> , 1994
pUCP21	<i>Ap lacp/o</i>	West <i>et al.</i> , 1994
pUCP28T	<i>Tm lacp/o</i>	Schweizer <i>et al.</i> , 1996
pUCP30T	<i>Gm lacp/o</i>	Schweizer <i>et al.</i> , 1996
pZR1	<i>pcaIJFBDKCHGquiBCX'</i> on a 11.0-kbp <i>EcoRI</i> fragment in pUC18	Doten <i>et al.</i> , 1987b
pZR2	<i>pcaK'CHGquiB'</i> on 2.4-kbp <i>HindIII</i> fragment in pUC18	Doten <i>et al.</i> , 1987b
pAN2	<i>catM'BCIJFD</i> on 5-kbp <i>EcoRI</i> fragment in pBR322	Shanley <i>et al.</i> , 1986
pPAN30	<i>ΔcatM'BJ'FD30</i> on 3.6-kbp <i>EcoRI</i> fragment in pUC19	Doten, 1987a
pBK391	<i>xylLT</i> on 2.1-kbp <i>PvuII/SmaI</i> fragment in pUC19	This lab
pBK188	<i>xylXYLTE</i> on 5.0-kbp <i>EcoRI</i> fragment in pBR322	This lab
pBK692mut3	<i>xylLTE</i> on 3.9-kbp <i>PvuII/ EcoRI</i> fragment in pUC19	This lab
pBK692mut5	<i>xylLTE</i> on 3.9-kbp <i>PvuII/ EcoRI</i> fragment in pUC19	This lab
pPW596	<i>pvuII/ SmaI</i> fragment in pUC19	This study
pPW1000	<i>ΔxylL'T159</i> on 1.9-kbp on a <i>EcoRI/HindIII</i> fragment in pUCP30T	This study
pRAY	6.1-kbp, <i>Gm^r</i>	Segal & Elisha, 1997

Table 3. (Cont.)

Strain or plasmid	Relevant phenotype/genotype	Source or reference
Plasmids (Cont.)		
pHS500	Linearized pRAY on 6.1-kbp <i>Bam</i> HI fragment in pUC19	Segal & Elisha, 1999
pWS1001	Linearized pUCP30T inserted into <i>Bam</i> HI site of pRAY	This study
pWS1100	<i>Bam</i> HI linearized pUCP30T with $\Delta xyLL'T159$ on a 1.9-kbp <i>Eco</i> RI/ <i>Pst</i> I fragment in pRAY	This study
pPW800	$\Delta xyLL'T159$ on 1.9-kbp <i>Pvu</i> II/ <i>Eco</i> RI fragment in pUCP21	This study
pBK801	<i>xyLTE</i> on 3.9-kbp <i>Pvu</i> II/ <i>Sma</i> I fragment in pUCP30T	This study
pPW703 pDKR1	$\Delta pyrBC'149$ on a 3.6-kbp <i>Pst</i> I fragment in pUCP21 RP1::TOL construct with the upper and <i>meta</i> -cleavage operons, Sm ¹⁰⁰⁰ Km ¹⁰⁰ Tc ⁵⁰ Ap ¹⁰⁰⁰	This study Kunz, DA
pMJS-29 pMJS291 pRK2013	<i>pyrBC'</i> on a 3.8-kbp <i>Pst</i> I fragment in pUC18 $\Delta pyrBC'149$ on a 3.6-kbp <i>Pst</i> I fragment in pUC18 Km ^r , Ap ^r , 6.3-kbp <i>ColE1</i> fragment, RK2 replication origin, self-transmissible	Schurr, 1995 This study Figurski & Helinski, 1979

liter volume or on LB agar containing 1.5% BBL® Granulated Agar. The pH was adjusted to 7.5 before sterilization. Cultures of *Pseudomonas* were grown on LB broth, LB agar, or *Pseudomonas* minimal broth (Kunz & Chapman, 1981) or minimal agar with 1.6% Difco Bacto-Agar® at 30 °C. Supplements were added as needed at the following concentrations: ampicillin (Ap), 50 µg/ml; gentamicin (Gm) 10 µg/ml; kanamycin (Km) 100 µg/ml; tetracycline (Tc) 50 µg/ml; X-Gal, 0.006%; isopropyl-β-D-thio-galactopyranoside (IPTG), 1 mM; tryptophan (Trp), 50 µg/ml; *meta*-toluate (*m*-tol), 5 mM. All *A. calcoaceticus* and *E. coli* liquid cultures were incubated at 37 °C in a New Brunswick™ Series 25 Incubator/Shaker at 250 rpm. *Pseudomonas* liquid cultures were incubated at 30 °C on a New Brunswick™ G-10 Gyrotory shaker in a Lab-Line™ Environmentally Controlled Room at 250 rpm.

Long-term storage of cultures

Bacterial strains were stored for extended periods of time at or below -80 °C. All bacterial strains were grown in 5 ml liquid cultures of the appropriate selectable medium. The cultures were incubated overnight at the appropriate temperature at 250 rpm. Five hundred microliters of an overnight culture were added to a sterile microcentrifuge tube containing 500 µl of sterile 80% glycerol. The tube was stored at -80 °C after vortexing the contents.

Bacterial strains were maintained on minimal medium plates containing the appropriate carbon source and amino acid or antibiotic for daily use.

Enzymes and chemicals

All enzymes and chemicals were obtained commercially at the highest purity available.

Gram stains (Benson, 1990)

Gram stains were performed to identify each strain's ability to retain the dye crystal violet. A small amount of bacterial culture grown for 16 to 18 hours was smeared onto a glass microscope slide with an inoculation loop. The smear was then covered with crystal violet and allowed to stand for 20 seconds. After 20 seconds, the stain was briefly washed off using distilled water. The smear was then covered again with Gram's iodine solution and allowed to stand for 1 minute. The Gram's iodine was poured off and the slide was flooded with 95% ethyl alcohol until the solvent flowed colorlessly from the slide. The action of the alcohol was stopped by briefly rinsing the slide again with distilled water. The safranin counter stain was then applied and allowed to stand for another 20 seconds. After a final rinse with distilled water, the slide was blotted dry with bibulous paper and allowed to air dry. Examination of the slide was performed under oil immersion at a magnification of 1000X.

Oxidase test (Benson, 1990)

The production of oxidase is a very significant test for the differentiating certain groups of bacteria. Enterobacteria are oxidase-negative and most species of *Pseudomonas* are oxidase-positive. A small sample of overnight bacterial culture grown

on a solid medium plate was removed with an inoculation loop and smeared onto a piece of Whatman No.2 filter paper. The filter paper was then soaked with oxidase test reagent containing a one percent solution of tetramethyl-*p*-phenylenediamine hydrochloride. An oxidase-positive reaction is indicated by a change from an initial pinkish color to dark red and finally black. If there is no noticeable color change, the organism contained is deemed oxidase-negative.

Selective plating

All strains used were tested to demonstrate the appropriate phenotype in response to chemically restrictive media. Appropriate phenotypic responses were demonstrated by the presence or absence of bacterial growth on media containing a sole carbon source, an antibiotic, or a specific amino acid. Supplements were added to the media, as needed, at the following concentrations: Ap, 50 µg/ml; Kn, 100 µg/ml; Gm, 10 µg/ml; Tc, 50 µg/ml; Trp, 50 µg/ml; 5 mM *m*-tol; 10 mM succinate; 5 mM Ben; and 5 mM POB.

Complete digestion of DNA with restriction endonucleases

Restriction endonuclease digestions were performed individually for each sample. All restriction reaction mixtures contained the appropriate amounts of New England Biolabs 10X reaction buffer necessary to give a final concentration of 1X in the solution, the appropriate restriction enzyme, deionized distilled water, and the DNA which was to be acted upon by the restriction enzyme. Digestions were carried out in a water bath at the appropriate temperature for the required period of time. Digested DNA was analyzed

by agarose gel electrophoresis, stained with ethidium bromide, and viewed under ultraviolet illumination.

Partial DNA digestion by restriction endonucleases

It was necessary to establishing the conditions by which partial digestion of donor DNA could be accomplished. In order to determine this, a reaction mixture containing 20 μg of DNA and a 1X concentration of the appropriate restriction enzyme buffer was prepared. The final volume of the reaction mixture was 150 μl . Nine microcentrifuge tubes were labeled and 30 μl of the reaction mixture was dispensed into tube 1, and 15 μl each into tubes 2 through 9. Twenty units of a specified restriction enzyme was added to tube number one and the contents were mixed thoroughly. After mixing, 15 μl of tube number one was added to tube number 2 and mixed thoroughly. The two-fold serial dilution was continued through tube number 8. Tube number 9 was used as an undigested control. Using a two-fold serial dilution creates a range of concentrations that vary from 2.5 U/ μg of DNA in tube number 1 to 0.002 U/ μg in tube number 8.

All 9 tubes were incubated at the temperature specified by the manufacturer for the enzyme for a period of 60 minutes. Reactions were terminated by adding 3 μl of 0.1 M EDTA pH 7.0 per 15 μl of reaction volume and enough 5X agarose loading buffer to bring the final concentration to 1X.

Analysis of the partial digestion was performed by gel electrophoresis through a 0.7% TAE low melting temperature agarose gel at 30 volts for 20 hours. Lambda DNA completely digested with *Hind*III and pBR322 completely digested with *Hinf*I were used

as size markers. The gel was stained for 15 minutes in a 50 µg/ml solution of EtBr to visualize the digested DNA. The appropriate size DNA bands were removed from the gels with a razor blade while being illuminated with UV radiation on a Fotodyne Model 3-3300 UV illuminator. Recovery of partially digested duplex DNA was performed as stated below.

Recovery of duplex DNA from low-melting temperature agarose gel

The recovery of DNA from agarose gelatin was performed using an Epicenter Technologies Gelase® DNA recovery kit. Individual agarose pieces containing partially digested duplex DNA were weighed and placed in a microcentrifuge tube. The weights were recorded and assumed to be equivalent to milliliters of gel solution. Enough 50X Gelase® buffer was then added to the tube to make a 1X final concentration. The tubes were placed at 75 °C to dissolve the low-melting temperature agarose and cooled to 45 °C in a water bath for 1 hour. At this point, 1.0 µl of Gelase® enzyme at a concentration of 50 U/µl was added to each tube. The solution was vortexed, the contents collected by brief centrifugation, and incubated for an additional hour in a 45 °C water bath. The total volume was then increased to 100 µl with ddH₂O. Finally, a standard DNA precipitation protocol was used to recover the DNA. This protocol consists of two phenol extractions to remove any enzymes or leftover agarose, followed by an ether extraction. The DNA is precipitated by adding a 1/10 volume of 3.0 M NaAcetate solution and 3 volumes of ice-cold 100% EtOH. The precipitated DNA is washed with a solution of 70% EtOH to

remove any residual salts before it is vacuum desiccated to remove the EtOH and resuspended in either sterile ddH₂O or 1X TE buffer for later use.

Quantitative and qualitative identification of DNA

DNA absorbs UV radiation maximally in the range of 257 nm to 260 nm. This allows DNA samples to be characterized and quantified by spectroscopy. An absorbance of 1.0 at a wavelength of 257 nm is equivalent to a concentration of 50 µg/ml of duplex DNA. Samples were diluted at a ratio of 1:50 or 1:100 and the absorbance scanned from 320 nm to 220 nm using a Beckman DU-50 spectrophotometer. The maximum absorbance at 257 nm to 260 nm can then be used to produce an approximation of the solution's DNA concentration. The following is an example of a typical calculation.

0.5 absorbance units @ 257 nm X dilution factor of 50 = 25 absorbance units total

25 absorbance units X $\frac{50 \mu\text{g/ml}}{1 \text{ absorbance unit}}$ = 1250 µg duplex DNA/ml

1250 µg / 1000 µg/mg = 1.25 mg

1250 µg/ml = 1.25 mg/ml or 1.25 µg/µl

A second method for quantification of DNA yield and purity involves gel electrophoresis, ethidium bromide staining, and control DNAs of known quantities. The presence of ethidium bromide (EtBr) allows DNA to be visualized by ultraviolet illumination. When exposed to UV radiation the EtBr emits an orange visible

florescence. DNA samples contained in a gel matrix were soaked in a solution of EtBr at a concentration of 50 µg/ml for 15 minutes. Markers of known DNA size(s) and concentration(s) were also present in the matrix. Comparison of the experimental samples to the provided markers allows an estimation of size and concentration to be made. Visualization of the DNA sample also allows a reference quantification to be made with the results of spectrophotometric analysis. It is possible that degraded DNA and RNA may be present in the sample. Both of these contaminants would be included in the spectrophotometric analysis and would be seen with EtBr and easy to differentiate from the duplex plasmid or high molecular weight chromosomal DNA.

Horizontal agarose gel electrophoresis

One method used to analyze many different DNA samples was horizontal agarose gel electrophoresis. To prepare an agarose gel, the TBE running buffer is made first. This buffer is made by adding 10.8 g of Tris base, 5.5 g of boric acid, and 10 ml of 0.25 M EDTA, pH 8.0 to a final volume of 1 liter. The quantity of buffer made is dictated by the number and size of agarose gels required to analyze the DNA and the number of gel chamber that must be filled to electrophoresis the gels.

Agarose gels can vary in the content of agarose, with the variance depending upon the size range of the DNA being analyzed. The analysis of smaller fragments requires a higher percentage of agarose to separate the DNA bands efficiently than the analysis of large DNA fragments. Percentages of agarose used in these experiments ranged from

0.5% to 2.0%. Agarose gel solutions was made in Erlenmeyer flask of an appropriate size for the desired volume. The contents for a typical 1% agarose gel are as follows:

1.0 g	electrophoretic grade agarose
100.0 ml	1X TBE buffer

The flask was weighed before the contents were heated in a microwave until the solution boiled. After the solution has boiled long enough to completely dissolve the agarose the solution was again weighed. Distilled deionized water was added to the solution to replace water that has evaporated during the heating process. Enough water was added to return the flask to its original weight. This was done to maintain the percentage of agarose in the agarose gel solution. After a brief cooling period, the gel solution was transferred to a gel form that contains a well-forming comb. Solidification occurred within approximately 30 to 45 minutes. After the gel had solidified, the comb was removed and the gel was placed in an electrophoresis buffer chamber. The chamber contained enough 1X TBE agarose electrophoresis buffer to cover the agarose gel by one or two mm. Wells in the gel were cleaned by using a Pasteur™ pipette to flush them with 1X TBE running buffer from the electrophoresis chamber. The gel is now ready to load the DNA sample.

DNA samples that were to be analyzed by agarose gel electrophoresis were mixed with an agarose gel loading buffer. The buffer was made at a 5X concentration so that it could be diluted in the sample to a final concentration of 1X. The buffer contained

25.0% glycerol, 0.5% SDS, 1% bromophenol blue, 0.1% xylene cyanol, and 50.0 mM EDTA. Samples were loaded onto the agarose gel by a micropipettor. After loading, the gel was electrophoresed at 80 to 100 V for a specified period of time. Voltage and running time were varied as necessary for the analysis of given DNA samples. For higher resolution agarose gel analysis a longer running time at a lower voltage was used. Longer running times were also necessary for gels containing a higher percentage of agarose. The DNA was visualized by soaking the gel in a solution of 50 µg EtBr per ml, briefly destaining in ddH₂O, followed by exposure to UV radiation on a UV transilluminator. Destaining reduced the amount of background fluorescence seen under UV radiation produced by residual EtBr. The gels were photographed with a Kodak MP-4™ transilluminator system or digitally stored with a Kodak Digital Science® 1D LE computerized imaging system.

Calf intestinal phosphatase treatment of vector DNA

Cloning vectors were treated with calf intestinal phosphatase (CIP) after being subjected to restriction endonuclease digestion in order to remove the 5' phosphate group from the DNA. This prevents the vector from closing upon itself and significantly reduces the amount of background that does not contain an insert. Background of this nature can be quite high in a ligation and subsequent transformation experiment that uses an untreated vector. A typical enzymatic treatment was performed by placing the digested vector DNA into a sterile 0.5 or 1.5 ml microfuge tube and adding the following:

5.0 - 50.0 μl	linear DNA (typically at a concentration of 100 ng/ μl)
10.0 μl	10X NEB Buffer #3
0.5 - 5.0 μl	calf intestinal phosphatase (0.5 U/ μg vector DNA)
<u>35.0 - 84.5 μl</u>	ddH ₂ O
100 μl	total volume

The reaction mixture was placed in a water bath at 37 °C for 1 hour. The enzyme and salts were removed from the sample by two phenol extractions, followed by an ethanol precipitation. The prepared vector was then resuspended at a final concentration of either 50 ng/ μl or 100 ng/ μl and stored at -20 °C until use.

DNA ligation

Recombinant DNA studies require that the desired genes be inserted into an appropriate cloning vector for replication *in vivo*, expression of the sequences coding proteins, and conjugation between organisms. This is achieved by ligation. The digested vector and target DNAs must be free from all traces of active restriction enzymes and these enzymes were routinely removed by phenol extraction. An extraction with diethyl ether and subsequent ethanol precipitation removed traces of the organic solvents, which also inhibit ligation. The following components were added to a sterile microfuge tube: A ratio of three moles of vector for every mole of target DNA, 1 μl of T4 DNA ligase at 1 U/ μl , 4 μl of 5X ligation buffer, and sterile deionized distilled water up to a final volume of 20 μl . Incubations were carried out at room temperature (23-26 °C) for 4 to 16

hours, depending upon the type end (either sticky or blunt) left by the digestion. The mixture was then diluted with an equal volume of TE buffer in preparation for subsequent transformation procedures. The ligation mix was then transferred to 4 °C for short periods of time or to -20 °C for long term storage.

Screening of recombinant plasmids

The transformants from the *E.coli* transformation experiments were screened on the basis of color formation and antibiotic resistance. *E.coli* colonies formed as a result of overnight growth are usually opaque and mucoid, and on LB agar they exhibit a white to off-white color. However, if an *E. coli* cell contains a functional β -galactosidase gene, and the medium contains the substrate X-Gal, the cell will produce a blue precipitate. The functional β -galactosidase protein product is produced by complementation of the α -subunit encoded by the vector plasmid and the β -subunit encoded by the bacterial chromosome. If the plasmid encoded α -subunit of the β -galactosidase gene is interrupted by target DNA, the α -subunit will no longer be made and the colony will not form a blue precipitate.

All colonies that were not blue in color were verified by replica plating them onto a master plate. The master plate was made by adhering an LB agar plate that contains Ap¹⁰⁰, 10.0 μ l of 100 mM IPTG, and 50.0 μ l of 2% X-Gal to a screening grid (Fig. 5). Individual colonies were then transferred to the plate with sterile toothpicks. This was done by carefully touching the colony and streaking it over a specific number on the grid. The plate was placed in an incubator at 37 °C overnight to grow. Any colonies that

produced a blue precipitate after the second screen were no longer analyzed as potential recombinants. The master plate also served as a method for the maintenance of a library of different recombinant clones. Colonies that were not blue were screened further by rapid plasmid isolation followed by restriction digestion to verify the presence and size of the insert.

Rapid analytical scale isolation of plasmid DNA by alkaline lysis (Birnboim & Doly, 1979)

Tubes containing liquid rich medium and appropriate selection were inoculated from an individual bacterial colony and grown overnight under the appropriate conditions. Cells were collected in a 1.5 ml microfuge tube by centrifugation at 10,000 x g for one minute. After decanting the supernatant, the cell collection process was repeated as many times as necessary in the same tube to collect all cells. The final bacterial pellet was left as dry as possible. The pellet was resuspended by vortexing in 100 µl of Solution A containing 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0), and lysozyme at 6.0 mg/ml. It is important that this solution be made fresh shortly before the experiment and kept on ice until used. The solution was stored at room temperature for 5 minutes before adding 200 µl of freshly prepared Solution B. Solution B contained 0.2 N sodium hydroxide (NaOH), and 1.0% sodium dodecyl sulfate (SDS). After the addition of Solution B, the tube was closed and its contents mixed by inverting the tube rapidly two or three times. The mixture was stored on ice for a period

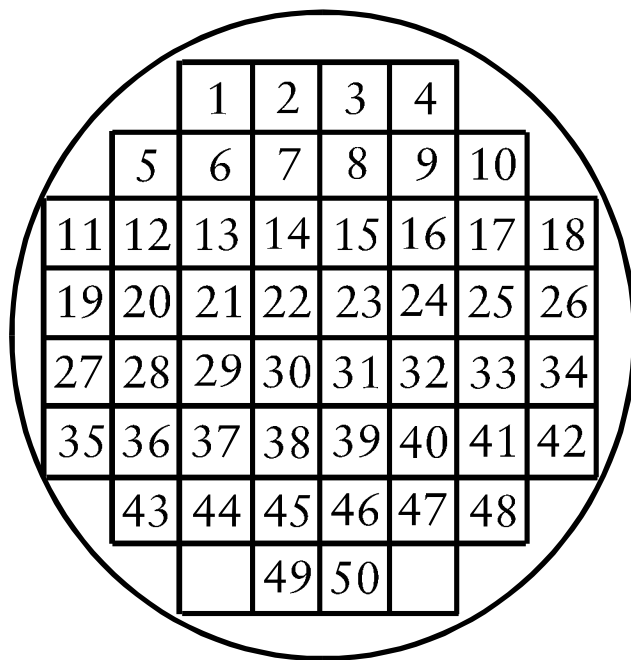


Fig. 5 Screening grid used for creation of master plates. The master plates served as tools for the phenotypic analysis of recombinant plasmids and strains and a method for the maintenance of libraries of different recombinant clones.

of 5 minutes. Next, 150 μ l of an ice-cold 5 M potassium acetate (\sim pH 4.8) was added and the contents were mixed by inverting the tube rapidly two or three times. The mixed contents were stored on ice for another 5 minutes. Centrifugation at 10,000 x g for 5 minutes at 4 °C in a microcentrifuge removed cellular debris and the supernatant was transferred to a fresh 1.5 ml microfuge tube. An equal volume of phenol:chloroform was added to the supernatant and the entire solution was mixed by vortexing. The aqueous and organic phases were separated by centrifugation for 2 minutes at 10,000 x g. In order to remove as much of the phenol as possible, the aqueous phase was transferred to another 1.5 ml microfuge tube. The phenol extraction removes much of the contaminating proteins from the DNA. Two volumes of ice-cold 100% ethanol were then added to the tube and the contents were mixed by vortexing. The mixture was then centrifuged in a 4 °C microcentrifuge at 10,000 x g for 10 minutes. The supernatant was subsequently removed with a drawn out Pasteur™ pipette. After the supernatant had been removed, an additional 1.0 ml of 70% ethanol was added to the tube. The tube was inverted briefly and centrifuged again at 10,000 x g for 5 minutes at 4 °C. The supernatant was removed using a drawn out pipette and all residual ethanol was then removed by drying the pellet briefly in a vacuum desiccator for 5 minutes. The dried pellet was then resuspended in TE buffer with heat-treated RNase A at 25 μ g/ml. Heating the resuspended pellet at 65°C for 8 minutes destroyed any contaminating RNA. The final plasmid preparation was then analyzed by quantitative ultraviolet spectrophotometry and agarose gel electrophoresis. It is possible that degraded DNA and RNA may be present in the sample. Both of these contaminants would be included in the

spectrophotometric analysis and would be seen with EtBr, easily differentiable from the duplex plasmid.

Preparative scale recombinant plasmid isolation

The method of Tanaka & Weisblum (1975) was used for large scale isolation of recombinant plasmids. A 5.0 ml tube of LB broth, containing the appropriate antibiotic to select for the recombinant plasmid, was inoculated from a single bacterial colony and grown overnight at the appropriate temperature. The overnight culture was used to inoculate a flask containing 50 ml LB broth. One liter of LB broth was then inoculated from the 50 ml overnight flask and once again grown overnight. When appropriate, liquid cultures contained ampicillin at a final concentration of 50 µg/ml and were incubated overnight in a 37 °C New Brunswick™ Series 25 incubator/shaker at 250 rpm. When using pUC-related vectors it is not necessary to add chloramphenicol due to the high copy number of the plasmid. The overnight culture was then collected in a pre-cooled Sorvall™ GS3 rotor by centrifugation at 6000 x g, 4 °C, for 6 minutes. The pelleted cells were then resuspended in 20 ml of cold 0.15 M sodium chloride per liter of overnight culture in Oak Ridge™ style centrifuge tubes and collected once again in a pre-cooled Sorvall™ SA600 rotor by centrifugation for 5 minutes at 6000 x g in a 4 °C centrifuge. All further steps in the preparative scale isolation procedure were performed in a cold room at 4 °C in order to reduce nuclease activities and obtain a maximum plasmid yield. The pellet was redissolved in 10 ml of cold 25% sucrose, 50 mM Tris-HCl, pH 8.0 and then vortexed until a uniform suspension was obtained. To this

suspension the following solutions were added at 5 minute intervals: 2.0 ml lysozyme (5 mg/ml), 4.0 ml 0.25 M Na₂EDTA (pH 8.0), and 5.0 ml 5 M NaCl with 2.0 ml of 10% SDS. After each addition, the solution was capped, mixed by inversion, and kept on ice for the remainder of the 5 minutes. Care was taken to avoid any violent shaking motions which might produce unnecessary amounts of foam and DNA shearing. After the final addition, the solution was left on ice in the cold room for a period of 1 1/2 hours, after which the tube was centrifuged in a 4 °C SA600™ rotor for 45 minutes at 16,350 x g. The tube was removed after the centrifugation and the supernatant was transferred to a graduated cylinder. An equal volume of cold isopropyl alcohol was added and the contents were mixed and transferred to a 250 ml centrifuge bottle and frozen solid in a -80 °C dry ice/ethanol bath. The frozen sample was thawed in a room temperature (23 °C) water bath and centrifuged in a 4 °C GSA™ rotor for 20 minutes at 8000 x g. The pellet was transferred to a 25 ml Erlenmeyer flask and resuspended in 8 ml of cold 10 mM Tris, 1 mM EDTA (pH 8.0) by using a stir bar and stirring on a magnetic stir plate while in the 4 °C cold room. DNase-free RNase was added to a final concentration of 20 µg/ml to destroy any remaining RNA. The solution was then centrifuged in a 4 °C SA600 rotor at 10,000 x g for 10 minutes to remove any denatured protein and other cellular debris and the pelleted material was discarded. To the supernatant, 1.04 g of cesium chloride (CsCl) per 1 ml of supernatant was added and allowed to completely dissolved. The solution was then dispensed into two 10.5 ml Sorvall Ultracrimp™ ultracentrifuge tubes leaving sufficient space to add ethidium bromide to a final concentration of 0.4 mg/ml. The tubes were then centrifuged at 118,700 x g for 40 hours

in a Sorvall™ T1270 rotor. After ultracentrifugation, the lower band (Fig. 6), as visualized by long-wave ultraviolet radiation, was removed using a twenty-gauge hypodermic needle and a 3 cc syringe. The EtBr-CsCl-plasmid solution was placed into a sterile 15 ml polypropylene conical tube and the ethidium bromide was extracted with TE-saturated isobutanol. The plasmid-CsCl solution was measured and 2 volumes of distilled water plus 9 volumes of ice-cold 100% ethanol were added and mixed well. This was transferred to a siliconized 30 ml Corex™ tube and centrifuged at 4 °C in a SA600™ rotor for 15 minutes at 8000 x g. The supernatant was then removed and the pellet was redissolved in 300 µl of 0.3 M sodium acetate. To this solution, 1 ml of cold 100% ethanol was added and the 1.5 ml microfuge tube was placed in a dry ice-ethanol bath for 2 minutes. After cooling the tube was centrifuged at 10,000 x g for 10 minutes at 4 °C. The supernatant was then removed with a pulled-out Pasteur™ pipette and the remaining pellet was washed with 1.0 ml of ice-cold 70% ethanol. The final supernatant was again removed with a pulled-out Pasteur™ pipette after centrifugation at 4 °C and 10,000 x g. Any residual ethanol was removed from the remaining pellet by centrifugation in a vacuum concentrator for 2 minutes. Finally, the pellet was redissolved in 500 µl of ddH₂O. Verification of plasmid isolation was obtained by electrophoresing a 2 µl sample on a 1% TBE agarose mini-gel and visualization was accomplished by staining with ethidium bromide. A final calculation of plasmid DNA concentration was made by spectrophotometric scanning of a diluted sample from 220 nm to 320 nm using a Beckman™ DU-50 UV-visible spectrophotometer.

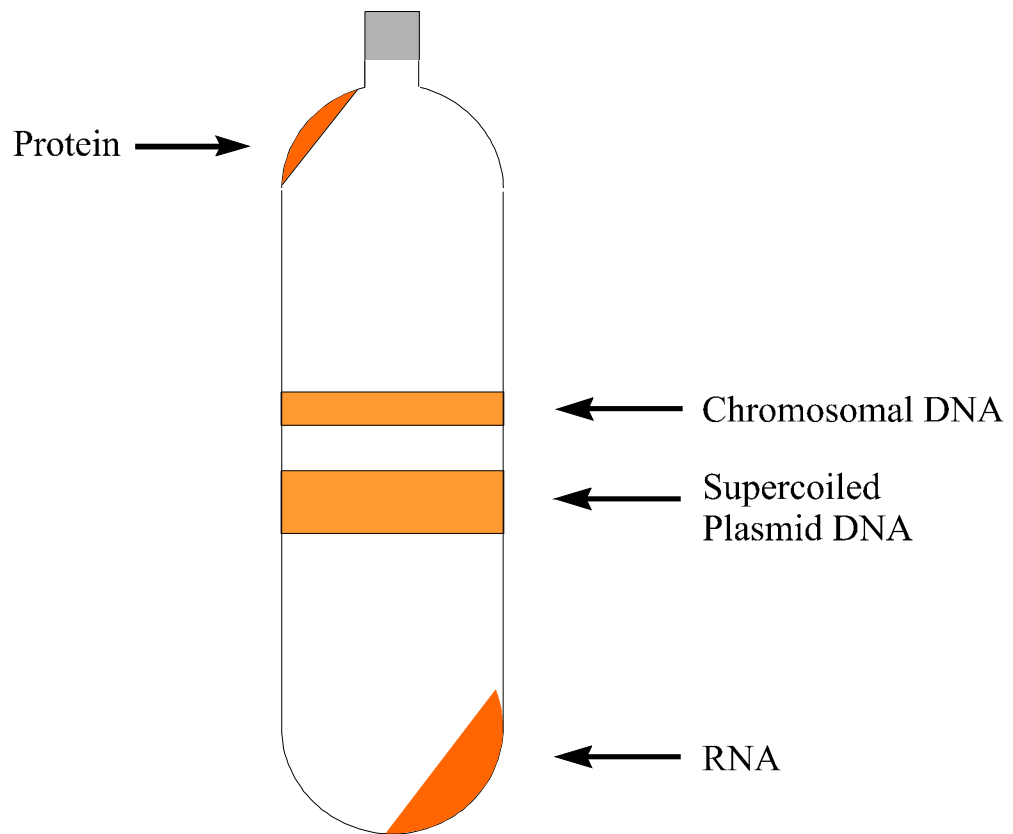


Fig. 6 Diagram of a CsCl gradient T1270 ultracentrifuge tube containing DNA bands after 40 hours of centrifugation at 118,700 x g. The upper band contains chromosomal DNA from bacterial cells. The lower band corresponds to the supercoiled plasmid of interest.

It is possible that RNA and degraded DNA may be present in the sample. Both of these contaminants would be included in the spectrophotometric analysis and would be seen with EtBr, and is easy to differentiate from the duplex plasmid or high molecular weight chromosomal DNA.

Rapid total DNA isolation (Adapted from Berns & Thomas, 1965)

The rapid genomic extraction was begun by pelleting 5 ml of a liquid overnight bacterial culture grown at 37 °C. Once the cells had been collected in a 1.5 ml microcentrifuge tube, they were washed by resuspending the pellet in 1X phosphate buffered saline (PBS) solution and collected again by centrifugation. The centrifugation in the first two steps is 10,000 x g for 2 minutes. Cell pellets were resuspended in 250 µl of 0.1 M NaCl, 10 mM Tris (pH 8.0), 10 mM EDTA. When preparing DNA from a large number of strains at one time, the pellets may be stored in a -20 °C freezer while awaiting further processing. A 10 µl volume of 10% SDS was added to the cell suspension and the microcentrifuge tube was heated at 65 °C in a water bath for 10 minutes. Following the SDS incubation, 25 µl of Proteinase K was added at a concentration of 5 mg/ml and the tube was heated a second time in a 37°C water bath for 1 hour. Incubation was followed by two extractions with an equal volume of phenol, and a single extraction by an equal volume of TE-saturated butanol. After the extractions, 100 µl of 7.5 M ammonium acetate was added and the solution was centrifuged for 15 minutes in a 4 °C Savant™ high speed centrifuge at 10,000 x g. The supernatant was added to a new sterile 1.5 ml microcentrifuge tube along with 750 µl of ice-cold 100%

ethanol. A large DNA precipitate was observed at this point. In order to fully precipitate all DNA, the tube is frozen for 30 minutes in a -80°C freezer for 30 minutes or placed in a dry ice-ethanol bath for 5 to 10 minutes. The DNA was pelleted by centrifugation in a 4°C Savant™ centrifuge at $10,000 \times g$ for 10 minutes. Ethanol was removed with a drawn out Pasteur™ pipette followed by 5 minutes in a Savant Speed Vac™ vacuum concentrator. The final DNA pellet is routinely resuspended in $250 \mu\text{l}$ of TE containing heat-treated RNase A at a concentration of $25 \mu\text{g/ml}$. Any RNA remaining in the sample was then degraded during heating the tube for 8 minutes in a 65°C water bath. This procedure was followed by spectrophotometric analysis using a Beckman DU®-50 series spectrophotometer to determine DNA concentration and purity. Any contaminants of RNA and degraded DNA present in the sample would be included in the spectrophotometric analysis and could be seen and differentiated from the plasmid DNA by horizontal gel electrophoresis and subsequent EtBr staining.

Southern transfer of DNA to nylon membranes (Adapted from Southern, 1975)

DNA can be faithfully transferred from an agarose gel to a nylon membrane that can be easily handled and probed by hybridization with radioactive probes. The desired DNA fragments, which have been digested with appropriate enzymes, were electrophoresed through a $11.5 \text{ cm} \times 21.0 \text{ cm}$ 0.7% TBE agarose gel at 32V for 18 to 20 hours. This gel was placed in a plastic tray containing 0.4 M NaOH , and gently shaken for 30 minutes. The NaOH acts to denature the DNA in the gel from its double-stranded form to single-stranded DNA. While the gel was shaking, filter paper and paper towels

were cut to gel size. To further prepare the membrane for transfer, it was soaked in 0.5 M NaOH, 0.5 M NaCl for 15 minutes. A sponge and three pieces of filter paper were also wet in the same solution. The denatured gel (well side down) was placed on top of the pre-soaked sponge and filter paper in a shallow plastic tray filled with 0.5 M NaOH, 0.5 M NaCl up to the level of the sponge, but not to the level of the gel. A large (10 or 25 ml) pipette was used to remove (by rolling) any air bubbles from the stack of components between each layer addition. The pre-soaked membrane was carefully placed onto the gel and all air bubbles were removed with the pipette. Another piece of pre-soaked filter paper was placed on top of the membrane and 2 to 3 inches of gel-sized dry paper towels were added to the stack. The entire stack was covered by a piece of 3/8" thick acrylic to apply evenly distributed pressure. Transfer of the DNA was allowed to proceed at room temperature for 3 hours with the damp paper towels replaced by new dry ones every hour. Sufficient 0.5 M NaOH, 0.5 M NaCl to keep the solution in contact with the sponge was maintained in the tray. The paper towels and filter paper were carefully removed after the transfer was complete and the membrane was washed once with 0.2 M Tris-HCl (pH 7.5), 2X SSC for 15 minutes with gentle shaking in a plastic tray. After the final wash, the membrane was allowed to dry, and the DNA was cross-linked to the membrane by exposing it to short wavelength ultraviolet radiation for 3 minutes at a distance of 10 centimeters.

Generation of ^{32}P -labeled DNA probes by polymerase chain reaction (Adapted from Mertz & Rashtchian, 1994)

Mertz & Rashtchian (1994) demonstrated that high specific activity α - ^{32}P - labeled probes can be generated by polymerase chain reaction. A region of the *xyLL* gene was amplified and used as a probe for restriction endonuclease digested DNA which had be transferred to a nylon membrane using the method of Southern (1975). CsCl-purified pBK391 was used as template for the *xyLLT* right/left primer set. The PCR reaction mixture consisted of:

8.0 μl	pBK391 CsCl-purified DNA at a concentration of 2.15 ng/ μl
5.0 μl	10X Fisher Scientific Assay Buffer A
1.3 μl	0.25 mM dGTP
1.3 μl	0.25 mM dCTP
1.3 μl	0.25 mM dTTP
10.0 μl	6000 Ci/mmol (α - ^{32}P) dATP, 10.0 mCi/ml initial specific activity
4.0 μl	primer <i>xyLLT</i> right
4.0 μl	primer <i>xyLLT</i> left
14.8 μl	ddH ₂ O
<u>0.2 μl</u>	Fisher Scientific Taq Polymerase at a concentration of 5u/ μl
50.0 μl	total volume

The mixture was assembled in a thin walled 0.6 ml microcentrifuge tube designed specifically for PCR. The PCR mixture was centrifuged briefly to collect the contents at the bottom of the tube and covered with 40 μ l of sterile mineral oil to prevent volatilization of the fluid during the thermal cycling process. The thermal cycler amplification profile was set for 1 cycle of 4 minutes at 94 °C for denaturation, to be followed by 35 cycles of 1 minute at 94 °C for denaturation, 1 minute at 65 °C for primer binding, 2 minutes of polymerase extension at 72 °C, and a final cycle of 4 minutes at 72 °C to extend any unfinished DNA segments. When the reaction procedure was completed, the thermal cycler held the samples at 4 °C until they were removed from the machine.

It was necessary to remove any residual free nucleotides, specifically radiolabeled ones, to prevent random binding during the hybridization protocol and the creation of excessive background in the subsequent autoradiograms. This was performed by using a Sephadex™ G-50 exclusion chromatography column. To begin assembly of the packed column, Sephadex™ G-50 was equilibrated in 1X TE buffer overnight. A 1.0 ml sterile syringe was plugged with fibers of siliconized glass wool to the 0.1 cc indicator. The plugged syringe was then filled with Sephadex™/TE suspension and placed in a 15 ml conical tube. The syringe/conical assembly was centrifuged at 1600 x g in a Sorvall™ T6000B for a period of 4 minutes. Liquid collected in the bottom of the conical was removed and the procedure repeated until the column was packed to a volume of 1.0 ml. A 50.0 μ l volume of 1X TE is applied to the packed column and it was then centrifuged

at 1600 x g for a period of 4 minutes, after which the column was ready for application of the sample. The PCR-generated DNA sample, free of any unincorporated nucleotides, was applied to the column and centrifuged as before. The purified sample is collected by placing a 1.5 ml microcentrifuge (tube with the lid removed) at the bottom of the conical tube prior to centrifugation.

Recovered radioactivity was determined by transferring a 2.0 μ l of the purified sample to a Beckman® LS 7000 Liquid Scintillation System and counting with an open window. An amount containing 1.0×10^6 to 1.0×10^7 cpm was routinely used in the hybridization protocol.

Hybridization of membranes with radiolabeled DNA probes

Hybridization was achieved through the use of a Techne Hybridiser HB-1D®. The nylon membrane and 30 ml of hybridization solution containing 80.0 ml 50% polyethylene glycol, 30.0 ml 20X SSPE (3.0 M NaCl, 0.2 M NaH_2PO_4 , 0.02 M EDTA), and 276.0 ml of 10% SDS were pre-hybridized at 65 °C for 30 minutes. This allows the hybridization solution to warm to the desired temperature and prevents random binding of the probe to the membrane. While the membrane and solution were warming, the radiolabeled probe was denatured by heating to 100 °C for 5 minutes. The probe was placed on ice until the pre-hybridization was complete and then injected into the hybridization tube using a micropipettor. The sealed hybridization tube was incubated at a temperature of 63 °C for 16 hours. The membrane was carefully removed from the tube and the radioactive solution was disposed of properly. This was followed by three

washes of the membrane to remove unbound or non-specifically bound probe. Two washes consisted of 15 minutes in 2X SSC, 0.1% SDS at room temperature, and a third high stringency wash for 30 minutes with 0.1X SSC, 0.1% SDS at 65 °C. The membrane was blotted dry on filter paper and sealed in plastic wrap after the washes were complete. It was necessary to exposing the membrane to X-ray film at a temperature of -80 °C in conjunction with the use an intensifying screen in order to visualize the areas where the probe bound successfully. The time required for exposure the was dependent upon the amount and distribution of radioactivity bound to the membrane.

Preparation of crude chromosomal transforming DNA

The method used to prepare chromosomal transforming DNA was modified from Marmur (1961). Cells were collected from 1.5 ml of overnight liquid culture by centrifugation in a microcentrifuge tube for 2 minutes at 10,000 x g. The cell pellet was resuspended in 500 µl of 0.05% SDS, 0.15 M NaCl, 0.015 M NaCitrate. The DNA was released from the cell by heating the cells in a water bath at 60 °C for a period of one hour. The sterile crude lysis solution was then stored at -20 °C. Storage at -20 °C resulted in no detectable loss of transforming activity throughout this study.

Preparation of transforming plasmid DNA

All plasmid DNA, used in transformation experiments, was prepared by the preparative scale recombinant isolation technique and purified on a CsCl gradient.

Triparental mating protocol

Triparental matings of *A. calcoaceticus*, *E. coli* DH5 α (harboring a pUCP30T derivative) and *E. coli* HB101 (harboring pRK2013) were performed from liquid cultures grown under appropriate conditions on the appropriate medium for 18 to 24 hours. A 250 μ l aliquot of each overnight culture was combined in a 1.5 ml microcentrifuge tube. The cell suspension was then pelleted by centrifugation at 10,000 x g for 2 minutes. The cells were resuspended in 1.0 ml of sterile 0.15 M NaCl to remove any residual medium or antibiotics. After the 0.15 M NaCl wash, cells were collected by centrifugation at 10,000 x g for 2 minutes and resuspended in 500 μ l of 1X PBS. A 150 μ l aliquot of the cell suspension was then spotted on an LB plate and allowed to soak in for 30 minutes. Plates were incubated overnight at 37 °C. The following day, the resulting cell growth was removed with an inoculation loop and spread onto selective media for subsequent single colony isolation and identification.

Conjugation protocol

Conjugations of *Acinetobacter* and *Pseudomonas* were performed using overnight liquid cultures grown under appropriate conditions. All selection for phenotypic traits of specific transconjugants was done on minimal medium plates with a sole carbon source and a specific antibiotic or amino acid. The general conjugation procedure consisted of a single cross between recipient and donor cells. The donor cells were used in a 2 to 1 ratio with respect to the recipient cells. Cells were placed in close proximity to one another by vacuum filtration of overnight cell suspensions and collection onto a 25 mm diameter

0.45 µm nitrocellulose filter using a Millipore Pyrex vacuum filtration apparatus. The entire filter was placed in the center of an LB plate and grown overnight at 30 °C or 37 °C, depending upon the preferred growth temperature for the recipient organism. The following day cells were removed from the membrane with an inoculation loop and streaked onto a selective medium for subsequent isolation and identification of transconjugants.

***Acinetobacter calcoaceticus* competent cell preparation**

Competent cells were prepared by inoculating a 5.0 ml overnight culture with the appropriate strain. All cultures of *A. calcoaceticus* were grown at 37 °C in liquid minimal medium (Ornston & Stanier, 1966) supplemented with Hutner's Metals 44 (Cohen-Bazire *et al.*, 1957) using 10 mM succinate as the carbon and energy source in a New Brunswick™ Series 25 Incubator/Shaker at 250 rpm. The following day a 50 ml flask of liquid medium was inoculated with 1 ml of the overnight culture and incubation continued at the same temperature and rpm. The optical density of the culture was monitored using a Beckman™ DU-50 spectrophotometer at a wavelength of 600 nm until an absorbance of 0.1 to 0.15 was reached. The cells were used immediately.

***E. coli* competent cell production**

Competent cells were prepared by inoculating a 5 ml overnight culture with *E. coli* DH5α. All cultures were grown at 37 °C on LB broth (containing 10 g Bacto Tryptone™, 5 g Difco™ Yeast Extract, 10 g NaCl, in a 1 liter volume) and shaken at 250

rpm in a New Brunswick™ Series 25 Incubator/Shaker. The following day 50 ml of liquid LB was inoculated with 500 µl of overnight culture and the growth was monitored in a Beckman™ DU-50 spectrophotometer until an OD at 550 nm = 0.45. The culture was then transferred to a sterile 50 ml conical tube and placed in an ice water bath for 20 minutes. The tube was centrifuged in a 4 °C Sorvall™ RT6000B centrifuge at 2200 x g for 10 minutes to collect the cells. The pellet was resuspended in 4 ml of TSS solution and placed on ice until needed.

***A. calcoaceticus* transformation protocol**

Competent *A. calcoaceticus* cells were diluted 1:100 with sterile ddH₂O. The desired amount of transforming DNA and 20 µl of 1:100 competent cells were added to a sterile 1.5 ml microcentrifuge tube. The final volume was then brought up to 60 µl with sterile ddH₂O. The entire 60 µl were spotted onto a minimal medium plate containing 10 mM succinate as the carbon source and incubated overnight at 37 °C. The following day 200 µl of minimal medium with no carbon source was spotted onto the bacterial growth and retrieved as best as possible with a micropipettor. The collected solution was then added to a 1.5 ml microcentrifuge tube containing 700 µl of sterile ddH₂O and vortexed thoroughly. From this solution, a dilution series ranging from 100-fold dilution to a 10,000-fold dilution of cells was made with sterile ddH₂O and the 1:2000, 1:5000, and 1:10,000 dilutions were pipetted onto selective plates and spread with a sterile glass “hockey stick” spreader. The plates were incubated overnight at 37 °C and scored for

transformants the following day.

***E. coli* transformation protocol**

Transformation of competent *E. coli* cells by previously prepared recombinant DNA molecules was achieved by transferring 200 µl of competent *E. coli* DH5α cells, grown to OD₅₅₀ = 0.45 into a sterile 1.5 ml microcentrifuge tube along with 100 ng of DNA. The tube was left in an ice bath for 10 minutes and then heat-shocked at 42 °C in a water bath for 2 minutes. One ml of sterile LB was added and the tube was incubated for 45 minutes at 37 °C in New Brunswick™ Series 25 Incubator/Shaker at 250 rpm. Varying cell concentrations were then spread plated with a sterile glass “hockey stick” on LB plates containing 10 µl of 100 mM IPTG and 50 µl of 2% X-Gal. These plates were incubated overnight at 37 °C.

DNA template preparation by the colony PCR method (Adapted from Zon *et al.*, 1989)

DNA can be amplified directly from bacterial colonies by PCR (Zon *et al.*, 1989). The preparation of template DNA by this method is faster and more convenient than traditional protocols. A single bacterial colony was placed in a 0.6 ml microcentrifuge tube containing 100 µl of sterile ddH₂O. After a brief vortexing to suspend the cells evenly, the tube was placed in a thermal cycler and elevated to a temperature of 100 °C for a period of 10 minutes. Any condensation inside the tube after boiling was collected by another brief centrifugation. Two microliters of the boiled bacterial cell solution

contained sufficient DNA template for most PCR reaction mixtures, and this amount was regularly used in PCR protocols.

DNA amplification by polymerase chain reaction (PCR)

Primers to amplify genes (allele) or groups of genes (alleles) were ordered from Biosynthesis, Inc. (Fig. 7). These DNA primers were designed so that they “point at each other” across a targeted region of a specific template DNA. Polymerase chain reactions were performed using a Perkin/Elmer/Cetus™ model 480 DNA thermal cycler. The PCR reaction mix consisted of ddH₂O, both left and right primers (5.0 nmol each), Fisher Scientific 10X Assay Buffer A at a final concentration of 1X, 0.25 mM of each dNTP, between 5 and 35 ng of DNA, and 1.0 unit of Fisher Scientific *Taq* DNA polymerase. This was assembled in a thin walled 0.6 ml microcentrifuge tube designed specifically for PCR. The PCR reaction mixture was centrifuged briefly in a microcentrifuge to collect the contents to the bottom of the tube and covered with 30 µl of sterile mineral oil to prevent volatilization of the mixture during the thermal cycling process. The thermal cycler amplification profile was set at 1 cycle of 4 minutes at 94 °C for denaturation, to be followed by 30 cycles of 1 minute at 94 °C denaturation, 1 minute at 65 °C primer binding, 2 minutes of polymerase extension at 72 °C, and a final cycle of 4 minutes at 72 °C to extend any unfinished DNA segments. When the reaction procedure was completed, the thermal cycler held the samples at 4 °C until they were removed from the machine. PCR products were visualized under ultraviolet radiation after electrophoresis of the amplified DNA through a 2% TBE agarose gel and subsequent staining with EtBr.

Primer Sets

<i>xyLL</i> Right	5' CGGTTTCAAAGGCTAGGCAGGCGG 3'
<i>xyLL</i> Left	5' GGTCGGCTGCTGCTGGTCGACCGT 3'
ΔxyL' Right	5' CGACGCACTTCGGCCTCGATCTCG 3'
pDKR1 <i>xyLL</i> Left	5' GCGGCGCCTGCAAATGCTTCGCCG 3'
<i>xyE1</i> Right	5' GCGAATTCGTCGCCGTACATCAGACAGTGC 3'
<i>xyE1</i> Left	5' GTTATGAGGTGCGTGAGCGGATTAGCGGCC 3'
<i>xyE2</i> Right	5' GATGTCGATGTGGCTGGCCAGGGATTTGG 3'
<i>xyE2</i> Left	5' GCGAATTCTGAGAACTGTTCCTGAGGTGCTCC 3'
<i>xyE3</i> Right	5' GGTTTGACCACCACGGTGTTGCCG 3'
<i>xyE3</i> Left	5' GGAGGAGCGACGTACACGAGGTCC 3'
Mut3 Forward	5' TTGCGCAGGCATCACCCAGAGCT 3'
Mut3 Reverse	5' GCGGTCCATCTCGATCAGGCCGAG 3'
pPAN30 Right	5' TCCGCACGTA CTGCTGCCAATCCG 3'
pPAN30 Left	5' CGCTGTCGGCAGTATTGCATCGGC 3'

Primer Sets (Continued)

catC Int Right 5' GAAGACGGATGACGATTGAGCGCC 3'

catC Chr Left 5' GGTTTGACTGACCGTTGCAATCCG 3'

pyrBC' *BstE*Δ Right 5' CACTGCGGATGTTGCCTTTGCGGC 3'

pyrBC' *BstE*Δ Left 5' TCGACGTGGTGATCATGCTGCGCC 3'

Fig. 7 Nucleotide sequence of oligonucleotide primers used for PCR amplification.

Primers were synthesized by Biosynthesis, Inc. (Lewisville, TX).

PCR product preparation for DNA sequence determination.

PCR products were prepared utilizing a US Biochemicals PCR Product Pre-Sequencing Kit. This kit contains exonuclease I, at a concentration of 10 U/ μ l in 20 mM Tris-HCl, pH 7.5, 5 mM 2-mercapoethanol, 50% glycerol, and shrimp alkaline phosphatase, at a concentration of 2 U/ μ l in 25 mM Tris-HCl, pH 7.6, 1 mM MgCl₂, 0.1 mM ZnCl₂, 50% glycerol. PCR products were prepared for sequence determination by adding the following to a 0.5 ml microcentrifuge tube:

5.0 μ l of PCR amplification mixture

1.0 μ l of exonuclease I (10.0 U/ μ l)

1.0 μ l of shrimp alkaline phosphatase (2.0 U/ μ l)

7.0 μ l Total

The contents of the tube were mixed and incubated for a period of 15 minutes at 37 °C, followed by another 15 minutes at 80 °C in a thermal cycle. The 80 °C incubation period inactivates the enzymes which might interfere with the sequencing reactions. Enzymatic preparation of PCR products requires that a modified sequencing primer annealing protocol be used. The modified protocol is performed by adding 5 to 10 pmol of sequencing primer to the 7 μ l total volume of the pre-sequencing treatment solution. The final volume was brought up to 10 μ l with ddH₂O. After mixing, the tube was placed in a thermal cycler and heated to 100 °C for 3 minutes. The denatured sequencing template

was placed on ice to snap-cool the primer onto the template and remained in the ice-water bath until it was used for the sequence analysis protocol.

Sanger dideoxyribonucleic acid sequence analysis of PCR-generated DNA products
(Adapted from Sanger *et al.*, 1977)

The Sanger method of deoxyribonucleic acid sequencing utilizes the incorporation of a nucleotide (dNTP) analog, dideoxynucleotide (ddNTP), to terminate the elongation of a complementary DNA strand synthesized from a DNA template. Four termination reactions are performed simultaneously from a single DNA template. Each reaction contains a specific dideoxynucleotide (ddGTP, ddATP, ddCTP, or ddTTP), that creates distinct fragment lengths. The termination of DNA strand synthesis is dictated by incorporation of the dideoxynucleotide. When these fragments are electrophoresed through a polyacrylamide gel, a difference in length of one nucleotide can be distinguished, as well as the specific dideoxynucleotide that caused termination. Analyzing the polyacrylamide gel by autoradiography allows the DNA sequence to be elucidated by reading the four lanes, each corresponding to a specific termination mix, from the smallest length fragment at the bottom of the gel, to the largest fragment at the top. Enzymatic synthesis of these strands is initiated by the polymeric elongation from a primer that binds to a specific region of the template DNA.

The primer for elongation in the termination reactions must be annealed to the template before elongation can take place. The annealing of the sequencing primer is accomplished by mixing the following in a 0.5 ml microfuge tube:

6 μ l	treated PCR template
1 μ l	sequencing primer at a concentration of 5 pmol/ μ l
2 μ l	Sequenase version 2.0 sequencing reaction buffer
<u>1 μl</u>	100% DMSO
10 μ l	total volume

The mixture was incubated for 3 minutes at 100 °C in a thermal cycler. During the incubation water evaporated from the reaction mixture and collected at the top of the tube. This was recovered by a brief centrifugation. After centrifugation, the mixture was immediately cooled in an ice water bath, and remained on ice until use.

A single 0.5 ml microfuge tube, or a set of two or four tubes were prepared for each template to be sequenced. The tubes served as individual termination reaction for the different dideoxynucleotides. Forty-eight terminations were done during each sequencing session. It was convenient to use different color tubes when more than one termination mix per template was being performed. This was done in order to avoid the confusing termination tubes for a template set. The colors used were green = G, orange = A, blue = T, and yellow = C when multiple termination reactions were necessary. The termination reaction mixture consisted of the following:

2.5 μl ddNTP (ddGTP, ddATP, ddCTP, or ddTTP)

1.1 μl 100% DMSO

3.6 μl total volume

It is important to make sure the mix is at the bottom of the tube before starting the sequencing protocol.

The necessary amount of labeling solution to compete 48 terminations was prepared next. The following was added to a 0.5 ml microfuge tube:

13.0 μl DTT (0.1 M)

22.8 μl ddH₂O

5.2 μl dGTP labeling mix

8.0 μl ³⁵S-dATP

47.0 μl total volume

Once again, the contents were collected at the bottom of the tube by a brief centrifugation after vortexing.

At this point, an 8-fold dilution of the Sequenase™ version 2.0 enzyme was made in 0.5 ml microfuge tube using the following components supplied by the manufacturer:

21.5 μl Sequenase™ dilution buffer

1.7 μl pyrophosphatase

3.3 μl Sequenase™ version 2.0 enzyme

26.4 μl total volume

Two microliters of this diluted Sequenase™ was added to each reaction mixture at the appropriate time during the sequencing protocol. To begin the sequencing protocol, 2.5 μl of annealed template was transferred to a new 0.5 microfuge tube. Then, 0.875 μl of DTT/labeling mix/³⁵S-dATP was added to each annealed primer/template. From this point, a timed sequencing protocol was used to ensure the proper distribution of enzyme and labeled template mixtures to the termination tubes. An overview of the timed sequencing protocol can be seen in Fig. 8.

Denaturing polyacrylamide gels for sequence determination (Sanger & Coulson, 1978)

The polyacrylamide gels used for sequence determination were designed to resolve radioactive DNA fragments differing in size by a single nucleotide. All DNA fragments on the gel possess one end in common that begins with the primer. The other fragment ends differ in the fragment set which was created by the dideoxynucleotide termination reactions. There are four factors that affect the resolution of a band on a DNA sequencing gel.

Sequencing time table

<u>Time</u>	<u>Action</u>	<u>Temp</u>
0	Warm termination tubes for templates 1 through 16	37°C
1:00	0.5 µl of “enzyme” mix to TEMPLATE 1. Mix	Room
1:15	0.5 µl of “enzyme” mix to TEMPLATE 2. Mix.	Room
1:30	0.5 µl of “enzyme” mix to TEMPLATE 3. Mix.	Room
1:45	0.5 µl of “enzyme” mix to TEMPLATE 4. Mix.	Room
2:00	0.5 µl of “enzyme” mix to TEMPLATE 5. Mix.	Room
2:15	0.5 µl of “enzyme” mix to TEMPLATE 6. Mix.	Room
2:30	0.5 µl of “enzyme” mix to TEMPLATE 7. Mix.	Room
2:45	0.5 µl of “enzyme” mix to TEMPLATE 8. Mix.	Room
3:00	0.5 µl of “enzyme” mix to TEMPLATE 9. Mix.	Room
3:15	0.5 µl of “enzyme” mix to TEMPLATE 10. Mix.	Room
3:30	0.5 µl of “enzyme” mix to TEMPLATE 11. Mix.	Room
3:45	0.5 µl of “enzyme” mix to TEMPLATE 12. Mix.	Room
4:00	0.5 µl of “enzyme” mix to TEMPLATE 13. Mix.	Room
4:15	0.5 µl of “enzyme” mix to TEMPLATE 14. Mix.	Room
4:30	0.5 µl of “enzyme” mix to TEMPLATE 15. Mix.	Room
4:45	0.5 µl of “enzyme” mix to TEMPLATE 16. Mix.	Room
5:00	3.5 µl to TEMPLATE 1 to termination tube. Mix.	37°C
5:15	3.5 µl to TEMPLATE 2 to termination tube. Mix.	37°C
5:30	3.5 µl to TEMPLATE 3 to termination tube. Mix.	37°C
5:45	3.5 µl to TEMPLATE 4 to termination tube. Mix.	37°C
6:00	3.5 µl to TEMPLATE 5 to termination tube. Mix.	37°C
6:15	3.5 µl to TEMPLATE 6 to termination tube. Mix.	37°C
6:30	3.5 µl to TEMPLATE 7 to termination tube. Mix.	37°C
6:45	3.5 µl to TEMPLATE 8 to termination tube. Mix.	37°C
7:00	3.5 µl to TEMPLATE 9 to termination tube. Mix.	37°C
7:15	3.5 µl to TEMPLATE 10 to termination tube. Mix.	37°C
7:30	3.5 µl to TEMPLATE 11 to termination tube. Mix.	37°C
7:45	3.5 µl to TEMPLATE 12 to termination tube. Mix.	37°C
8:00	3.5 µl to TEMPLATE 13 to termination tube. Mix.	37°C
8:15	3.5 µl to TEMPLATE 14 to termination tube. Mix.	37°C
8:30	3.5 µl to TEMPLATE 15 to termination tube. Mix.	37°C
8:45	3.5 µl to TEMPLATE 16 to termination tube. Mix.	37°C
9:00	Rest or catch up.	
10:00	Warm termination tubes for templates 17 through 32	37°C
	Repeat	
19:00	Rest or catch up.	
20:00	Warm termination tubes for templates 33 through 48	37°C
	Repeat	
29:00	Rest or catch up.	
30:00	4.0 µl Stop solution to tubes 1 to 16	Room
35:00	4.0 µl Stop solution to tubes 17 to 32	Room
40:00	4.0 µl Stop solution to tubes 33 to 48	Room

Fig. 8 Summary of timed DNA sequence determination protocol. The PCR-generated templates were subjected to the sequence analysis protocol in sets of forty-eight (for single terminations), twenty-four (for double terminations), or twelve (for all 4 ddNTP terminations) per sequencing session.

1. The purity of the polyacrylamide gel.
2. The diffusion of DNA fragments prior to, during, and after electrophoresis.
3. The vertical distance separating the bands.
4. The intensity of radioactive emissions from the ^{35}S -label incorporated in the DNA fragments.

Gel purity is maintained by using molecular biology grade chemicals. Diffusion of DNA fragments horizontally is minimized by quick loading of the sample onto the gel and applying voltage across the gel as soon as possible. Lateral diffusion may also be prevented by running the gel at a high voltage and drying the gel after electrophoresis. The gel solution is also filtered prior to pouring to remove any solid materials that may interfere with DNA fragment migration during electrophoresis. The thickness of the gel also can be controlled in such a way that diffusion of the radioactive signal is minimized during autoradiography.

Acrylamide sequencing determination gels were poured 12 to 18 hours before electrophoresis to ensure complete polymerization of the polyacrylamide matrix. A cassette consisting of two glass plates (52 cm x 41 cm x 0.6 cm) was prepared by cleaning the glass plates with commercial glass cleaner followed by a second cleaning with 95% ethanol. The sides of the glass plates that were to face outward were labeled as such. One inside surface was coated with a solution of 5% dichloromethylsilane dissolved in heptane. This coating was applied twice. The plates were heated in a drying oven for 15 minutes at 100 °C between the application of the coats. The final coating was allowed to

dry before the cassette was assembled. To assemble the cassette, the two glass plates were placed on top of one another with the pre-designated outside surfaces in the appropriate positions. A 0.25 mm Delrin™ spacer was then placed between the edges of the plates on the end that will be the top of the gel during electrophoresis and clamped into place by metal binding clips. Two more spacers were then inserted in from the sides of the gel cassette. These spacers run vertically lengthwise on either side and were also secured in position by 6 to 8 metal binding clips. At this point, the cassette was checked to ensure that no particulate matter, that might be incorporated into the gel matrix when poured, was trapped between the plates. A funnel shaped reservoir was made from pre-warmed (37 °C) Plastocene™. This was constructed in manner that would allow the majority of the polyacrylamide gel solution to be poured into the gel cassette down the inside space of the cassette, next to the Delrin™ spacer, to prevent trapping air bubbles during the pouring process.

The polyacrylamide gel solution was prepared by adding the following components to a 200 ml beaker:

42.4 g urea

20.0 ml 30% acrylamide/1% bis-acrylamide

10.0 ml 10X TBE denaturing buffer

30.0 ml ddH₂O

A stir bar

After the solution was dissolved on a stir plate, the final volume was brought up to 100 ml by the addition of ddH₂O. The final solution was then purified by vacuum filtration into a 250 ml Erlenmeyer™ filtration flask through a Buchner™ funnel which contained a piece of Whatman™ No. 1 filter paper. A 0.12 gram portion of solid ammonium persulfate was added to the filtrate, the solution swirled and then the filtrate was de-gassed by sealing the flask and applying a vacuum for a period of one minute. The ammonium persulfate removes any free radicals which may inhibit the polymerization process after in the cassette. 20 µl of N, N',N'' -tetramethylenediamine (TEMED) was added to the de-gassed solution and gently swirled in a way that would not reintroduce any bubbles into the solution. This solution was poured into the gel cassette from the open end. During the pouring, the cassette was tilted so that the gel solution ran completely to the bottom along one side and then across the width of the gel. This method prevents trapping any air bubbles in the gel solution during the pouring process. When the solution reached approximated $\frac{3}{4}$ of the way to the top of the cassette, the cassette was then laid flat on 4 1" rubber stoppers which had been arranged in a rectangle on the working surface on. A small Delrin™ spacer was placed in the middle of the open end and approximately $\frac{1}{2}$ " into the gel area of the cassette. This prevents any pinching of the gel caused by the sagging of the glass plates under their own weight. The time for polymerization depended upon the temperature of the room, the extent of the gas removal from the gel solution, and the amount of TEMED added to the solution. Varying amounts of TEMED were used to compensate for the temperature of the room. The ends

of the gel were then wrapped in plastic cellophane wrap to decrease the likelihood of polymerization inhibition by contact with the air.

Prior to loading samples on the gel, the cellophane wrap, metal binding clips, and the Delrin™ spacers in the ends of the cassettes were removed. The gel was placed upright in a sequencing gel electrophoresis stand, and upper and lower chambers of the sequencing stand were filled with 1X TBE denaturing sequencing buffer. 1X TBE denaturing sequencing buffer contains 10.0 mM Tris, 1.2 mM boric acid, and 0.1 mM EDTA. Enough buffer was used in each chamber to ensure the buffer level was above the exposed end of the gel on top, and the bottom of the gel was submerged about 5 cm in the buffer solution. A Pasteur™ pipette was used to squirt buffer and thus displace any air bubbles which may have been trapped between the plates, above the surface of the gel and between the gel and the buffer solution in the lower chamber. After the cassette was set up in the gel chamber, it was electrophoresed for at least one hour at 50 Watts. Pre-electrophoresis of the gel removed excess charged particles from the gel ahead of the samples to be loaded. This also aids in the resolution of individual DNA bands.

In order for the sample to be loaded, shark tooth well forming combs were placed on the top of the gel. Samples were placed in a 90 °C water bath for two minutes to denature any DNA secondary structures that might effect the DNA's migration during electrophoresis. The samples were placed in an ice-water slurry until they could be loaded onto the gel. Loading of the samples was done using a Hamilton™ syringe with a flat point 32 gauge needle. 2.0 µl to 3.5 µl of each sample was loaded in an order that was optimal for the analysis of the sequence to be determined. Control sequencing

reactions were also loaded for comparison on the autoradiograph. The running time and voltage of electrophoresis for each gel varied, but a constant 50 Watts of power was maintained. The distance of migration desired determined the length of the running time. Migration distance was estimated by measuring the distance traveled by the marker dyes in the loading buffer.

Autoradiography of sequence determination gels

The gel cassettes containing the sequence analysis gels were removed from the electrophoresis apparatus and dismantled after the gel was electrophoresed for the appropriate amount of time. Disassembly required the removal of all metal binding clips and Delrin™ spacers. The plates were pried apart with the use of a metal spatula and the gel was adhered to a piece of Whatman 3MM™ filter paper. The filter was cut to the appropriate size (14" x 17") before the disassembly process was begun. Excess gel was trimmed away with a razor blade and discarded. Once the gel had adhered to the filter paper, it could be removed from the glass plate. Plastic cellophane was stretched over the gel, and the overhanging cellophane was also removed with a razor blade. The gel was then transferred to a gel slab dryer, and dried under a vacuum at a temperature of 85 °C until it was stiff. This usually required between 45 and 90 minutes. The urea contained in the gel quenches the radioactive emissions necessary to expose the X-ray film. A leaching solution of 12% methanol and 10% glacial acetic acid was utilized to remove the urea. After removing the cellophane, the gel was soaked in the solution for 15 minutes. The

gel was again covered with cellophane wrap and placed on the slab gel dryer, under a vacuum at 85 °C, until the gel was completely dry.

The dried gel, adhered on filter paper, was exposed to X-ray film in an autoradiography cassette at room temperature produced an autoradiograph. Loading of the gel/filter paper was done in a darkroom after the cellophane had been removed. The exposure length varied, depending on the amount of radioactivity in the gel and the type of X-ray film used. The length of time for exposure was adjusted to produce the best resolution on the autoradiograph and a consistent band intensity.

CHAPTER III

RESULTS AND DISCUSSION

Verification of bacterial strains

All strains used in experiments were first validated with respect to Gram stain, oxidase test, antibiotics resistance, carbons source utilized, and amino acid auxotrophy to verify the correct phenotype before any experimentation took place. Only the strains which responded appropriately were utilized for further experimentation.

Determination of ability to transfer plasmids between *Acinetobacter* and *Pseudomonas* by conjugation

It was essential to verify that pDKR1 could be readily transferred into and out of *A. calcoaceticus*. *A. calcoaceticus* was only to be used as a vehicle for modifying pDKR1. If the modified plasmid could not be transferred back to an appropriate genetic background for any number of possible reasons, there would be no point in continuing with the proposed approach. A reason might be as simple as the loss of plasmid transfer function due to ineffective promotion of transfer factor expression in the new host bacterium. Therefore, it was necessary to design experiments that would demonstrate our ability to transfer pDKR1 into *A. calcoaceticus*, maintain it there in a stable fashion (preferably as an autonomous replicon) and then return it to a recipient strain of *P. putida*

where the TOL functions could be expressed. The results of experiments to validate the protocol are described below.

Conjugation of pDKR1 into ADP1

P. putida PaW630 cells (harboring plasmid pDKR1) were conjugated with *A. calcoaceticus* ADP1 cells by placing the bacteria in close proximity to one another on a nitrocellulose filter disk. The disk was placed on an LB medium plate and cells were cultured for 18 to 24 hours at 37 °C. Aliquots of the conjugation mixture were spread on plates of minimal medium containing 10 mM succinate as the carbon and energy source with the antibiotic kanamycin at 100 µg/ml to select for transconjugants. Selection was also carried out with minimal medium plates containing 5 mM *meta*-toluate as the sole carbon and energy source. Colonies that demonstrated resistance to kanamycin were again tested for resistance to all pDKR1-encoded antibiotic resistance markers to increase the probability of obtaining an *A. calcoaceticus* strain carrying the entire pDKR1 TOL plasmid. As expected, selection of *A. calcoaceticus* carrying pDKR1 on *meta*-toluate minimal medium was not successful, probably due to major differences between the TOL promoters and sigma factors used by *A. calcoaceticus*. Colonies selected for use in subsequent experiments demonstrated all appropriate phenotypic antibiotic resistances.

Isolates were further subjected to an oxidase test (see Materials and Methods) to assure that the presumptive *Acinetobacter* strains with pDKR1 were not simply mutant strains of the donor *Pseudomonas*. Finally, total DNA was isolated from transconjugant strains and used to transform ADP6 from a POB⁻ phenotype to POB⁺. The phenotypic

reversion demonstrated that the *Acinetobacter* genetic material was contained in the transconjugant strains.

Verification of pDKR1 TOL genes in *Acinetobacter* strains

Additional tests to demonstrate the presence of the pDKR1 TOL genes in strains of antibiotic resistant *Acinetobacter* isolated following conjugation with PaW630 were performed. This was necessary because the TOL⁺ phenotype is not expressed in *A. calcoaceticus*. Several different approaches were used to verify the existence of the TOL genes in transconjugant *A. calcoaceticus*.

The first approach utilized large plasmid and cosmid isolation procedures in to attempt to isolate pDKR1 from recipient strains. None of these procedures provided conclusive, reproducible results indicating the presence of pDKR1. Though unfortunate, this was not completely unexpected since the pDKR1 plasmid is a very large, single copy plasmid which is not readily isolated by current rapid plasmid preparation protocols.

The second approach utilized was total DNA isolation from transconjugant strains and subsequent hybridization with radiolabeled TOL DNA probes. The isolated DNA was subjected to restriction digestion by the enzymes *XhoI* and *PstI* and loaded onto an agarose gel. The gel was electrophoresed and the DNA transferred to a nylon membrane by the Southern (1975) transfer method. Control DNAs of *A. calcoaceticus* ADP1, *P. putida* PRS2000, and *P. putida* PaW30 containing pDKR1 were also digested with the restriction endonucleases *XhoI* and *PstI* and loaded onto the same gel. A *xylLT* gene fragment contained in pUC18 was ³²P-labeled by PCR amplification (see Materials and Methods) and used to probe the membrane. The resulting autoradiograph (Fig. 9)

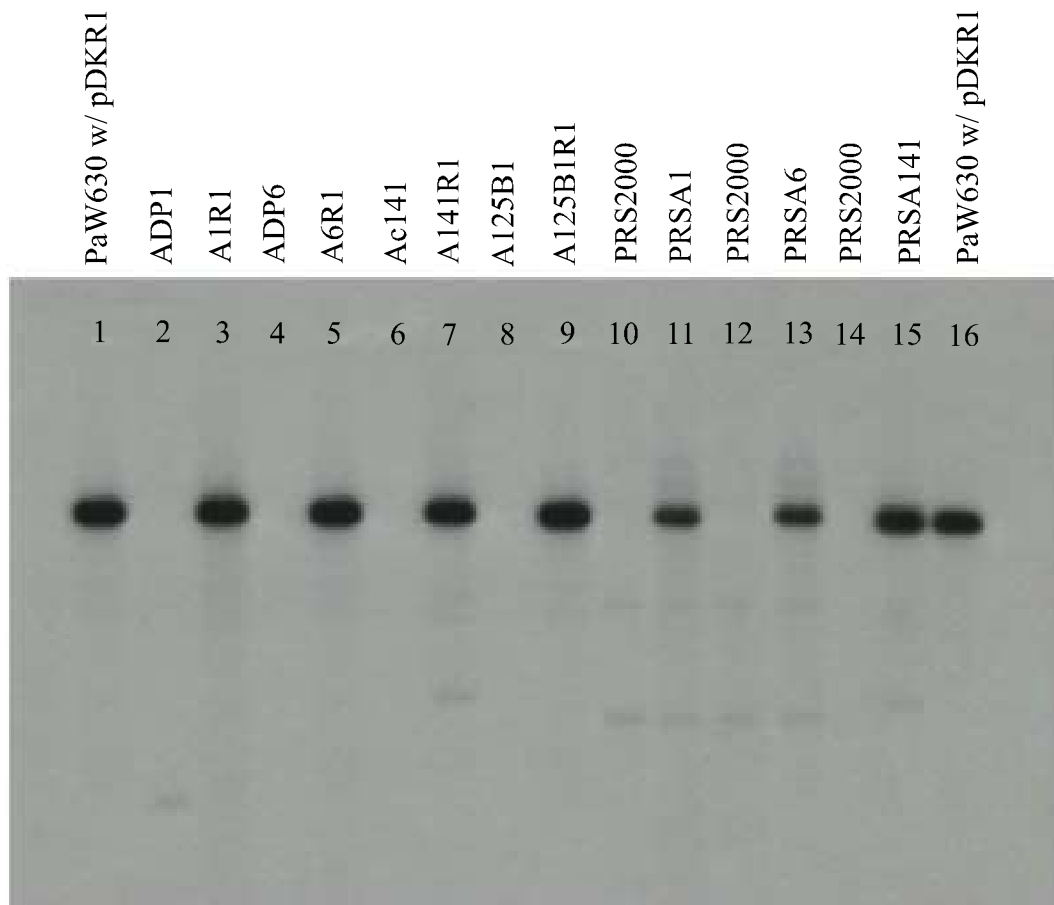


Fig. 9 Representative autoradiograph showing DNA fragments hybridized by ³²P-labeled *xyLT* PCR product. Total DNA isolates were from *P. putida* PRS2000, *P. putida* PaW630 containing pDKR1, *A. calcoaceticus* ADP1, samples of *A. calcoaceticus* ADP1 containing pDKR1, samples of *A. calcoaceticus* ADP6 containing pDKR1, and *P. putida* PRS2000 containing pDKR1. Prior to electrophoresis and Southern transfer, DNAs were digested with the restriction endonuclease *Pst*I. The autoradiograph shows radioactive bands in transconjugant lanes which, based upon size and complementarity to the probe, are consistent with being derived from pDKR1. No hybridization of the probe to total DNA from *A. calcoaceticus* ADP1 or the wild-type strain of *P. putida* PRS2000 is observed.

shows the expected radioactive bands in the transconjugants, consistent with each carrying the pDKR1 TOL region. The autoradiograph also shows no hybridization of the probe to total DNA from *A. calcoaceticus* ADP1 or the wild-type strain of *P. putida* PRS2000.

An additional approach used to screen for the presence of TOL genes in the transconjugant *A. calcoaceticus* was PCR amplification using TOL specific primers (see Materials and Methods). The primers were used to amplify the locus from total DNA isolates of *Acinetobacter* transconjugants demonstrating pDKR1 antibiotic resistant phenotypes. Total DNA isolated from wild-type *A. calcoaceticus* ADP1, *P. putida* PaW630 containing the pDKR1 plasmid, and *P. putida* PRS2000 was also amplified by PCR. The PCR amplification products were analyzed by viewing under UV illumination after gel electrophoresis and subsequent ethidium bromide staining. The products were found to be consistent with known TOL amplification products (Fig. 10).

Isolates were further subjected to oxidase test to assure that the *Acinetobacter* strains assumed to contain the pDKR1 were not just mutant strains of donor *Pseudomonas*. Finally, total DNA was isolated from transconjugant colonies and the DNA was used to transform ADP6 from a POB⁻ phenotype to POB⁺. The phenotypic reversion from POB⁻ to POB⁺ demonstrates that the *Acinetobacter* genetic background was contained in the transconjugant strains. *Acinetobacter calcoaceticus* ADP1 transconjugants, verified to contain pDKR1, were then given the A1R designation with additional numbers to

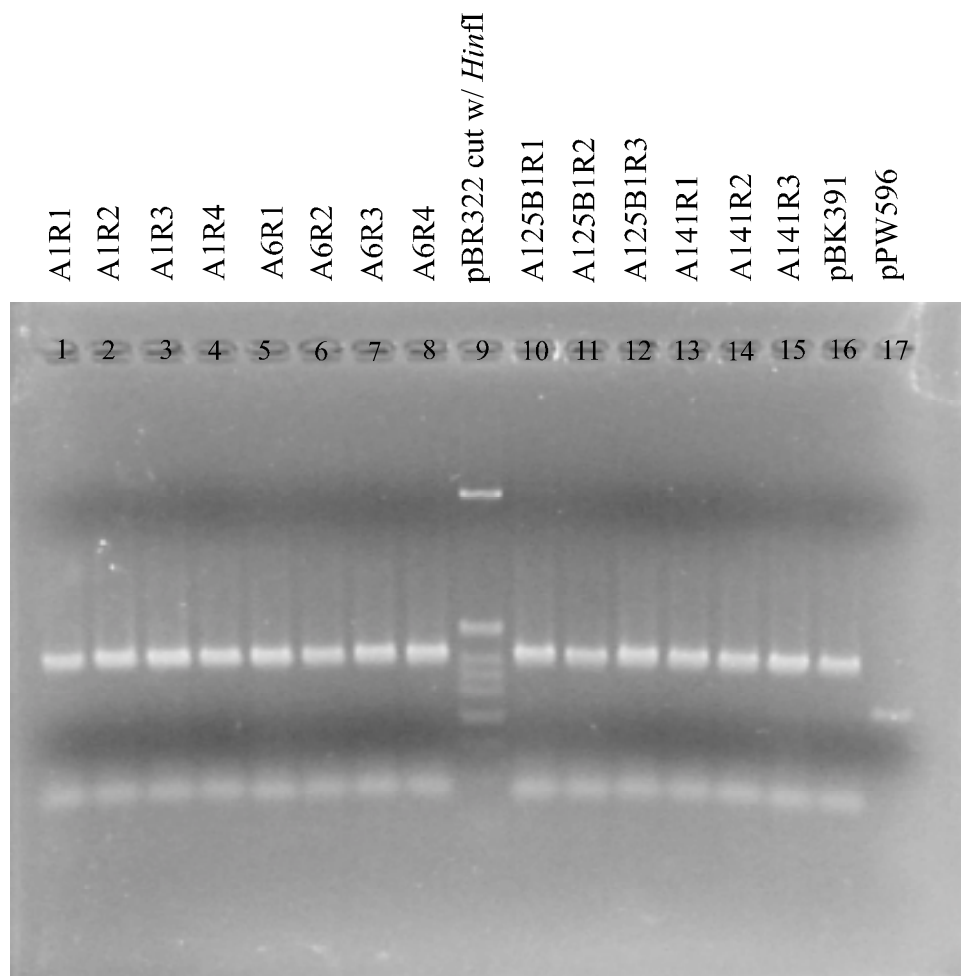


Fig. 10 Verification of TOL gene sequences in ADP1 recipient strains by PCR amplification. Total DNA was isolated from Km^r *A. calcoaceticus* strains ADP1 and ADP6 after conjugation with *P. putida* PaW630 containing TOL plasmid pDKR1 and used as template for amplification of the targeted *xyiL* region. Samples were electrophoresed on a 1% TBE agarose gel, stained with EtBr and recorded photographically. Lanes 1-4 are PCR amplification products of ADP1 isolates that contain pDKR1 and lanes 5-8 ADP6 isolates harboring pDKR1. Lanes 10-12 are A125B1 isolates containing pDKR1 and lanes 13-15 are Ac141 strains containing pDKR1 (described in **Generation of strains for transformation experiments**). Lanes 16 and 17 are PCR amplification products from pPW596 and pBK391 DNAs (mutant and wild-type *xyiL*, respectively) used for size reference and positive PCR amplification controls. Lane 9 is a λ DNA digested with *HindIII*.

identify specific isolates. The set, beginning with A1R1 and continuing through A1R20, was frozen for long-term storage.

Accumulated data suggested that multiple encoded pDKR1 genes were present. However, it was possible that the plasmid might have been integrated into the chromosome. Therefore, it was necessary to demonstrate that the plasmid was a distinct replicon, or at least transferable by conjugation to further clarify the analysis.

Verification of pDKR1 conjugal transfer from *A. calcoaceticus* to *P. putida*

In order to determine whether pDKR1 could be transferred by conjugation from *A. calcoaceticus* strains into a wild-type recipient strain of *P. putida* PRS2000, A1R transconjugants were conjugated with PRS2000 (see Materials and Methods). The wild-type *P. putida* PRS2000 did not demonstrate any of the pDKR1 phenotypes prior to the conjugation procedure. The *P. putida* transconjugants were analyzed to verify the appropriate phenotypic traits. These included growth on 10 mM succinate minimal medium plates containing the antibiotics tetracycline or kanamycin, as well as growth on minimal medium plates containing *meta*-toluate as the sole carbon source. All primary pDKR1 phenotypes were conferred to the *P. putida* PRS2000 transconjugants, including the ability to grow on TOL pathway substrates as sole carbon and energy sources. The conferring of TOL phenotypes also proved that pDKR1 had been present in *A. calcoaceticus*. Additional verification of pDKR1's presence in *P. putida* was accomplished through hybridization to radiolabeled DNA probes and PCR amplification of specific *xyl* genes. All results indicated the presence of pDKR1 in *P. putida* PRS2000.

Optimization of transformation protocol

A. calcoaceticus strain ADP6, with its phenotypic conversion from POB⁻ to POB⁺, was used as a model system to outline and optimize a protocol for the eventual transformation of pDKR1 in order to determine the most efficient method to transform *A. calcoaceticus* cells. The analysis of factors which affect the transformation rate of ADP6 served multiple functions. It helped provide a better understanding of and experience with the cellular systems that would ultimately be used for protocol development. It also performed a quality control and assurance function by establishing a verified transformation protocol and thus eliminating much of the experimental process as a variable when dealing with transformation of novel DNA constructs. Many factors can play a role in transformation efficiency. Those considered to be the most relevant were examined and are discussed in the sections below.

Determination of optimal growth phase for *Acinetobacter* transformation

Competence variation as a function of growth phase has been speculated to occur by many investigators (Ahlquist *et al.*, 1980; Ahmadian-Tehrani, 1990; Cruze *et al.*, 1979; Herzberg *et al.*, 2000; Juni & Janik, 1969; Lorenz *et al.*, 1992; Palmen *et al.*, 1993; Palmen & Hellingwerf, 1997; Porstendorfer *et al.*, 1997). However, the published experimental results conflict on the specifics of this point (Ahlquist *et al.*, 1980; Ahmadian-Tehrani, 1990; Cruze *et al.*, 1979; Juni, 1972; Lorenz *et al.*, 1992; Palmen *et al.*, 1993; Porstendorfer *et al.*, 1997). Experiments to measure competence as a function of growth phase were performed using phenotypic conversion of ADP6 from POB⁻ to POB⁺ as an indicator of transformation.

Cells were grown in both LB broth or liquid minimal medium with 10 mM succinate. ADP6 cells suspensions were withdrawn at multiple time points during the growth curve, diluted 100-fold, and transformed using a standardized transformation protocol (see Materials and Methods) with pZR2 donor DNA at a concentration of 10.0 µg/ml. Recipient *A. calcoaceticus* cells were found to be competent over the entire cell cycle. Two periods of elevated competence were observed (Fig. 11). The first occurred as the cells entered log phase and the second during entry into stationary phase, with the highest frequency of transformation obtained during the first period. These times have recently been demonstrated to correspond to the induction and expression of genes encoding products necessary for the competent state (Herzberg *et al.*, 2000; Porstendorfer *et al.*, 2000).

Dependence of transformation efficiency on donor DNA concentration

It was necessary to determine the minimum donor DNA concentration that would provide the maximum transformation rate. Not all DNA molecules in a donor DNA sample encode the actual sequence required to transform a given locus, DNA concentration can be relevantly considered in two ways. Either by simple mass per volume (micrograms of DNA per milliliter of solution), or by copy number concentration (the number of DNA molecules per milliliter which are homologous to the site being transformed). The initial analysis was strictly based on the mass of total donor DNA utilized in the transformation experiment.

A. calcoaceticus ADP6 cells were grown in liquid 10 mM succinate minimal medium at a temperature of 37 °C to an OD reading of between 0.1 and 0.125 at

Competence Development as a Function of Growth Phase

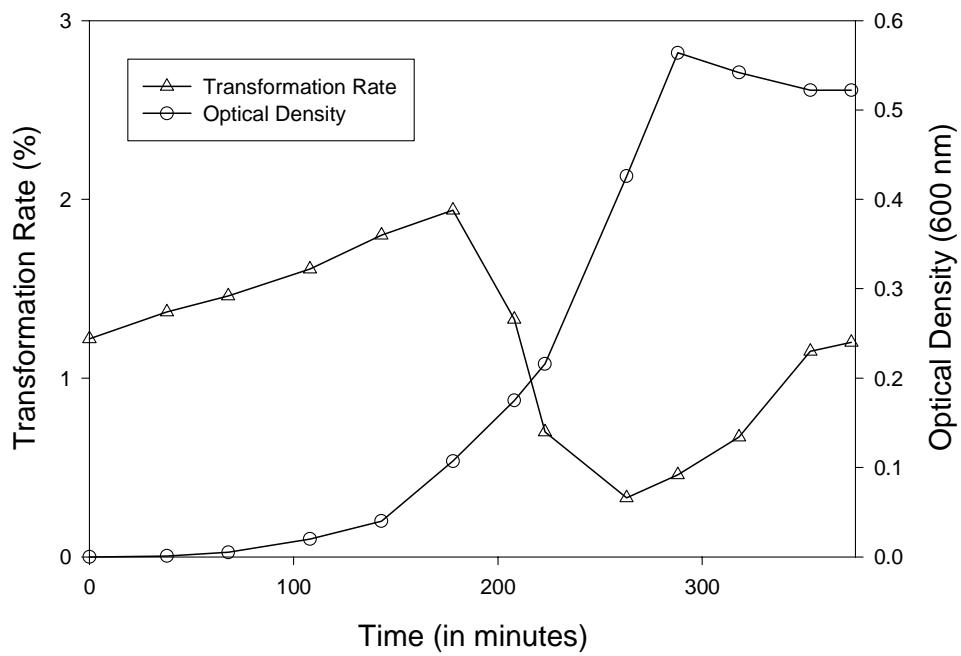


Fig. 11 Competence development in *A. calcoaceticus* ADP6 as a function of growth phase. Samples were taken and transformations were performed at each of the times shown. Transformation efficiency is represented by unfilled triangles and OD (600 nm) of the bacterial culture is depicted by filled circles.

600 nm. Cells were transformed from POB⁻ to POB⁺ phenotype using a standardized transformation protocol (see Materials and Methods). Three types of transforming DNA were utilized for the experiment and the concentration of DNA in the transformation mixture was varied over a range of 0.1 µg/ml to 20.0 µg/ml.

Transformation efficiency reached a maximum of approximately 2% with pZR1 as the donor DNA, 1.3% with pZR2 as the donor and 1% with ADP1 genomic DNA as donor (Fig. 12). All three frequencies saturated as the donor DNA reached a concentration of 5 about µg/ml indicating that the DNA uptake system, or the recombinational/transforming cellular apparatus, can handle a limited quantity of DNA. Some questions are also raised by the data. Since the maximum rate of transformation is different for each type of donor DNA, it was concluded that further investigation into the characteristics contributing to the ultimate effectiveness of a donor DNA molecule was necessary. There are obvious differences between plasmids and genomic DNAs beyond simply the DNA sequences. The first to be explored was the effect of the donor molecule conformation on transformation rate.

Dependence of transformation efficiency on donor DNA conformation

The conformation of a donor DNA molecule could have an effect on the DNA's ability to enter and transform the cell. In order to determine the effect(s) attributable to the closed circular supercoiled conformation of the donor DNA, plasmid DNAs in their linear and closed circular supercoiled forms were compared as donor DNA molecules. Plasmids were linearized by digestion with restriction endonucleases which also released

Dependence of Transformation Efficiency on DNA Concentration

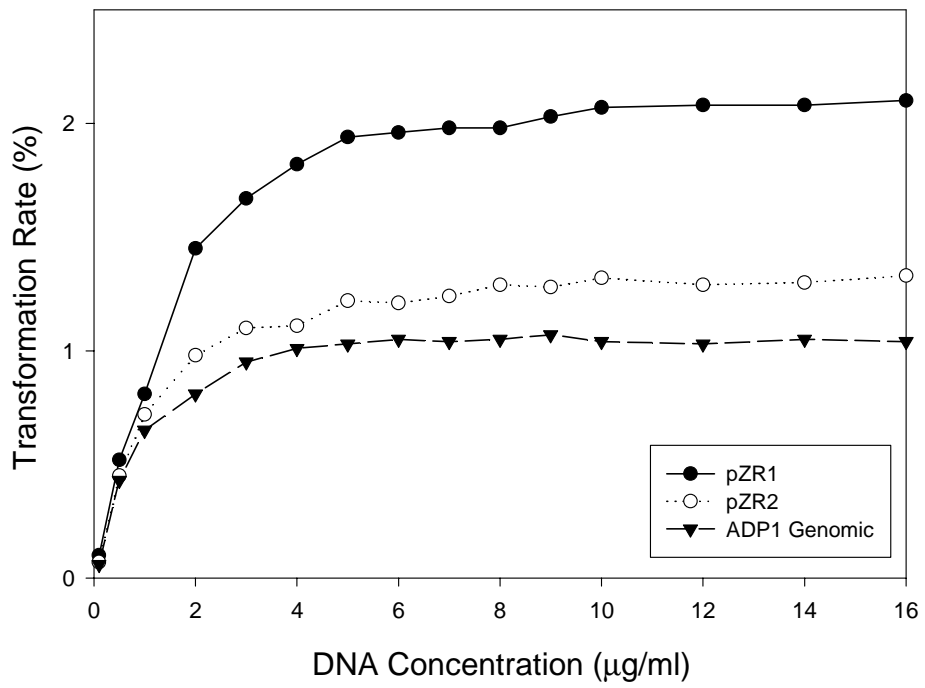


Fig. 12 Transformation efficiency for competent *A. calcoaceticus* cells as a function of donor DNA concentration. Three different transforming DNAs were utilized. Transformation efficiencies obtained with pZR1 are represented by filled circles, pZR2 efficiencies are represented by unfilled circles, and ADP1 genomic DNA efficiencies by solid triangles. A maximum of approximately 2% was obtained with pZR1.

the insert from the vector. It is important that the donor sequence, homologous with the site of recombination, remain intact after the endonuclease cleavage and therefore, endonucleases were chosen such that no cuts would be made within the *pca* operon (the site of transformation for the POB^- to POB^+ reversion tested).

Recipient *A. calcoaceticus* ADP6 cells were grown on minimal medium containing 10 mM succinate at 37 °C until an OD of 0.1 at 600 nm was reached. As before, the standardized *Acinetobacter* transformation protocol (see Materials and Methods) and a range of donor DNA concentrations from 0.1 µg/ml to 10 µg/ml was used. Plates were scored for colony growth after incubation and the number of CFU on selective plates was compared to the total viable cell count on non-selective plates. Transforming efficiencies were calculated and the results can be seen in Fig. 13.

Supercoiled donor pZR1 and pZR2 DNA molecules each provided a somewhat higher transformation rate than the same DNA in linear form. Current models for DNA translocation into *Acinetobacter* cells require the conversion of donor DNA to linear single strand form upon entry into the cell. Any advantage supercoiled DNA might have over linear donor DNA molecules would therefore be likely to occur prior to internalization, unless for unknown reasons the circular molecules have an independent mechanism for crossing the plasma membrane. It is possible that the lack of a free end reduced the susceptibility of the molecule to spurious extracellular exonuclease activities. Resistance of supercoiled donor DNAs to extracellular exonucleases or endonucleases would provide a mechanism to maintain a longer-term extracellular reservoir of

Dependence of Transformation Efficiency on Donor DNA Conformation

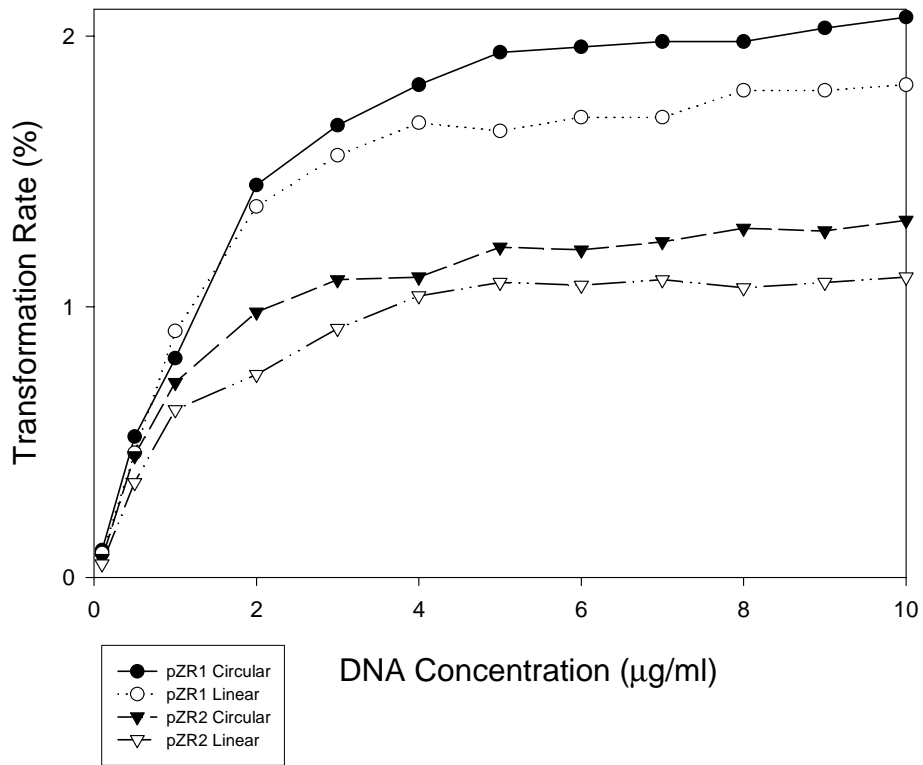


Fig. 13 Transformation efficiency for competent *A. calcoaceticus* cells as a function of donor DNA conformation. Two types of transforming DNA (pZR1 and pZR2) in two different conformations (linear and supercoiled) were utilized. Linearized samples were treated with the appropriate restriction enzyme to remove the cloned fragment as a single fragment. Transformation efficiencies obtained with supercoiled DNA are represented by filled symbols, while linear DNA efficiencies are represented by unfilled symbols. A maximum rate of approximately 2% was obtained above approximately 5 µg/ml of supercoiled pZR1 donor DNA.

transforming DNA that that obtained using DNA of a linear form. Providing recipient cells an extended period of exposure to donor DNA molecules would allow an increased opportunity for the transforming molecules to be taken up by the recipient cell.

Alternatively, if internalized DNAs have a short useful half-life, then continued uptake of donor DNA molecules over time might allow one of them to eventually assimilate and cause transformation of the relevant locus. Consistent with this idea, the closed circular nature of the DNA molecule seems to play its small role in determining the transformation rate produced by donor DNA molecules primarily at saturation concentrations. At low concentrations, where all donor DNA might be removed by DNA uptake conformation, seems to make little difference.

Dependence of transformation efficiency on donor DNA size

It was anticipated that the size of the donor DNA molecule and also the related gene dosage issue (discussed later) would also play major roles in determining the transforming activity of a DNA sample. Donor DNA size might affect transformation efficiency in several ways. These include the ability to transport the molecule across the plasma membrane of the cell or the size of the sequence homology region flanking either side of the recipient sequence being transformed. Therefore, a comparison of the transformation efficiency of ADP1 genomic DNA digested with *EcoRI* to the efficiency of undigested ADP1 genomic DNA was carried out. This begins to address the issue of donor DNA molecule size, since both the total amount of DNA and the gene dosage of the two samples can be maintained at similar levels. The plot for *EcoRI*-digested ADP1

genomic DNA shows a reduced transformation efficiency when compared to uncut ADP1 genomic DNA in Fig. 14. A reduction in donor DNA molecule size clearly decreases the transforming activity of the DNA. After *EcoRI* digestion, an average DNA fragment size reduction of approximately three-fold produces a 20% reduction in transformation efficiency. The decrease in transforming activity of the *EcoRI*-digested ADP1 genomic sample suggests that competent *Acinetobacter* cells take up only a limited number of DNA molecules. Therefore, if the molecules are larger, there is a better chance that one of the fragments taken up will contain the transforming locus. In as much as the saturating transforming frequency of the digested samples never reach the maximum transformation efficiency of uncut, it seems likely that the number of molecules taken up, not the mass that can be taken up, is the primary limiting factor. However, it does not suggest that only a single molecule is taken up. If only a single transforming molecule were taken up, one would expect to see a three-fold drop in transformation rate corresponding to the three fold increase in the number of DNA fragments in the digested sample. Similarly, pZR1 is a more efficient donor DNA than the smaller pZR2, and this will be discussed further in the gene dosage section below.

Transformation efficiency as a function of gene dosage

Donor DNA molecule concentration must always be considered in the context of gene copy number in addition to simple total mass. Gene copy number in a given quantity of DNA varied widely between pZR1, pZR2 and genomic ADP1 DNA samples

Dependence of Transformation Efficiency on Donor DNA Size

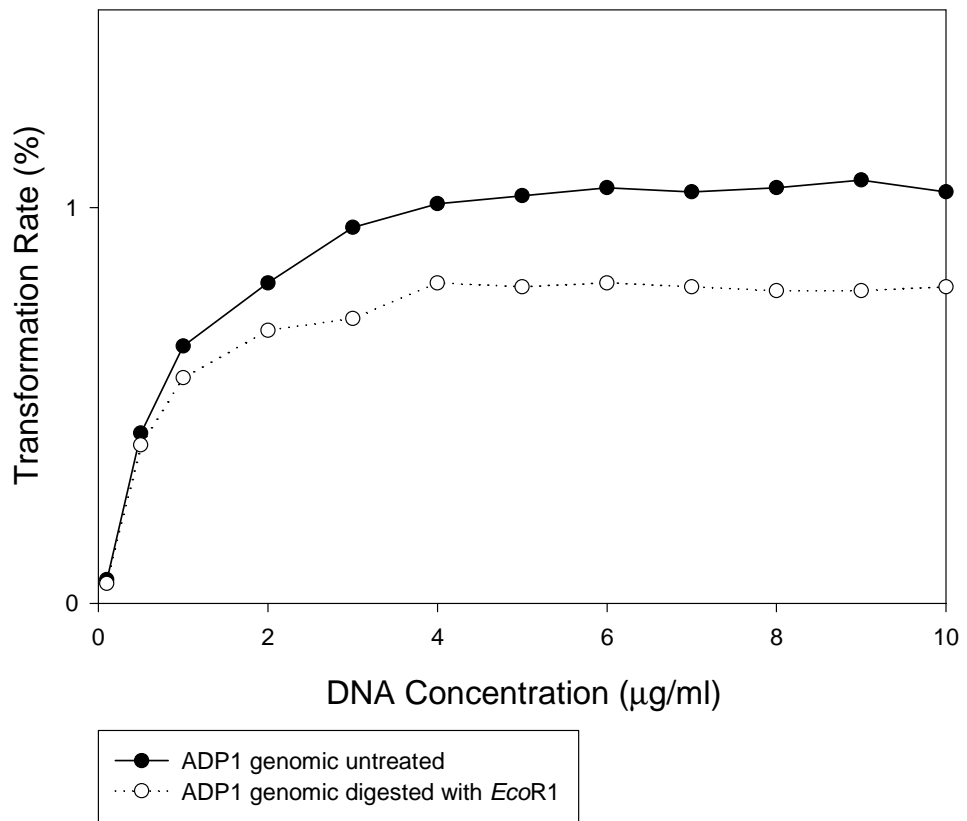


Fig. 14 Transformation efficiency for competent *A. calcoaceticus* cells as a function of donor DNA size. Genomic ADP1 DNA digested with *EcoRI* and undigested genomic DNA (randomly sheared during isolation) were utilized as donor DNA. Transformation efficiencies obtained with undigested donor DNA sample were approximately 20% higher than with *EcoRI*-digested DNA.

due to the difference in the complexity of the donor DNAs. One microgram of chromosomal DNA is very different from one μg of a 13.7 kbp pZR1 DNA with respect to the number of DNA molecules that are capable of transforming the specified locus. *A. calcoaceticus* ADP1 genomic DNA only contains a single copy of the *pca* sequence necessary to transform the mutant ADP6 locus per genome equivalent of DNA fractured into more than one hundred pieces. A plasmid, which consists of a specific cloned DNA fragment (the *pca* operon in the case of pZR1), has a much higher percentage of relevant sequence per μg of DNA and each molecule encodes the relevant locus. The same is even more true of pZR2, since pZR2 contains only the portion of the *pca* operon required to correct the mutation in ADP6 and little more. This means that a given quantity of pZR2 is equivalent to more copies of the transforming sequence than an equal amount of pZR1 or genomic *A. calcoaceticus* DNA. Therefore, it was logical to investigate the effect of gene dosage on the transformation rate.

It was determined that analysis of gene dosage would be best accomplished by linearizing plasmid DNA samples with restriction endonucleases which cut out the cloned *A. calcoaceticus* sequence, thereby eliminating any effects attributable to the closed circular conformation of the donor DNA or the presence of non-homologous flanking sequences. Calculations were performed to determine the amount of each transforming donor DNA sample which would provide a set equivalent number of copies of the relevant transforming DNA. All transformation rates should be approximately similar at an equivalent gene dose if gene dosage is the major factor in determining transformation efficiency. It is logical to conclude that any remaining differences in the

effectiveness of the transforming DNA samples would be attributable to DNA size or other inherent characteristics of the molecule.

Transformation experiments were performed using the standard *Acinetobacter* transformation protocol (see Materials and Methods) and ADP6 as the recipient strain. Transformation efficiency was based upon the frequency of reversion from POB⁻ to POB⁺. Donor DNAs utilized for transformation were *Eco*RI-digested pZR1 (linear pUC19 plus an 11.0 kbp insert), *Hind*III digested pZR2 (linear pUC18 plus the 2.4 kbp insert), and ADP1 chromosomal DNA digested with *Eco*RI.

A difference in the transformation efficiency between donor DNA types at an equivalent gene dosage was observed (Fig. 15) indicating transformation efficiency is not simply a function of the number of transforming DNA molecules encoding the sequence necessary to transform a specific locus. Other factors also play important roles. Surprisingly, ADP1 DNA is several orders of magnitude more efficient, on a per *pca* operon basis, than is either pZR1 or pZR2. This is true even though the relevant genomic fragment is presented to recipient cells with an entire genome equivalent of competing nontransforming DNA (not homologous to the *pca* operon).

The effect of gene dosage is not the only observation that can be made from the data. A comparison of pZR1 with pZR2 transformation rates, both in a linear conformation and at an equivalent gene dosage, further supports the hypothesis that the size of the donor DNA molecule has a substantial effect on transformation efficiency. Since in both cases all molecules encode the wild-type *pca* sequence necessary for the

Transformation Efficiency as a Function of Gene Dosage

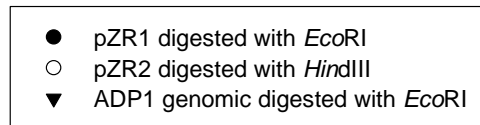
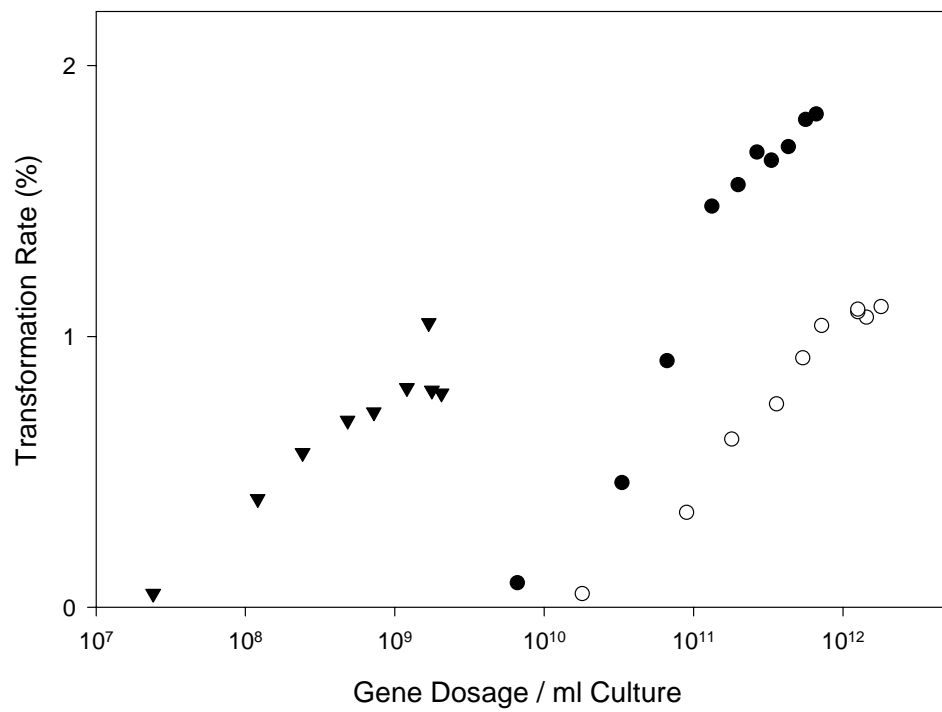


Fig. 15 Transformation efficiency for competent *A. calcoaceticus* cells as a function of gene dosage. Three different linearized donor DNAs were utilized. Efficiency of transformation for equivalent gene doses of *Eco*RI-digested pZR1 (linear pUC19 plus an 11.0 kbp insert) is represented by filled circles, *Hind*III- digested pZR2 (linear pUC18 plus the 2.4 kbp insert) by unfilled circles and *Eco*RI-digested ADP1 genomic DNA [3.78 Mbp per genome (Gralton *et al.*,1997)] by filled triangles.

POB⁻ to POB⁺ transformation, the difference in efficiency must relate to the size differential of 11.1 kbp versus 2.4 kbp. Melnikov & Youngman (1999) state that the length of donor DNA molecule with homology to flanking regions of the locus being transformed affects general recombination. A comparison of the plots of transformation efficiency of pZR1 and pZR2 shows a increased efficiency of pZR1 over that of pZR2. Therefore, the amount of homologous sequence to the site of recombination contained in the *pca* clones also must have an effect on the ability of the molecule to be integrated onto the chromosome during the transformation process. The greater size of sequence homology on either side of the donor locus, the more sequence alignment there is to aid in the integration event, thus increasing transformation efficiency. Regions of homology are larger in pZR1 (which contains the entire *pca* operon) than in that of pZR2. Following this line of logic, a genomic *A. calcoaceticus* DNA sample would contain the largest regions of homology to the recipient locus. As mentioned before, ADP1 genomic DNA and pZR1 digested with *EcoRI* are the same size but not the same transforming efficiency, so there are other factors effecting efficiency of a donor DNA molecule.

Chromosomal DNA acts as a much better transforming DNA than you would expect based upon concentration or gene dosage. Compared to pZR1 and pZR2 samples, genomic DNA samples contain approximately 350 to 1000 times the amount of non-transforming (i.e. no *pca*) competing DNA per relevant *pca* sequence. The presence of competing DNA does seem to have some effect on *A. calcoaceticus* transformation (Ahmadian-Tehrani, 1990). Therefore, it is suspected that only a limited amount of DNA can be taken up per cell. Most cells would likely never take up enough DNA to find a

specific locus if there is a very low dosage of the corresponding gene in the transforming DNA sample. For example, if a purified cloned gene containing only the necessary transforming sequence is used, it would only be necessary to take up a single DNA molecule for successful gene conversion. The gene dosage of ADP1 genomic DNA required to produce an equivalent transformation rate to pZR1 or pZR2 donor DNAs is two to three orders of magnitude less (as indicated in Fig. 15). This drastic increase in efficiency, even with the competing DNA fragments present, might be due to the presence of a post-replicative modification. For example, a unique methylation pattern could prevent cellular nucleases from degrading a sequence or enhance the rate of some other step in the transformation process. Survival of a donor DNA strand may not only be a matter of importance until entry into the cell, but it may continue to be relevant once it is inside. This effect would not be seen when comparing pZR1 to pZR2, as neither would contain the unique pattern. The differences seen between the two plasmids used in this case is solely an issue of the size of the homologous flanking region.

Increased transformation efficiencies as a result of dilute lysis solution with detergent

The routinely obtained transformation efficiency observed in this study were low compared to rates sometimes reported by others (Doten *et al.*, 1987b). An analysis of literature reviewing *Acinetobacter* transformation studies revealed a number of differences in the method of donor DNA isolation. In some instances, the reported transformation rates were substantially higher than the ones observed in this study (e.g. Doten *et al.*, 1987b). Also, my earlier results suggested that the use of crude cellular lysates as donor DNAs provided a higher transformation frequency than that obtained

with CsCl-purified donor DNAs. Although DNA in a crude lysate might be expected to be in particularly large fragments and this in itself is a definite factor, further experimentation was performed to determine if a component of the cell lysate transferred to the transformation mix was also increasing the efficiency.

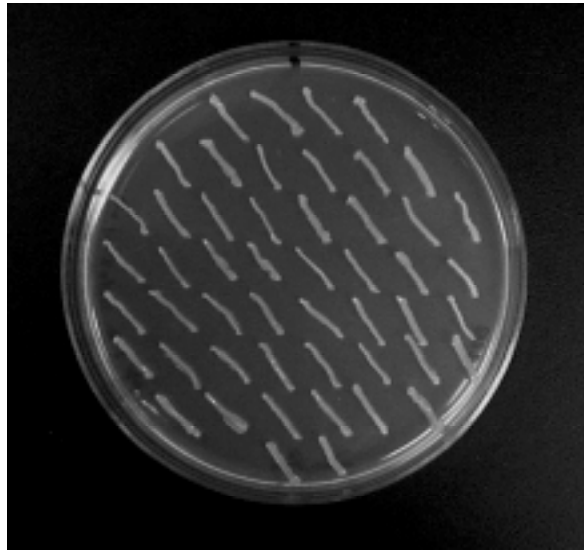
We questioned whether the addition of a small amount of detergent-based cell lysis buffer (used to make crude lysates) would increase the transformation efficiency of CsCl-purified donor DNA to levels similar to those obtained with DNA in the crude cellular lysate. It should be noted that many early transformations which included lysis buffer exhibited no cell growth after treatment due to near complete recipient cell death attributed to the effects of the detergent on the plasma membrane. Over time, an optimal lysis buffer concentration was determined, much as an LD-50 would be, to avoid this level of cell death. An optimal level of recipient cell survival leading to the maximum number of transformants was in the range of 5-10% of input cells. The addition of “lysis solution” to the transformation mixture gave an order of magnitude increase in transformation rates.

When including lysis solution in the transformation mixture, the bacterial cells occasionally formed individual colonies instead of the usual area of confluent growth at the site of inoculation. This was due to the reduced number of total viable cells. Individual colonies were isolated when possible and screened immediately for the presence of transformants. Therefore, it was not necessary to recover and dilute the confluent spot of cell growth as in previous transformation experiments and the original *Acinetobacter* transformation protocol was modified to accommodate this phenomenon.

When individual colonies were not present, confluent growth was diluted and individual isolates obtained before phenotypic screening. Isolates were grown on both non-selective and selective media and the transformation efficiency was calculated by dividing the total number of individuals isolated by the number of isolates positive for growth on selective medium.

Another interesting phenomenon was observed while recording the experimental results of the dilute lysis solution transformations. Transfer to selective plates indicated that 100% of the colonies from non-selective plates contained transformants. However, many replicated patches on the selective plates grew as only a few distinct colonies (Fig. 16 B) in the inoculated area instead of the expected confluent growth pattern (Fig. 16 A). Therefore, it did not appear that 100% of the cells in each colony had in fact been transformed. This result suggests that in many cases the transformation event(s) are taking place after the onset of colony formation. It is important to note that *A. calcoaceticus* is a metabolically capable organism and sometimes form “micro-colonies” on agar plates, thus allowing one cell of a “micro-colony” to be transformed later on and produce a large transformed colony. This phenomenon could cause inaccuracy in calculation of transformation rates and might explain some differences observed. It is probably not as important in understanding transformation mechanistically as it is from understanding transformation operationally. It is very important to re-screen colonies on selective medium to ensure a pure colony. The “micro-colony” issue can also be addressed by the use of auxotrophic strains and will be discussed as appropriate in subsequent sections.

A



B

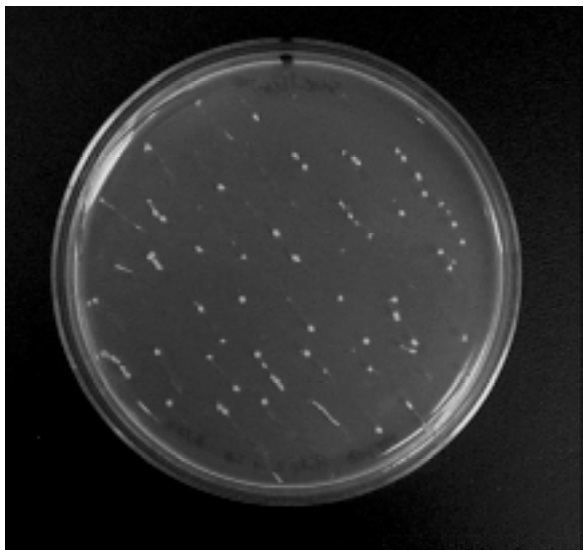


Fig. 16 Replica plates demonstrating the effect of dilute lysis solution in the transformation mixture on transformation rate. Individual isolates were patched onto non-selective (10 mM succinate minimal medium) and selective (5 mM POB minimal medium) agar plates using the master plate grid (see Material and Methods). **A.)** Confluent growth in the inoculated areas. **B.)** Individual colonies in the inoculated areas indicating the bacterial colony selected for patching was not comprised of a single pure transformant clone.

Summary of optimization of transformation protocol

Individual factors such as growth phase of the recipient cell, donor DNA concentration, conformation, size and gene dosage were addressed in order to optimize conditions and obtain maximum transformation efficiencies. The data generated by this study indicate that the highest transformation rates are obtained using recipient cells entering log phase and closed circle donor DNA at high gene dosage. Donor DNA must also have the largest amount of homologous sequence possible flanking the locus being transformed, but the constructs do not have to be so large as to make it difficult to engineer or prevent easy modification of the donor DNA. For example, pZR1 as donor DNA provides a higher transformation rate than pZR2, yet plasmids the size of pZR1 (13.7 kbp) are still manageable and pZR2-sized (5.1 kbp) plasmids still provide transformation, although at a lower rate. These conditions are readily obtained and therefore are not obstacles to general application of an approach for engineering large plasmids, such as producing the transforming molecules by subcloning. The complete basis for high transforming ability of *A. calcoaceticus* genomic DNA is still not clear, but will continue to be addressed in the following sections. At this time, a mechanism has not been identified that simulates the unidentified properties of genomic DNA for a cloned DNA fragment.

Engineering of TOL genes for the generation of donor and target constructs

Doten (1987b) found that specific “targeted” sequence deletions could be inserted into the chromosome of *Acinetobacter* by natural transformation. He demonstrated that

small segments DNA could be genetically engineered *in vitro*, then used as donor DNA for transformation and the DNA would be taken up and exchanged for the homologous sequence in the cell. Our ability to engineer genes in the large TOL plasmid would obviously benefit from being able to carry out similar manipulations using natural transformation in *A. calcoaceticus*. Therefore, subclones containing mutant TOL segments for use as donor DNAs in transformation experiments were constructed. Desired features of the mutant TOL DNA segments included the ability of the modified locus/allele to be screened for in a simple and rapid manner. A detailed nucleotide sequence of the TOL *meta*-cleavage operon and a restriction map elucidated by a previous doctoral student in our laboratory (Hares, 1998) allowed the selection of recombinant TOL segments which could be easily mutated. These were used to construct mutant TOL segments to transform pDKR1 in *A. calcoaceticus*.

Generation of pPW596 containing Δ *xyLL*'T159

An *E. coli* DH5 α strain containing the plasmid pBK391 was acquired from Doug Hares (1998) in order to produce this construct. pBK391 contains the *xyLLT* region on a 2.1-kbp *PvuII/SmaI* fragment in pUC19. The plasmid was used to create a 159 bp internal deletion in *xyLL* by *XhoI* restriction digestion. *XhoI* digestion cut out a 159 bp segment of DNA between two *XhoI* sites internal to *xyLL*. After digestion, the two flanking *XhoI* sites were ligated. The ligation DNA (mixture) was then transformed into competent *E. coli* DH5 α cells and the presence of the deletion was confirmed by rapid plasmid isolation (see Materials and Methods) and agarose gel analysis (Fig. 17). Lane

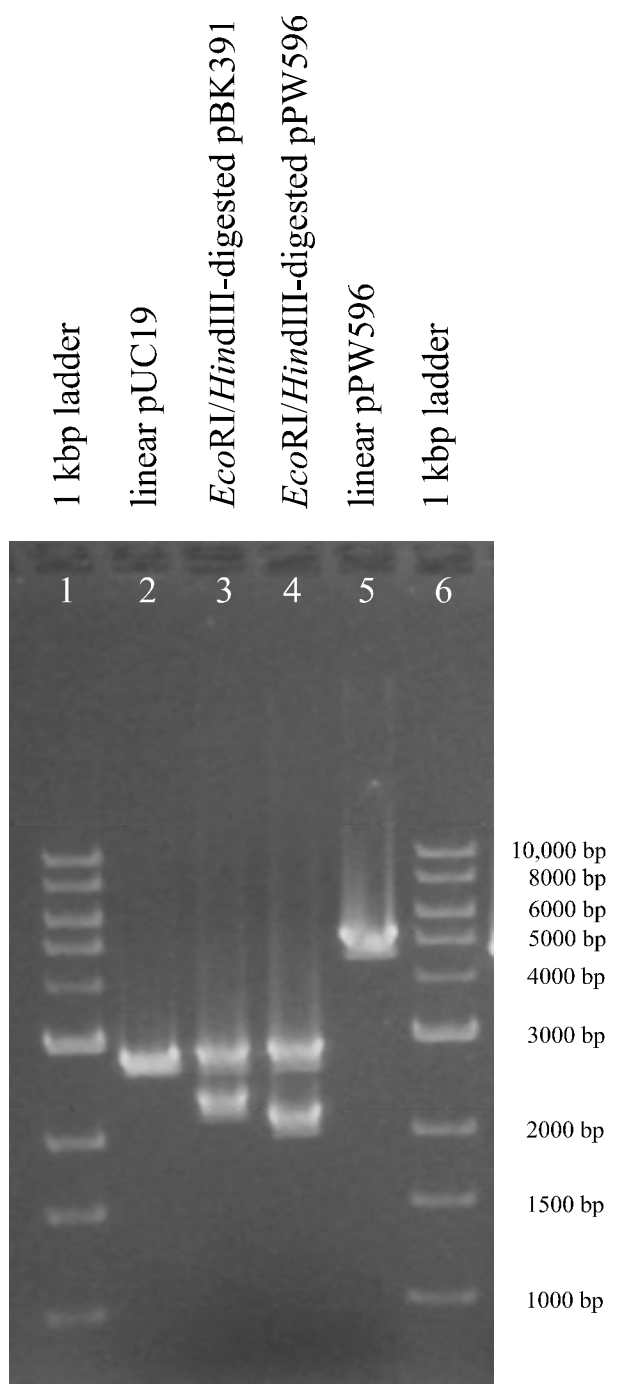


Fig. 17 Agarose gel demonstrating the presence of the $\Delta xyiL'T159$ fragment in pPW596. Samples of pBK391 and pPW596 were digested with *EcoRI* and *HindIII*, loaded onto a 1% TBE agarose gel and electrophoresed in 1X TBE buffer for 90 minutes at 83 V. A reference size marker was loaded onto lanes 1 and 6. Linearized pUC19 vector (lane 2) and *EcoRI/HindII* digested pBK391 (lane 3) were used as controls. The presence of a deletion in *xyiL* is indicated by the smaller size of the pPW596 insert (lane 4) as compared to the wild-type pBK391 insert (lane 3).

three contains pBK391 with the wild-type *xyiL*. Lane four contains *xyiL* with the internal 159 bp *XhoI* deletion. Presence of the deletion was verified by the size reduction of the insert contained in the multiple cloning site of pUC19. A preparative scale plasmid isolation (see Materials and Methods) was performed to obtain the plasmid DNA containing the *XhoI* deletion used for further experimentation. The subcloned *xyiLT* region fragment containing the 159 bp *xyiL* deletion was given the designation $\Delta xyiL'T159$ and pUC19 containing $\Delta xyiL'T159$ was designated pPW596. This construct was made to determine if a deletion can be inserted into pDKR1 by transformation in *A. calcoaceticus*.

Subcloning of $\Delta xyiL'T159$ into pUCP30T

Transfer of plasmids, containing engineered segments of DNA, by conjugation from *E. coli* and *P. putida* to *A. calcoaceticus* provides an advantage in experimentation. It has been previously demonstrated by this study that both *A. calcoaceticus* and *P. putida* have the ability to transfer and maintain, in a stable fashion, a plasmid containing a broad host range integrate. pDKR1 was chosen over pDK1 for its RP1 cointegrate, as well as for the transfer functions RP1 possess. These functions overcome several problems encountered when dealing with a large plasmid such as pDKR1, primarily size. In the case of *A. calcoaceticus*, natural and artificial transformation are not mechanisms capable of internalizing intact plasmids because internalized DNA is linearized upon entry and remains in a non-active linear form once inside the cell. Therefore, it was necessary to put all targets for use in subsequent experimentation in a form which could be conjugated into *A. calcoaceticus*.

The transfer of plasmids to *Acinetobacter* by conjugation allows a variety of targets to be utilized for demonstrating transformation of plasmid DNA molecules. One such recombinant DNA molecule, was to be the $\Delta xyL'T159$ fragment. Not only will $\Delta xyL'T159$ be used as a donor DNA, but in the appropriate vector, it could also be used as a target. Such a target will allow us to analyze any differences in the ability to repair a deletion over inserting one. Therefore, pUCP30T (Schweizer *et al.*, 1996) was obtained from Daniel Kunz. The vector contains a broad host range origin derived from pRO1600 (Jansons *et al.*, 1994) and the multiple cloning site of pUC, as well as transfer functions and antibiotic resistance markers. The $\Delta xyL'T159$ insert was removed from pPW596 by restriction digestion with the enzymes *EcoRI* and *HindIII*. This was then purified by vertical agarose gel electrophoresis (see Materials and Methods). pUCP30T was also digested with *EcoRI* and *HindIII* and the $\Delta xyL'T159$ fragment inserted by ligation. Fig. 18 shows *EcoRI/HindIII*-digested pUCP30T in lane 2, purified $\Delta xyL'T159$ *EcoRI/HindIII* fragment in lane 3 and the newly created plasmid in lane 4. The new plasmid was designated pPW1000.

Subcloning of pRAY into pUCP30T

The transformation system of *Haemophilus influenzae* requires the donor DNA to contain recognition sequences for successful uptake and transformation of the recipient cell. Similarly, *E. coli* contains Chi sites which are specific nucleotide sequences that create hot spots of increased homologous recombination. Genomic DNA from ADP1 has an increased transforming activity well beyond what would be logically predicted from

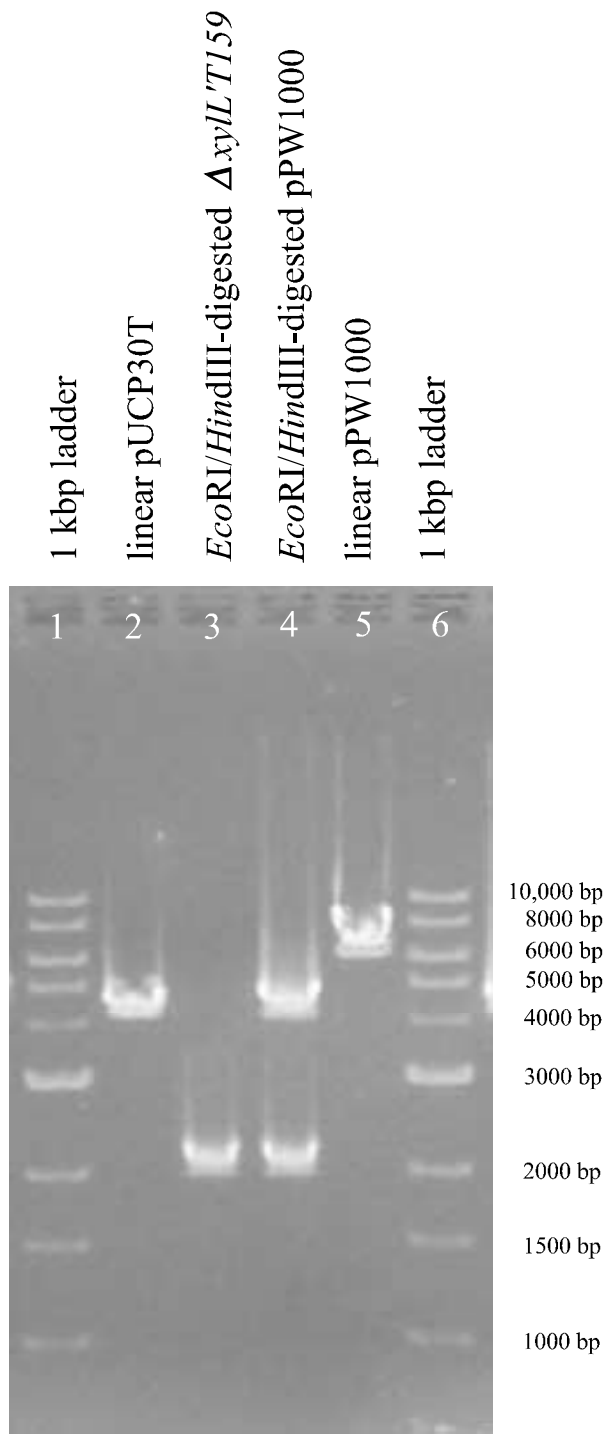


Fig. 18 Agarose gel demonstrating the presence of the $\Delta xyL'T159$ fragment in pPW1000. Plasmid DNA was digested with *EcoRI* and *HindIII*, loaded onto a 1% TBE agarose gel along with appropriate markers and electrophoresed in 1X TBE buffer for 90 minutes at 83 V. Reference size markers are in lanes 1 and 6. Linear pUCP30T is in lane 2 and purified $\Delta xyL'T159$ fragment is in lane 3. The newly created plasmid is in lane 4.

concentration and size alone. It is reasonable to suggest that any characteristic that might identify ADP1 genomic DNA to the recipient cell as an appropriate DNA donor for transformation would be conferred to many or all DNAs which are resident in *A. calcoaceticus* cells for an extended period of time (e.g. phage or plasmid as in *H. influenzae*). Therefore, a resident *A. calcoaceticus* plasmid, pRAY fused to pUC19 (pHS500), was acquired from Heidi Segal (Segal & Elisha, 1999) to see if it might be a better recipient vector due to the possibility of uptake sequences or a recombination requirement by *A. calcoaceticus*. The pRAY component was removed from pHS500 by *Bam*HI restriction digestion and the transfer functions and a broad host range origin of pUCP30T were added by ligation. *Bam*HI-linearized pRAY can be seen in the lane three of Fig. 19. Lane two contains *Bam*HI-linearized pUCP30T and lane four shows pRAY removed from pUCP30T by a double restriction endonuclease digestion with the enzymes *Eco*RI and *Pst*I. The new plasmid was designated pWS1001 and this was used as a vector for TOL constructs in *A. calcoaceticus* as described below.

Subcloning of $\Delta xyL'T159$ into pWS1001

A final step in creating a plasmid suitable for determining influence of possible specific *Acinetobacter* sequences on transformation efficiency was the addition of the $\Delta xyL'T159$ marker downstream of pRAY in pWS1001. A double digestion of pWS1001 with *Eco*RI and *Kpn*I provided a unique site of opening within the multiple cloning site and the necessary sticky ends for the insertion of $\Delta xyL'T159$. However, the $\Delta xyL'T159$ fragment is not flanked by a *Kpn*I site for creation of the matching end necessary for

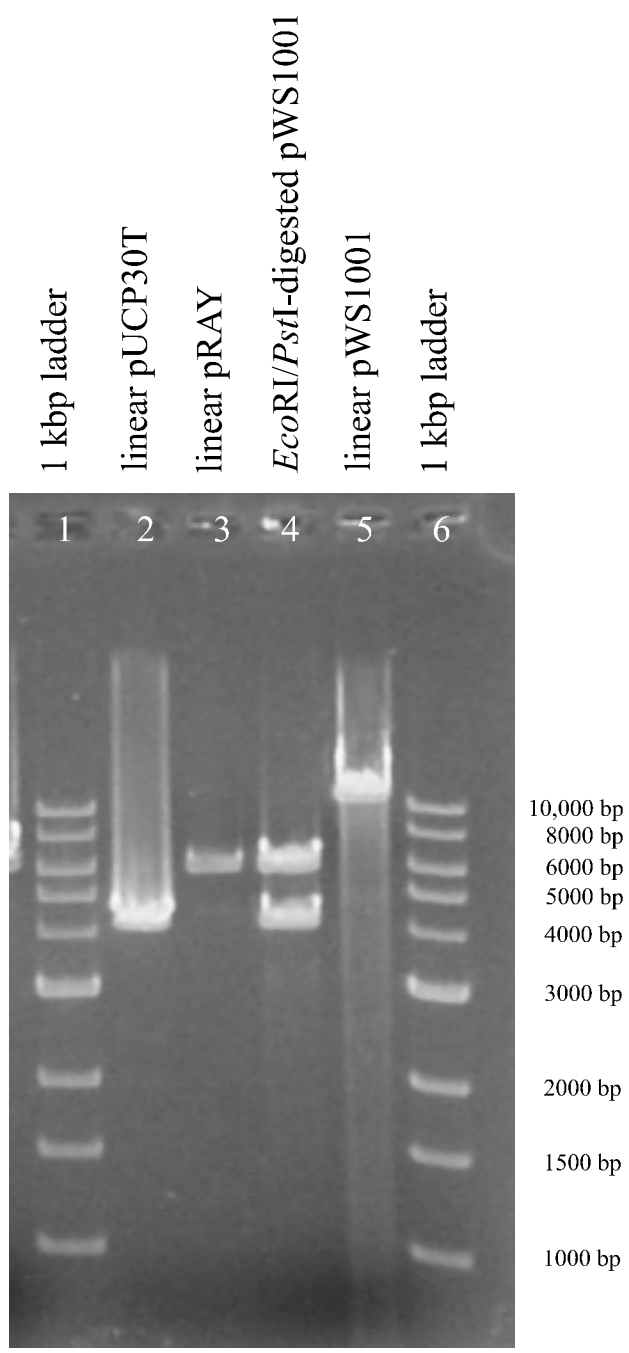


Fig. 19 Agarose gel demonstrating the presence of pRAY in pWS1001. Digestion of pWS1001 with the restriction enzymes *EcoRI* and *PstI* removes pRAY from the pUCP30T vector. The digested sample, along with appropriate size markers, was loaded onto a 1% TBE agarose gel and electrophoresed in 1X TBE buffer for 90 minutes at 83 V. Reference size markers are in lanes 1 and 6. Linear pUCP30T is in lane 2 and *Bam*HI-linearized pRAY is in lane 3. pWS1001 (with the inserted removed by digestion) is in lane 4 and linearized pWS1001 is in lane 5.

ligation of the insert to be successful. Therefore, an 8 bp DNA adapter was designed to join the 5' overhang of the insert's *Hind*III sticky end to the 3' overhang of the plasmid *Kpn*I site during the ligation process. The ligation mixture was then transformed into competent *E. coli* DH5 α cells and the presence of the $\Delta xyL'T159$ marker was confirmed by plasmid isolation (see Materials and Methods) and agarose gel analysis (Fig. 20). Lane 2 contains pUCP30T linearized with *Eco*RI, lane three contains the pWS1001 linearized with *Eco*RI and lane 4 contains the plasmid isolated after ligation of the insert $\Delta xyL'T159$ with the pWS1001. The insert shown in lane 4 was removed by a double restriction digestion with the enzymes *Eco*RI and *Pst*I. Lane 5 contains the new plasmid linearized with *Eco*RI. The new plasmid was given the designation pWS1100.

Generation of strains for transformation experiments

Previous experimentation (this study) has demonstrated the ability to transfer pDKR1 into *A. calcoaceticus* and maintain it there in a stable fashion. Other strains of *A. calcoaceticus* were created to provide improved markers for transformation studies. Many were created to address specific concerns as indicated below.

Generation of A125B1 by natural transformation

One of our objectives was to determine if a segment of a plasmid-encoded gene could be deleted by transformation of that strain with donor DNA molecules from which this segment has been removed. As an initial step in this process, the experiments of Doten *et al.* (1987a) were partially replicated using *A. calcoaceticus* chromosomal

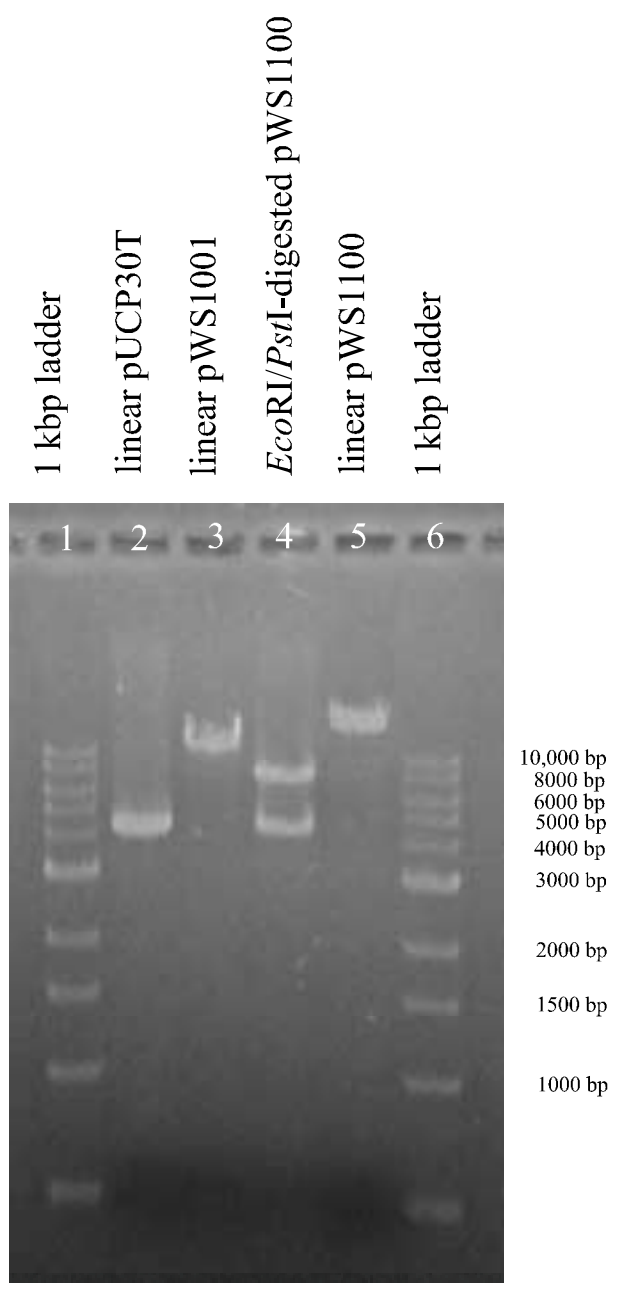


Fig. 20 Agarose gel showing the $\Delta xyLL'T159$ fragment in pWS1001. Digested samples, along with appropriate reference markers, were loaded onto a 1% TBE agarose gel and electrophoresed in 1X TBE buffer for 90 minutes at 83 V. As before, lanes 1 and 6 contain reference size markers. Lane 2 contains linear pUCP30T. Lane three contains *EcoRI*-linearized pWS1001. The pWS1100 insert shown in lane 4 was removed by a double restriction digestion with *EcoRI* and *PstI*. Lane 5 contains *EcoRI*-linearized pWS1100.

markers. Replication of the experiment would serve as validation of the optimized protocol, as well as establish a reference to which later transformation could be compared. Two strains, *A. calcoaceticus* ADP125 and *E. coli* JM107 containing the plasmid pPAN30, were acquired from Nicholas Ornston's laboratory (Yale University). ADP125 carries a deletion in the *pcaIJ* region of the *pca* operon. This mutation is required for the genetic stability of any mutation that might be transformed into the corresponding region of the *cat* pathway, as the *cat* and *pca* gene sequences are very similar and can contribute to the restoration of each other's function (R. C. Doten and L.N. Ornston, Abstr. Annual Meet. Am. Soc. Microbiol. 1985, K39, p. 178). The plasmid donor DNA pPAN30 ($\Delta catM'BJ'FD30$ on 3.6 kbp *EcoRI* fragment in pUC19) was created from pAN2 (*catM'BCIJFD* on 5 kbp *EcoRI* fragment in pBR322) by removing of a 1.4 kbp segment of DNA between two *SalI* restrictions sites internal to the cloned fragment. Plasmid donor DNA was prepared using the preparative scale plasmid isolation protocol of Birnhoim and Doly (1979). *A. calcoaceticus* ADP125 was transformed using the standard *Acinetobacter* transformation protocol (see Materials and Methods). Transformants were screened for the acquired Ben⁻ phenotype using a master plate screening grid with 10 mM succinate minimal medium plates as a non-selective control, and with 5 mM benzoate minimal medium plates for selection against the Ben⁻ phenotype. A total of 1,150 colonies from 11 different transformation experiments were screened, yielding seven colonies displaying a Ben⁻ phenotype. This represents a transformation rate of 0.6%, a 30-fold decrease from that reported by Doten *et al.* (1987a), but in line with transformation efficiencies reported for *A. calcoaceticus* by

other investigators (Cruze *et al.*, 1979; Juni, 1972; Kok *et al.*, 1999; Lorenz *et al.*, 1992; Palmen *et al.*, 1993; Porstendorfer *et al.*, 1997). The ADP125 transformants demonstrating a Ben⁻ phenotype were designated A125B1 through A125B7 and frozen for long-term storage.

Generation of A6R1, A125B1R1 and A141R1 by conjugation

The ability to screen for a phenotypic conversion concurrently with a genotypic conversion speed the isolation of desired transformants although the frequency of transformation in *A. calcoaceticus* has been demonstrated to be sufficiently high (on occasion) to allow screening of transformants by genotypic rather than phenotypic properties (Doten *et al.*, 1987a). Cotransformation, or transformation with two donor DNAs concurrently, can serve important functions. Phenotypic conversion of a chromosomal locus by one of the donor DNAs contained in a transforming sample acts as a positive control for the transformation experiment by identifying recipient cells that were indeed in a competent state and that had been successfully transformed at one locus. It is reasonable to suggest that *A. calcoaceticus* does not have the ability to discriminate between two donor DNA molecules and therefore, transformation of one locus correlates with the uptake and transformation by the second donor DNA. The coupling of phenotypic selection and genotypic screening of markers can thus provide a significant advantage with the number of colonies which must be screened to find transformants at the second locus. For this reason, additional strains of *A. calcoaceticus* were created to provide a range of selectable markers for cotransformation studies.

Specific *A. calcoaceticus* strains were chosen to create suitable cotransformation marker systems. ADP6 was chosen because the establishment of the transformation protocol was based up the POB⁻ to POB⁺ conversion and this was therefore a well characterized system. A125B1 was chosen because it had been created through transformation, validating the transformability of the Ben locus and Ben⁺ is an easily selected phenotype. The Ben⁻ phenotype was also considered to be more genetically stable than the *pca* mutation in ADP6 due to the “double knock out” approach used, reducing the frequency of spontaneous phenotypic reversion which might be misinterpreted as a transformation event. *A. calcoaceticus* Ac141 is an arginine auxotroph and was utilized in an effort to reduce the “micro-colony” background on transformation plates by providing tighter selection than a block in a catabolic pathway (like that of ADP6 or A125B1).

Each strain used in cotransformation experiments was individually conjugated with *P. putida* (PaW630) containing pDKR1. As with ADP1, conjugations were carried out for 24 hours at 37 °C on LB media and aliquots were plated on minimal medium plates containing 10 mM succinate as the carbon source with the appropriate amount of kanamycin for selection. Colonies that displayed a resistance to kanamycin were analyzed using the same protocols used for verification of pDKR1 in ADP1 (see Verification of pDKR1 TOL genes in *Acinetobacter* strains). Verification included total DNA isolation of transconjugant strains and the subsequent hybridization with radiolabeled TOL DNA probes (Fig. 9), PCR amplification using TOL specific primers (Fig. 10), oxidase tests and transformation assays (phenotypic reversion of ADP6 from

POB⁻ to POB⁺ by using total DNA isolated from the transconjugant strains demonstrated that the *Acinetobacter* genetic background was present).

Colonies selected for use in subsequent experiments demonstrated all appropriate pDKR1 phenotypic antibiotic resistances and multiple isolates of each *A. calcoaceticus* transconjugant, verified to contain pDKR1, were then designated as A6R1-20 (being derived from ADP6), A125B1R1-20 (being derived from A125B1) and A141R1-20 (being derived from Ac141). These were frozen for long-term storage.

Generation of A6P1, A6P2, A141P1, A141P2, A141R1P1 by triparental mating

Triparental mating (see Materials and Methods) was used to transfer previously assembled recombinant DNA constructs, pPW1000 and pPW1100, into *A. calcoaceticus* strains ADP6, Ac141 and A141R1. Strain names were modified to include the designator P1 for those which contain pPW1000 (A6P1, A141P1 and A141R1P1) and the designator P2 for those which contain pWS1100 (A6P2 and A141P2).

The pPW1000 and pWS1100 plasmids, once transferred to *A. calcoaceticus*, were used as targets for transformation. Transformation of targets which varied in size and source provided additional data to aid in further refining the transformation parameters while continuing development of transformation-based site-directed mutagenesis protocols for large plasmids. A difference in the ability to transform the plasmid borne gene targets of pPW1000, pWS1100, and pDKR1 might be attributable to a physical characteristic of the DNA molecule and thereby provide a better understanding of *A. calcoaceticus* transformation. Specifically, comparisons could be made in regards to

plasmid size, G+C content of target gene and the presence of *Acinetobacter* sequences within the plasmid construct.

Transformations of the pDKR1 TOL plasmid with the standard *Acinetobacter* transformation protocol

Transformation of A1R1 with pPW596

The standard *Acinetobacter* transformation protocol (see Materials and Methods) was utilized to transform pDKR1 in the recipient strain A1R1 with the $\Delta xyL'T159$ marker of pPW596. Two experiments were performed, one using circular pPW596 as donor DNA. The second used linear pPW596 a donor DNA, with the $\Delta xyL'T159$ segment removed by *EcoRI* and *HindIII* digestion. Aliquots of the conjugation mixture were spread on minimal medium plates containing 10 mM succinate as the sole carbon and energy source and 100 μ g kanamycin per ml (in order to maintain the plasmid in the recipient strain). After incubation, the total cellular DNA of 100 isolates was prepared for experiments with each donor DNA type. PCR amplification using *xyL* region primers was performed and the PCR products were screened for incorporation of the $\Delta xyL'T159$ marker into the pDKR1 plasmid (Fig. 21). Total DNA isolated from ADP1, PaW630 containing pDKR1, wild-type PRS2000, and *E. coli* containing pPW596 (a control for PCR). All isolates were negative for the presence of $\Delta xyL'T159$ in pDKR1. The experiment was repeated a second time with similar negative results.

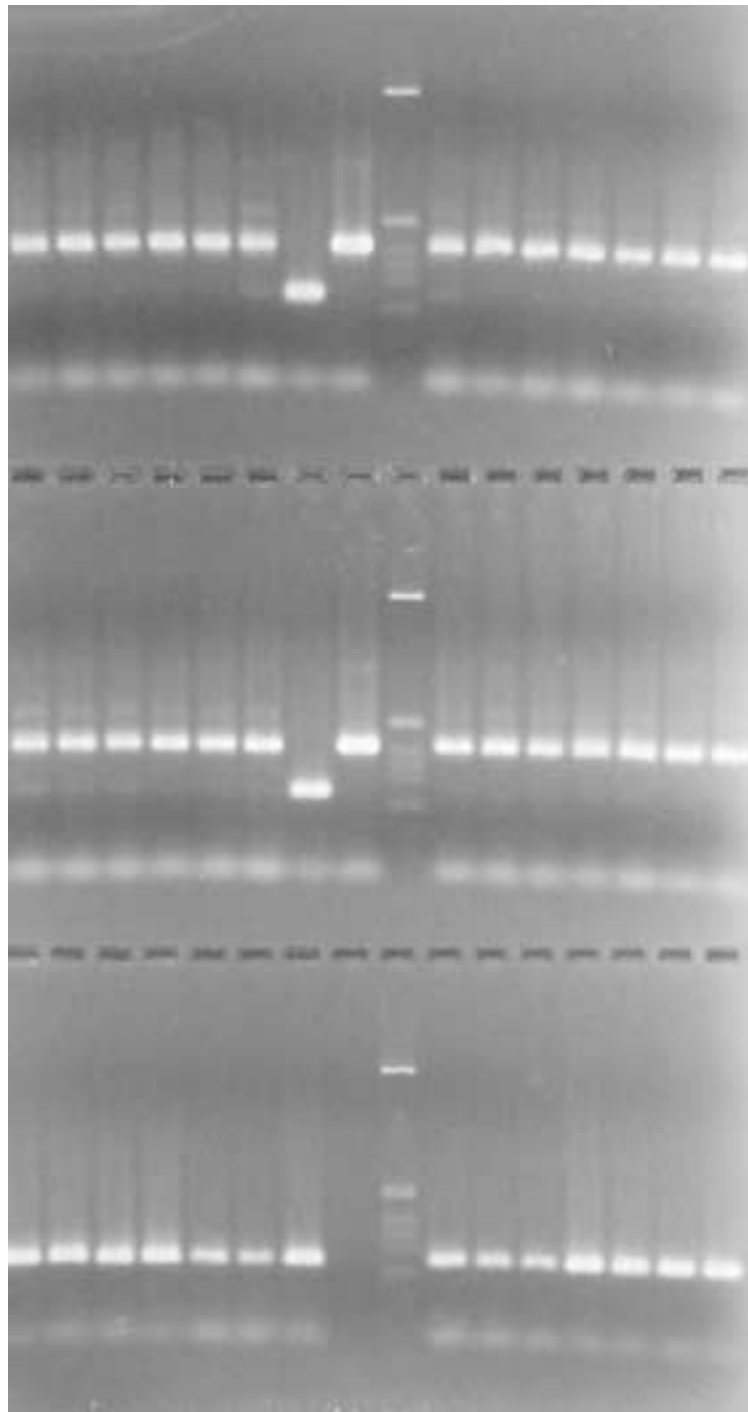


Fig. 21 Agarose gel demonstrating the absence of $\Delta xyiL'T159$ fragment in pDKR1. The TOL *xyiL* region targets for transformation was amplified by PCR. PCR products were loaded onto a 2% TBE agarose gel and electrophoresed in 1X TBE buffer for 150 minutes at 83 V, stained with EtBr and recorded as a digital image. PCR amplification products from pPW596 and pBK391 DNAs (mutant and wild-type *xyiL*, respectively) used for size reference and positive PCR amplification controls. *HinfI*-digested pBR322 was used for a size reference ladder. Results were obtained with other experiments which appear visually similar and are denoted (data not shown).

Cotransformation of A6R1 with pPW596 and pZR1 DNAs

A1R1 was not considered a suitable recipient strain due to the lack of any observable cotransformation marker indicating that cells had actually taken up donor DNA. A6R1 was utilized to alleviate this problem and the conversion of a POB⁻ phenotype to one of POB⁺ was used as an indication of transformation.

Cotransformations were performed using the standard *Acinetobacter* transformation protocol (see Materials and Methods) with circular pPW596 and pZR1 as the donor DNAs. Donor DNAs were added in equimolar concentrations to approximate equivalent gene dosage. Minimal medium plates containing 5 mM *para*-hydroxybenzoate (POB) and kanamycin at a concentration of 100 µg/ml were used to select transformants. A total of 150 colonies with a POB⁺ Km^r phenotype were selected and template DNA isolated from each individual by the colony PCR method (see Materials and Methods) on two occasions. PCR amplifications were performed using the *xyiL* region DNA primers and CsCl-purified plasmid DNAs from pPW596 and pBK391 as amplification controls. PCR products were screened by agarose gel analysis for the transfer of $\Delta xyiL'T159$ to pDKR1. Neither transformation experiment produced a modified pDKR1 plasmid (data not shown).

Cotransformation of A125B1R1 with pPW596 and ADP125 genomic DNA

Reversion of a single mutation in the *pca* genes is likely to occur by restoration of function from corresponding regions of the *cat* pathway (R. C. Doten and L.N. Ornston, Abstr. Annual Meet. Am. Soc. Microbiol. 1985, K39, p. 178). Strain A125B1R1 was

selected to increase the probability of obtaining a pDKR1 transformant in the event that reversion in the A6R1 cotransformations might be “masking” actual transformation events. Strain A125B1R1 provides a different, more genetically stable selectable marker by which to assess transformation. Cotransformations were carried out with genomic ADP125 DNA and circular pPW596 as the donor DNA molecules and A125B1R1 as the recipient. The transformation mixture was spotted onto a non-selective 10 mM succinate plate and incubated at 37 °C for 18-24 hours. Transformants were isolated by spreading dilutions of the resulting cell growth on minimal medium plates containing 5 mM benzoate as the sole carbon source and kanamycin at a concentration of 100 µg/ml. Plates were scored for cell growth and one hundred colonies with a Ben⁺ Km^r phenotype were selected for screening by PCR amplification using the *xyL* region DNA primers. Total DNA was isolated from each transformant, as well as from strains used for positive and negative controls. These included A125B1, A125B1R1, PaW630 containing pDKR1 and *P. putida* PRS2000. All transformants were negative for the presence of the $\Delta xyL'T159$ marker in pDKR1. The experiment was performed in triplicate and a second a third group of 100 transformants also did not contain pDKR1 with the $\Delta xyL'T159$ marker incorporated by transformation (data not shown).

Cotransformation of A141R1 with pPW596 and ADP1 genomic DNA

Given the frequency with which *pca* mutants (ADP6) and *cat* mutants (A125B1) can spontaneously revert or become “leaky” (causing micro-colonies due to carbon scavenging), we decided to use auxotrophy to prototrophy conversion (e.g. Arg⁻ → Arg⁺)

for cotransformation selection. This reduces background and increases potential for recovery of a double transformant. The standard *Acinetobacter* transformation protocol (see Materials and Methods) was performed using A141R1 as the recipient cell. Conversion of an Arg⁻ phenotype to Arg⁺ was used as a marker by which to judge successful transformation. Transformations were performed with genomic ADP1 DNA and circular pPW596 as the donor DNA molecules and A141R1. Transformants were isolated and subjected to selection on 10 mM succinate with kanamycin at a concentration of 100 µg/ml. One hundred colonies with a wild-type Km^r phenotype were prepared as templates for PCR amplification (using the *xyiL* region DNA primers) by the colony PCR method (see Materials and Methods). PCR products were screened for the presence of $\Delta xyiL'T159$ marker incorporation in pDKR1 by agarose gel analysis. One hundred transformants were shown to be negative for the presence of the marker on two separate occasions (data not shown).

Cotransformation of A125B1R1 with pBK692mut3 and ADP125 genomic DNA

Exchange of the small (159 bp) deletion of the $\Delta xyiL'T159$ fragment with the wild-type sequence of pDKR1 may, for some unknown reason, not be as favored as other donor DNA molecules. Transformation of pDKR1 with a single point mutation, providing the highest similarity to the target locus, could increase transformation efficiency. Therefore, the plasmid construct pBK692mut3 (from our laboratory reference collection) containing a point mutation in *xyiT* was utilized to investigate this hypothesis. The transversion of a cytosine residue to adenine (Fig. 22) causes the creation of a

(A)
TTC CGT TGC CTG CCG GAG

TGC → TGA
Cys Stop

Fig. 22 Nucleotide sequence of the *xylT* region mutated in pBK692mut3. The red cytosine residue was the nucleotide targeted for mutagenesis. The red nucleotide in parenthesis indicates the mutation incorporated. The change of cytosine to adenine caused a premature stop in the *xylT* ORF at this point.

nonsense codon from one coding for cysteine, thereby acting as a translational terminator. If successful conversion of pDKR1 was achieved, similar manipulations would allow the highly specific modification of genes, so this avenue of investigation was of great interest. The cotransformation experiment was performed with genomic ADP125 DNA and circular plasmid pBK692mut3 as the donor DNA molecules and A125B1R1 as the recipient *Acinetobacter* strain. Transformants were isolated on 10 mM benzoate minimal medium plates with kanamycin at a concentration of 100 µg/ml. One hundred and fifty colonies with a Ben⁺ Km^r phenotype were selected for PCR amplification using the mut3 region DNA primers and PCR amplification was performed on total DNA. The absence of a size difference between the two alleles required that incorporated pBK692mut3 point mutations into pDKR1 be identified by DNA sequence analysis of the PCR products. Sequence analysis indicated that all 150 transformants were negative for the presence of the pBK692mut3 point mutation (data not shown).

Modification of the standard *Acinetobacter* transformation protocol

A suitable explanation for our inability to isolate a pDKR1 transformant eluded us at this point. Several aspects of the *Acinetobacter* transformation protocol that might lead to reduced transformation efficiency of $\Delta xylL'T159$ were considered. Until this point, all transformations were performed with saturating concentrations of all donor DNA.

Ahmadian-Tehrani (1990) and Lorenz *et al.* (1992) have demonstrated that transformation efficiency is reduced in the presence of competing DNA. Our protocols

involving cotransformation of two loci seemed particularly susceptible to negative saturation effects, especially if one of the donor DNAs was preferentially utilized at the expense of the other. Total DNA levels were therefore reduced ten-fold to limit the possible effect of competition. A reduction of donor DNA concentration might increase the probability of less preferred donor DNA molecules being taken up (if the *A. calcoaceticus* transformation system is not truly indiscriminate of the source of the donor). Another parameter of the *Acinetobacter* transformation protocol that had not been previously investigated was the effect of anaerobic growth on cellular competence during transformation. It has been shown that moderately anaerobic growth conditions increase *A. calcoaceticus* transformation rates (Gerard O'Donovan, personal communication). Therefore, the standard *Acinetobacter* protocol was modified as described below to address these issues. The modified transformation protocol was first tested with ADP6, as it had been well characterized and familiarity with cellular response to the POB⁻ to POB⁺ conversion reduced the introduction of extraneous variables.

Cotransformation studies using model constructs

Previous characterization of *A. calcoaceticus* transformation has focused on properties of the donor DNA molecules and the competent state of the *A. calcoaceticus* cell being transformed. Properties of the target molecule might also be important to the process of transformation. With this in mind, strains A6P2 (A6R1 containing pWS1100), A141P1 (Ac141 containing pPW1000) and A141P2 (Ac141 containing pWS1100) were created to compare the efficiency of transforming different target plasmids. This would

provide a basis for understanding which, if any, characteristics of the recipient DNA have an effect on transformation efficiency. The effect target plasmid size might have on transformation rate was addressed by both plasmid constructs (pPW1000 and pWS1100 - 6.2 and 12.3 kbp, respectively). Each contains the conjugable vector pUCP30T and both are significantly smaller than pDKR1 (100 kbp). Any effect on transformation rate resulting from the presence of an *A. calcoaceticus* sequence in the target vector would be evident from the use of pWS1100, which contains 6.1 kbp of *A. calcoaceticus* plasmid DNA joined with the $\Delta xyL'T159$ fragment in the MCS of pUCP30T. Auxotrophic (Ac141) and catabolic mutant (ADP6) strains of *Acinetobacter* were used as hosts for the plasmid targets during cotransformation experiments. Transformation rate changes caused by host strain interaction with the target vector is inferred by a comparison of pWS1100 transformation rates in different *A. calcoaceticus* strains (Ac141 versus ADP6). Both plasmid targets contain the $\Delta xyL'T159$ fragment, thereby providing the ability to determine whether a difference in transformation efficiency would be observed by inserting a segment of DNA into a deleted target DNA instead of deleting a segment by transformation of a wild-type locus with a “deleted” donor. In each case, a segment of DNA in the heteroduplex will be looped out due to the lack of a complementary segment. However, in one case the “bulge” will be in the donor strand while the other will bulge out the recipient strand. Due to the conformation barriers which might exist when exchange occurs between two DNA molecules which are not homologous for a significant stretch (i.e. 159 bp), it was tested both ways.

Cotransformation of A6P2 with pBK391 and pZR1 DNAs

Strain A6P2 contains pWS1100 (pUCP30T joined with the $\Delta xyL'T159$ fragment and the *A. calcoaceticus* plasmid pRAY). A requirement for an *A. calcoaceticus* sequence as an identifier for the transformation apparatus transformation should be satisfied by the presence of pRAY in the target DNA. Conversion of the targeted plasmid locus by wild-type *xyLLT* (pBK391) would restore the mutant *xyLL* fragment back to wild-type. The modified *Acinetobacter* transformation protocol was used to cotransform A6P2 with pBK391 and pZR1 donor DNAs and the conversion of the recipient A6P2 cells from a POB⁻ to POB⁺ phenotype was used for selection of successfully transformed cells. Selection was on 10 mM POB with gentamicin at concentration of 10 µg/ml to ensure the continued presence of pWS1100. Two sets of one hundred and fifty transformants exhibiting a POB⁺ Gm^r phenotype were selected for PCR screening. DNA templates were prepared by the colony PCR method and PCR amplification was performed using the using the *xyLL* region DNA primers. PCR products were screened for conversion of the $\Delta xyLL'T159$ marker to a wild-type conformation by agarose gel analysis using pBK391 and pPW596 template PCR products as reference size markers. Conversion of the 200 bp “deleted” PCR product to the 360 bp wild-type size would indicate successful transformation of the plasmid. Both sets of transformants were negative for the incorporation of the wild-type TOL DNA (data not shown).

Cotransformation of A141P1 with pBK391 and ADP1 genomic DNA

Transformation studies using pPW1000 targets ($\Delta xyL'T159$ in pUCP30T) were performed in Arg⁻ strains of *Acinetobacter* (Ac141) for a comparison with transformation efficiency of plasmid targets containing the *A. calcoaceticus*' plasmid pRAY. A141P1 was cotransformed with circular wild-type *xyLLT* (pBK391) and genomic ADP1 donor DNAs using the modified *Acinetobacter* transformation protocol. Recipient cells were determined to have undergone transformation based upon the conversion of an Arg⁻ phenotype to Arg⁺. Transformants were then subjected to selection on 10 mM succinate with gentamicin at concentration of 10 µg/ml to ensure the presence of pPW1000. One hundred colonies with an Arg⁺ Gm^r phenotype were selected for PCR screening. PCR amplification using the *xyL* region DNA primers was performed on DNA templates prepared by the colony PCR method. Plasmid DNA from pBK391 and pPW596 were used as amplification controls and the plasmid PCR products were used as size reference markers. PCR products were screened for conversion of the $\Delta xyL'T159$ marker back to a wild-type conformation by agarose gel analysis. Both sets of one 100 transformants were negative for the incorporation of the wild-type *xyLLT* fragment (data not shown).

Cotransformation of A141P2 with pBK391 and ADP1 genomic DNA

A comparison of the transformation of A141P2 to A141P1 transformation rates addresses the possible effect *A. calcoaceticus* plasmid DNA sequences in the target pWS1100. Comparisons can be made between of A6P2 and A141P1 transformation rates, but the use of A141P2 eliminates uncontrolled variables introduced by comparing

experiments that use different cotransforming DNAs. A6P2 cotransformations used pZR1 donor DNA to transform a POB⁻ phenotype to POB⁺, whereas A141P1 and A141P2 both use genomic ADP1 donor DNA for the conversion of Arg⁻ to Arg⁺. The modified *Acinetobacter* transformation protocol was also used to transform recipient A141P2 cells containing pWS1100. Again, conversion from an Arg⁻ phenotype to a wild-type state was used as a selector of successful transformation. Transformations were performed with circular pBK391 and ADP1 genomic DNA as the donor DNAs. Transformants were isolated on 10 mM succinate with gentamicin at a concentration of 10 µg/ml. Two independent transformation experiments produced a set of one hundred transformants with an Arg⁺ phenotype. The transformants were screened by PCR amplification using the *xyiL* region DNA primers with DNA templates prepared by the colony PCR method. CsCl-purified pBK391 and pPW596 were used as controls for template amplification. Agarose gel analysis of transformant PCR products did not indicate the conversion of the $\Delta xyiL' T159$ marker back to the 360 bp wild-type sequence. Therefore, all transformants were considered to be negative for cotransformation of the targeted plasmid borne locus (data not shown).

Summary of cotransformation studies using model constructs

A variety of target plasmids were utilized to assess possible differences in transformation efficiency of plasmids. None of the transformation experiments demonstrated a significant increase in transformation efficiency above that previously observed with other targets (none of 200+ tested, therefore all experiments were less than 0.5%). Therefore, recipient plasmid size and the presence of *A. calcoaceticus* DNA

sequences in the target plasmid DNA can be ruled out as major contributing factors to the low plasmid transformation frequencies. With regard to transforming a locus where the donor and the recipient differ by the deletion of a sequence, it seems to make little difference in the transformation rate whether the deletion is in the recipient or the donor.

Cotransformation of A6R1 with pPW596 and pZR1 DNAs

The cotransformation of A6R1 was repeated using modified cotransformation conditions. Cotransformations were performed with circular pPW596 and pZR1. Equimolar amounts of the donor DNAs were used at 10% of the concentration required to saturate the system. The cotransformation mixture was incubated on a non-selective 10 mM succinate minimal medium agar plate at 37 °C for 70-80 hours under reduced oxygen content. The conditions were provided by covering the inoculated area with 0.7% sterile top-agar before the prolonged incubation. The resulting cell growth was collected by removing the top-agar with a sterile scalpel. Cells were then diluted and spread on minimal media plates containing 5 mM *para*-hydroxybenzoate (POB) and 100 µg of kanamycin per ml. Plates were scored for cell growth after an 18-24 hour incubation. A total of 150 colonies with a POB⁺ Km^r phenotype were selected and template DNA isolated from each individual by the colony PCR method (see Materials and Methods) PCR amplification was performed using the *xylL* region DNA primers and CsCl-purified plasmid DNAs from pPW596 and pBK391 were used as controls. PCR products were screened by agarose gel analysis for the transfer of $\Delta xylL'T159$ to pDKR1.

Three transformants were positive for the presence of the $\Delta xyL'T159$ marker in pDKR1 (Fig. 23 A, B, C).

It was unclear which change in the transformation protocol (DNA concentration or reduced oxygen) had provided the conditions for successful transformation of the TOL plasmid. Therefore, the experiment was repeated with the standard *Acinetobacter* transformation protocol, using the ten-fold reduction in donor DNA concentration. A total of 150 colonies displaying a $POB^+ Km^r$ phenotype were selected for PCR amplification using the *xyL* region DNA primers. Agarose gel analysis of the amplification products again revealed two transformants positive for the presence of the $\Delta xyL'T159$ marker in pDKR1. The standard *Acinetobacter* transformation protocol was thereafter modified to include the reduction of donor DNA concentration to 10% of saturation level after the replication of pDKR1 transformation.

Summary of pDKR1 transformation studies

The objective of modifying pDKR1 by transformation was the development of a mechanism for site-directed engineering of large bacterial plasmids. The novel approach was to create relatively small mutant donor DNAs (or clones) and to insert the mutant “allele” into corresponding locus of the larger target plasmid by transformation. Modification of pDKR1 in A6R1 was achieved by cotransformation with pZR1 and pPW596 DNAs. The average cotransformation efficiency of pDKR1 with the modified *Acinetobacter* transformation protocol was approximately 2%.

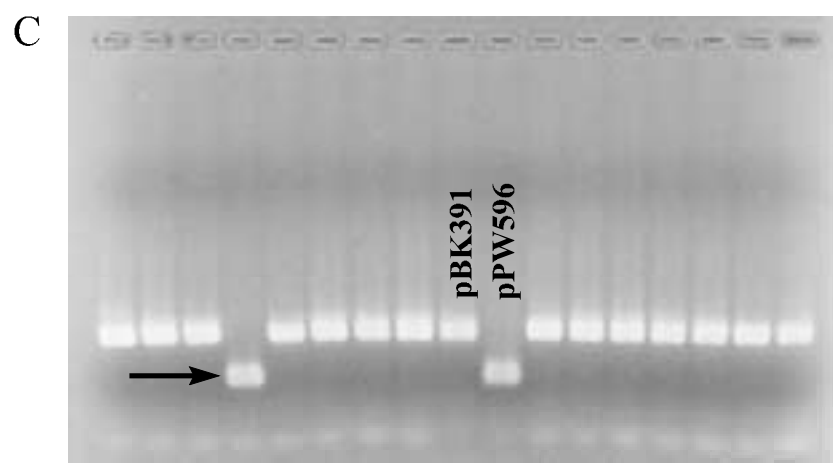
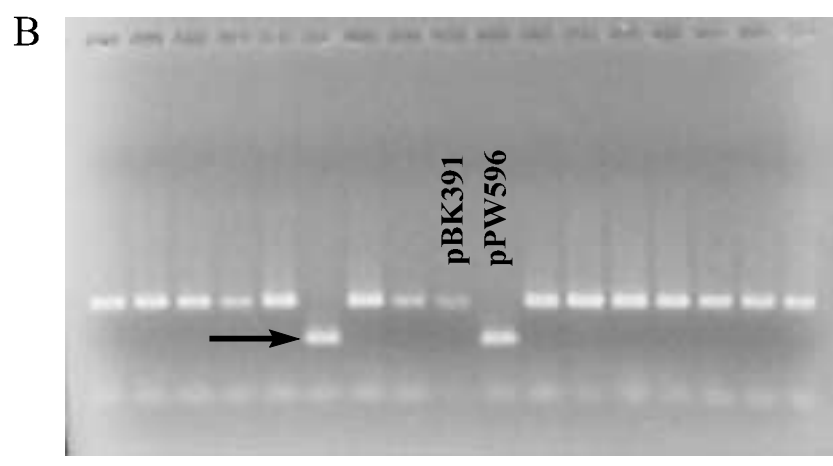
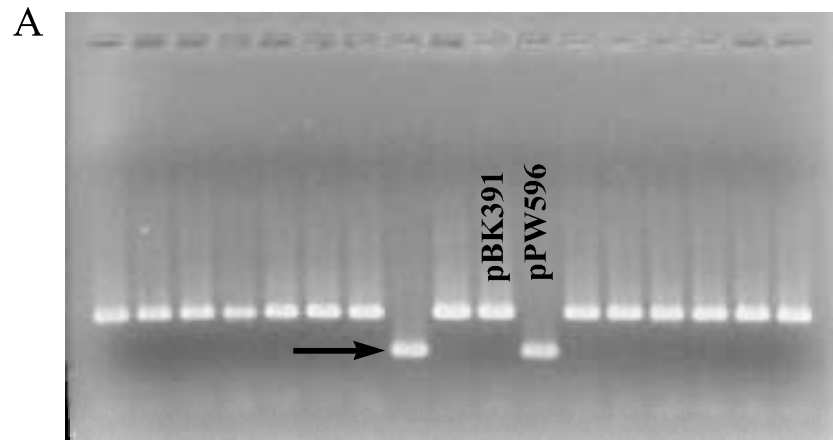


Fig. 23 A, B, C Agarose gel analysis demonstrating the incorporation of $\Delta xyL'T159$ into pDKR1. Transformant DNA was prepared for use as template for PCR by the colony PCR method (see Materials and Methods). PCR products were electrophoresed through a 2% TBE agarose gel at 83V for 150 minutes. The gels were stained with EtBr and recorded a digital images. Arrows indicate pDKR1 amplification products consistent with being derived from $\Delta xyL'T159$ transformed plasmids. Plasmids pBK391 and pPW596 were amplified as PCR controls and used as reference size markers.

Conjugation of modified pDKR1 into *P. putida*

Modified pDKR1 ($\Delta xyL'T159$) was transferred by conjugation from *A. calcoaceticus* strains to a recipient wild-type strain of *P. putida* PRS2000 which did not demonstrate any pDKR1 phenotypes prior to the procedure. The transconjugants were analyzed to verify appropriate pDKR1 phenotypic traits by growth on 5 mM *m*-toluate minimal medium plates containing the antibiotics tetracycline and kanamycin, as well as being subjected to an oxidase test. Also, transconjugant DNA templates produced by the colony PCR method (see Materials and Methods) were amplified using the *xyLLT* primer set. All strains selected for subsequent experimentation displayed the appropriate oxidase response, pDKR1 phenotypic responses and produced PCR products consistent with the presence of the $\Delta xyL'T159$ fragment. The strains were given the designation PR1 Δ , with an additional number indicating the isolate number.

Effect of $\Delta xyL'T159$ incorporation into pDKR1 on *m*-toluate metabolism

PR1 Δ 1 and PaW630 containing wild-type pDKR1 were grown in minimal medium broth containing 5 mM *meta*-toluate as the sole carbon and energy source and tryptophan at a concentration of 50 μ g/ml when necessary. The growth of cultures was monitored by the OD at 600 nm using a Beckman™ DU-50 spectrophotometer. The optical density of the culture was plotted as a function of time for the two cultures. Transformation with pPW596 and incorporation of the 159 bp deletion into *xyL* was expected to cause the loss of PR1 Δ 1's ability to use *m*-toluate as a sole carbon source. Although the effect was dramatic, it was not as absolute as expected. A significant

reduction in growth on *m*-toluate as a sole carbon and energy source is observed (Fig. 24). The residual growth is suspected to be the result of the bacteria using a chromosomally-encoded enzyme, possibly the *benD*-encoded NAD⁺-dependent *cis*-diol dehydrogenase or another related enzyme to provide a partial bypass of the block. It has been observed in *A. calcoaceticus*, which has many similar pathways for the catabolism of aromatic compounds, that BenD can act on compounds beyond its normal substrate and potentially compensate for missing enzymes (Neidle *et al.*, 1992). Whatever the mechanism, it requires an extended lag period for expression and provides only a brief period of growth before cell division ceases, possibly due to the buildup of a toxic byproduct.

Growth as a Function of Time
PR1 Δ 1 versus PaW630 w/pDKR1

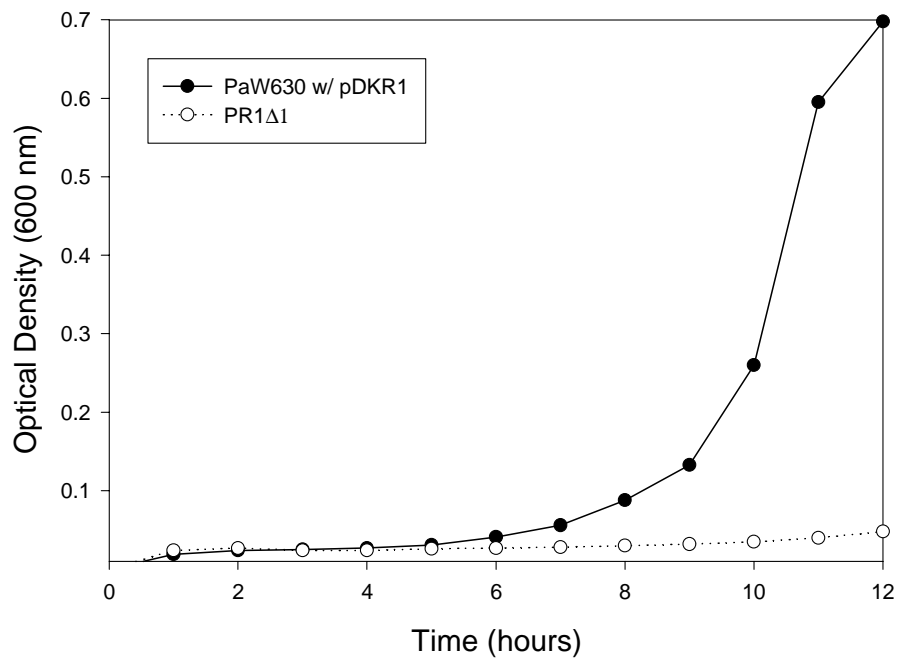


Fig. 24 Plot of PR1 Δ 1 and wild-type pDKR1 growth as a function of time using *m*-tol as the sole carbon and energy source. The effect of $\Delta xyL'T159$ incorporation into pDKR1 results in a significantly reduced ability of *P. putida* to metabolize *meta*-toluate.

CHAPTER IV

CONCLUSIONS

It has been previously shown that *A. calcoaceticus* exhibits a high rate of natural transformation (Doten *et al.*, 1987a). The original objective of this study was to use natural transformation to genetically engineer pathways encoded on large catabolic plasmids. RP1 encodes a broad host range origin, transfer functions and a variety of selectable markers (Nakazawa *et al.*, 1978), all of which are expressed in a wide range of bacterial organisms. An RP1::TOL cointegrate therefore makes the TOL plasmid more readily manipulated. Antibiotic resistance markers expressed in a wide range of hosts, including *A. calcoaceticus*, allow for stable maintenance and selection of the plasmid and transfer functions are essential for the mobilization of the plasmid between species/genera. TOL pDKR1 was thus chosen as a model system. It was desirable to develop a mechanism that is generally applicable to other large plasmids. The transformation of TOL pDKR1 was expected to encounter many or most problems that would be presented when modifying or expressing genes encoded by any large plasmid outside its native genetic background. The most important obstacles were considered to be:

1. Size - large plasmids are difficult to manipulate

2. TOL pDKR1 genes are not expressed in *Acinetobacter* and thus changes in phenotype are not observed
3. Many encoded genes utilize a very different codon usage pattern from that of resident *Acinetobacter* genes
4. The TOL *meta* operon is 60% (\pm) G+C

The high rates of transformation published by Doten *et al.* (1987a) prompted an immediate attempt at transforming pDKR1 and a genotypic screen for successful transformation by PCR. Transformation of pDKR1 was not detected. It was subsequently determined that a general characterization of *Acinetobacter's* transformation system was essential before a study of the transformability of plasmids transferred to *A. calcoaceticus* could be carried out. A previously published experiment (Doten *et al.*, 1987a) that performed transformation of a chromosomal locus was repeated. The intermittent reports of occasionally high levels of natural transformation were not obtainable under well controlled conditions. In the case of Doten *et al.* (1987a), 18% of transformants incorporated a deletion into the chromosome under non-selective conditions. After much effort, it was determined that the results of Doten *et al.* (1987a) could not be duplicated quantitatively, but only qualitatively. The desired result was obtainable, but not at the previously observed frequency. However, transformation rates were determined to be sufficiently high (0.5% - 5%) to justify pursuing our original proposal to modify pDKR1 *in vivo*, since it would be at best extraordinarily difficult, if not impossible, to make similar modifications by any other known mechanism. It is not

clear why we were never able to achieve the transformation frequencies reported by Doten *et al.* (1987a), although it is worth noting that similar levels of competence have not been reported by any other researchers, including our laboratory, since 1987. Completion of our own characterization of *A. calcoaceticus* transformation provided an enhanced understanding of the special considerations for our study of plasmid transformation (as discussed in the following paragraphs) and allowed the development of a standardized transformation protocol that includes optimization for the competency of recipient cells, as well as the types and quantities of donor DNAs.

Conformation, size and source of the donor DNA molecules affected the transformation rate of loci on both the bacterial chromosome and introduced plasmids. The effect of donor DNA conformation (circular vs. linear) on its transforming rate was demonstrated by a comparison closed circular plasmid DNA to the same plasmid DNA linearized by restriction digestion. On average, circular plasmid DNA transformed a selectable chromosomal locus more efficiently than linearized plasmid DNA. Comparison of transformation rates of different size cloned fragments demonstrated an increase in transformation efficiency directly related to the size of the homologous flanking DNA sequence of the donor DNA.

The source of the donor DNA also had an interesting effect on the rate of transformation. Significant differences between the efficiency of *A. calcoaceticus* chromosomal and *E. coli*-derived plasmid donor DNAs were observed. A donor sample of pZR1 contains a *pca* operon gene dosage approximately 350-fold higher than that of an equivalent mass of genomic ADP1 DNA. The difference between transformation

frequencies of restriction digested pZR1 and restriction digested genomic ADP1 DNAs is small in comparison (approximately 0.3%). It would seem as if donor pZR1 DNA, derived from *E. coli*, many lack some type of post-replicative modification, a methylation pattern for example, when compared to the genomic ADP1 DNA.

The total amount of donor DNA was determined to be critical to transformation. Natural transformation is protein driven and therefore saturation is a relevant concern. It has been demonstrated that the transformation system of *Acinetobacter* can be saturated (this study; Ahmadian-Tehrani, 1990; Cruze *et al.*, 1979; Palmen *et al.*, 1993; Palmen & Hellingwerf, 1997; Porstendorfer *et al.*, 1997). There are multiple points at which this saturation phenomenon might exert its effect and these will be discussed in later in the context of cotransformation.

Addition of a small amount of detergent-based lysis solution to the transformation mixture increased transformation efficiency. It also increased the lethality of the transformation protocol to recipient cells, but cells that survived had a much higher rate of transformation. It was unclear whether this elevated transformation rate was due to a detergent-induced permeability of the bacterial membranes followed by an increased influx of donor DNA molecules or the induction of an unidentified stress response which in turn increased the rate of successful transformation.

Experiments were performed to determine whether altering the plasmid target would cause a substantial increase in transformation, with the understanding that some of the changes might not be generally applicable to transformation protocols used for modifying large plasmids. Mechanisms for transformation requiring uptake sequences in

donor DNA have been demonstrated to be used by *H. influenzae* (Smith *et al.*, 1981) and specific sequences for recombination with bacterial chromosome, called Chi sequences in *E. coli* are well documented (Arnold *et al.*, 2000). In order for transformation to occur, donor DNA must be internalized through DNA uptake and assimilated into the locus being transformed. If the transformation system of *A. calcoaceticus* were to include a mechanism similar to *H. influenzae* or *E. coli*, a barrier preventing the modification of extra-chromosomal loci could be present. A barrier preventing the uptake of non-*A. calcoaceticus* DNA has not been reported to date. However, it is possible that only *A. calcoaceticus* sequences are subsequently incorporated into recipient DNA molecules and a general difference between transformation efficiency of *A. calcoaceticus* chromosomal DNA and extra-chromosomal DNA targets could exist, even though all types of donor DNA are taken up. Transformation of a plasmid containing *A. calcoaceticus* DNA sequence was utilized to identify such a requirement in the recipient vector for integration. The presence of *A. calcoaceticus* plasmid DNA sequence in the recipient plasmid being transformed did not have a major effect on the rate of transformation.

Overall, optimization of the transformation protocol demonstrated that conformation, size and source of the donor molecule each have an effect on transformation rates, but no effect is such a major consideration that it might unduly complicate transformation of a large plasmid target. All types of donor DNA can transform a given locus. Reduction of the total amount of donor DNA and the addition of dilute detergent solution significantly increased the observed cotransformation efficiency.

Parameters in the transformation protocol were optimized to obtain the highest possible rate of transformation before repeating experiments to transform pDKR1 .

It is always difficult to demonstrate that cells have been transformed without the use of a selectable marker. In *A. calcoaceticus*, the TOL⁺ phenotype usually conferred by the presence pDKR1 is not expressed. Therefore, it is difficult to score for transformation of a pDKR1 TOL locus. Internalization of donor DNA and the subsequent transformation of a marker locus increases the probability that a second donor DNA would be internalized and transform a second locus. Therefore, the standard transformation protocol was adapted to include cotransformation. Amino acid auxotrophs and mutants in catabolic pathways encoded on the host chromosome were used for this purpose. Cotransformation is also beneficial when making modifications, such as gene knockouts, for which no selection is available. Cotransformation uses a selectable locus as a marker for transformation and was utilized to enrich for transformation by excluding all cells that are not definitively competent and actively taking up DNA.

Saturating concentrations of donor DNA complementary to the selectable chromosomal locus produced decreased levels of plasmid transformation even when saturating levels of this DNA were also utilized. This suggests that saturation of the transformation system may decrease efficiency at one locus, by selection of one type of donor molecule over another. Thus, increasing the amount of donor DNA does not necessarily increase the amount of DNA entering the cell or the rate of transformation after saturating levels are reached. If genomic *A. calcoaceticus* DNA fragments are

preferentially utilized, either during internalization or integration into the site being transformed, the uptake of large quantities of genomic DNA can saturate the transformation system and essentially exclude other DNAs from the process. Therefore, the use of genomic ADP1 donor DNA to cotransform a selectable marker would exclude the uptake of any donor DNA homologous to the plasmid target. Target plasmid loci were in fact transformed less efficiently when genomic ADP1 cotransforming donor DNA was used and therefore its use was eliminated from the optimized transformation protocol. Logic dictated that equimolar quantities of similar, yet different donor DNAs should both have an equal opportunity for internalization by competent *A. calcoaceticus* cells. Cotransformation experiments exhibiting the highest rate of transformation utilized two plasmid donor DNAs of approximately the same size and conformation.

Transformation rates indicate that 1-2% of the pDKR1 target plasmids were transformed by the donor $\Delta xyiL'T159$ DNA. The result of transformation was deletion of a specific 159bp *XhoI* fragment from pDKR1. This plasmid has seven *XhoI* sites in the TOL *meta* operon alone, and therefore this modification could not have been readily achieved with traditional recombinant DNA techniques. Such methods require the isolation of the plasmid, partial digestion with restriction endonucleases (if available), ligation, isolation of the correct deletion (because of all the possible permutations) and the transformation of the plasmid back into a host cell. The optimized protocol for engineering large plasmids by natural transformation provides a mechanism for many modifications not readily achieved and the ability to specifically modify tiny segments of large replicons is a very powerful molecular tool. It should also be noted that the 1-2%

rate of pDKR1 transformation was very similar to the rate of transformation of an equivalent transformation of the *A. calcoaceticus* chromosome (0.5% - 5% for the *pca* deletion)

Transformation data present the opportunity for speculation about the nature of transformation in *A. calcoaceticus*. As previously mentioned, it is not well understood why *EcoRI*-digested chromosomal DNA transforms a chromosomal target locus several hundred times more efficiently, homologous molecule per homologous molecule, than a clone of the same size and DNA sequence produced in *E. coli*. It is not clear what is causing this phenomenon, but a plausible explanation might be the existence of an unidentified restriction modification system or a methylation pattern which is important in transformation. Cloned donor DNA fragments produced in *E. coli* would not contain the methylation pattern which might be important for internalization by *A. calcoaceticus*, survival of the DNA molecule once it has been internalized, or integration of the donor DNA into the genetic material of a recipient *A. calcoaceticus* cell. The ability to discriminate which donor DNA molecule is assimilated into the genetic material would provide an advantage to the recipient cell. Integration of the donor DNA molecule based solely upon a certain level of homology would include the risk of inserting DNA sequences that can not be properly expressed. This could be extremely detrimental to the cell and is probably the reason most known transformation systems provide some level of discrimination on the part of the recipient cell. A simple, yet logical, explanation for decreased transformation efficiency of non-*Acinetobacter* DNA is an intracellular

discrimination system that preferentially routes non-*Acinetobacter* DNA into the “nutrient” category. This provides an area for further speculation.

Acinetobacter's indiscriminate DNA uptake system could allow internalization of exogenous DNA to serve multiple functions. Most extracellular DNA encountered by *A. calcoaceticus* cells might not be homologous to any *A. calcoaceticus* sequence.

However, non-selective DNA uptake by *A. calcoaceticus* does suggest that uptake of such DNA is viewed as essential (or at least a useful). DNA uptake by a non-specific mechanism provides a mechanism to bring in extracellular DNA which could be routed into either of two pathways. One pathway could lead to degradation and the formation of a nutritional reservoir of nucleotides used for building new genetic material, while homologous DNAs could be directed to the transformation apparatus. Therefore, *A. calcoaceticus* takes up all DNAs, but when competence is induced, the fate of some of the internalized DNA can be different than DNA that is utilized as a nutritional source. Internalized homologous DNA would not be degraded and one strand would be used as donor DNA during the cell's competent state. Induction of *A. calcoaceticus*' DNA internalization system (which is usually discussed in terms of competence development) might also include a nutritional override which would induce a simultaneous high level of cellular transformation competence. *A. calcoaceticus* is a metabolically and nutritionally versatile organism. DNA synthesis is very energetically demanding. Internalization of DNA does not require expenditure of energy by the *A. calcoaceticus* cell, meaning no energy is expended to obtain a ready source of nucleotide monophosphates. Many nucleotides/nucleosides could be lost prior to uptake if extracellular nucleases were used

to degrade DNA. If DNA is first internalized, substrate acquisition is therefore more efficient. This DNA uptake system may be another example of how *A. calcoaceticus* is designed to maximize the extraction of nutrients from its environment and therefore it is highly efficient at recovering DNA.

Hypothesizing that extracellular DNA is viewed by the cell as nutritional source and indiscriminate internalization of large amounts of DNA provides an advantage for the cell is not unfounded. A number of naturally transformable bacteria use DNA as carbon, nitrogen and energy sources (Stewart & Carlson, 1986). *Haemophilus* only integrates about 15% of donor DNA internalized, whereas *Bacillus* and *Streptococcus* integrate about 50% and degrade the remainder (Stewart & Carlson, 1986). An apparatus for internalizing any DNA molecule encountered could be partially geared toward nutrition, as cellular production of separate apparatuses to perform the same function would not be efficient. It is possible to hypothesize that natural transformation systems originally evolved for nutritional reasons rather than internalizing DNA for the purpose transformation. DNA uptake in *A. calcoaceticus* could be viewed as a “salvage system” for extracellular DNA (nucleotides). *A. calcoaceticus* lacks uridine kinase, uridine hydrolase, uridine phosphorylase, 5'-nucleotidase, cytidine deaminase, CMP glycosylase, and cytosine deaminase (Beck, 1995). Nutritional internalization of exogenous DNA as observed in bacterial species not capable of natural transformation usually involves degradation by extracellular nucleases, conversion to nucleosides by a phosphatase activity and internalization for use through salvage pathways. The inability of *Acinetobacter* to utilize many of these substrates would make a compensatory mechanism

useful. Nucleotides from degraded DNA would satisfy nutritional requirements of the *A. calcoaceticus* cell reducing the metabolic cost of DNA synthesis by nearly two thirds. Selective DNA uptake would decrease the effectiveness of such a system. If such a role were important as (or more important) to cellular metabolism as genetic transformation, then it is possible that an additional mechanism might exist for the induction of genes, the products of which would subsequently increase transformation rates. Average transformation frequencies for *A. calcoaceticus* ranging from 0.5% (Cruze *et al.*, 1979; Juni, 1972; Kok *et al.*, 1999; Lorenz *et al.*, 1992; Palmen *et al.*, 1993; Porstendorfer *et al.*, 1997) to 18% (Doten *et al.*, 1987a) represent an approximately 40-fold range of expression. The same range of increase is observed in many operons when comparing basal level expression to induced expression. This variability is at least suggestive that the transformation system remains poorly understood and that conditions yielding maximum competence are unidentified and only accidentally encountered. Perhaps an association of transformation with nucleotide scavenging may explain some of this variability, since single mutations in key metabolic operons could then readily induce high levels of competence.

A. calcoaceticus provides a unique mechanism for the study of otherwise unmanageable constructs, although the system is not as efficient as originally hypothesized. This study has demonstrated that natural transformation can be utilized to genetically engineer encoded pathways of large catabolic plasmid genes. The ability to specifically modify tiny segments of large replicons is a very powerful molecular tool

and the use of natural transformation for genetic engineering is generally applicable to modification of other large replicons.

APPENDIX

Acinetobacter Transformation Protocol (Modified)

Day 1

1. Inoculate 500 μ l of recipient *A. calcoaceticus* in 50 ml of liquid minimal medium with the appropriate carbon source.
2. Monitor cell growth to an absorbance of 0.1 to 0.15 at 600 nm.
3. Dilute cells to 1.0×10^{-2} with 1X PBS
4. Add 10 to 60 ng of transforming DNA, 3.5 μ l of lysis buffer and 20 μ l of the 100-fold recipient cell dilution to a 0.6 ml microfuge tube and increase the volume to 60 μ l.
5. Spot the transformation mixture on a non-selective agar plate.
6. Incubate overnight at 37°C.

Day 2

1. Transfer as much cell growth as possible to a microfuge tube containing 1.0 ml of sterile 1X PBS and fully resuspend the cells.
2. Make a 1.0×10^{-2} to 1.0×10^{-4} dilution series from the cell suspension.
3. Spread 50 μ l and 100 μ l of the 1.0×10^{-4} dilution on selective and non-selective plates.
4. Incubate overnight at the appropriate temperature.

Day 3

1. Score plates for cell growth.

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