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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

OBSERVATIONS OF GENETIC TRANSFORMATION IN NUTRITIONALLY DEFICIENT MUTANTS OF *CANDIDA PSEUDOTROPICALIS*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

BERNARD LEE FRYE

Norman, Oklahoma

1972

OBSERVATIONS OF GENETIC TRANSFORMATION IN NUTRITIONALLY DEFICIENT MUTANTS OF *CANDIDA PSEUDOTROPICALIS*

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OBSERVATIONS OF GENETIC TRANSFORMATION IN NUTRITIONALLY DEFICIENT MUTANTS OF *CANDIDA PSEUDOTROPICALIS*

CHAPTER I

INTRODUCTION

The phenomenon of transformation was first observed in 1928 by F. Griffith (9) in his experiments involving pneumococcus. Although the first character studied was that of a specific polysaccharide synthesis, the process of transformation has now been expanded to include all characters that can be conveniently investigated (27). It was not until 1944 that O. T. Avery and others (2) helped to demonstrate the "transforming substance" to be deoxyribonucleic acid (DNA). Avery reported the transformation of a rough colony of pneumococcus into a smooth colony by adding DNA from the donor. The smooth colony had encapsulated cells while the rough colony had non-encapsulated cells, and in his study the capsules of the transformed cells demonstrated the same serologically type-specific polysaccharide. Since then genetic transformation has been observed in a number of bacterial genera (27 & 33). It occurs regularly in several

species of Hemophilus, Neisseria, Pneumococcus, Bacillus and Streptococcus. Attempts to find transformation in Escherichia, Salmonella, or Shigella have been only partly successful. The occurrence has been only briefly observed despite numerous attempts to find transformations in the enteric bacteria, and this fleeting observation has never been regularly reproduced (27). The reciprocal genetic transformation of streptomycin-susceptible cells to streptomycin-resistant cells between a coccus Neisseria catarrhalis and a rod Moraxella nonliquefaciens, representing a genetic exchange between members of different species, has been reported (42). Rensburg (45) reported the isolation of biologically active DNA from Proteus mirabilis which could transform auxotrophic mutants to prototrophy, while Raina and Modi (26) published information on genetic transformation in Rhizobium. Dobrzański, Osowiecki, and Jagielski (7) noted the occurrence of transformation between Staphylococci as DNA donors and Streptococci as recipient cells, and Kloos (16) reported the ability by members of the genera Micrococcus, Sarcina, and Staphylococcus to transform auxotrophs of Micrococcus lysodeikticus to prototrophy. Tyeryar and Lawton (44) reported transformation in auxotrophic mutants of Pasteurella novicida, while Colman (6) noted that cultures of Streptococcus milleri incorporated DNA from either S. milleri, S. viridans, or S. sanguis. In bacteria, genetic transformation involves the incorporation of DNA by the recipient cells and

the subsequent integration, replication, and function of that segment of genetic material.

Transformation studies also have been conducted on fungi. Sen, Nandi, and Mishra (29) observed the event in Aspergillus niger by studying reversions occurring in nutritionally deficient mutants. This reversion rate was affected if the donor DNA was treated with ultraviolet irradiation, heat, or deoxyribonuclease (DNase). The experiment also demonstrated a response to concentration changes of donor DNA; however, the frequency of transformation was lower than that usually found in bacteria. This lower frequency of transformation was attributed to a number of factors; the growth of bacteria differ from that of the fungi, environmental factors, the nature of competence in fungi, and the method of DNA extraction.

Shamoian, Canzanelli, and Melrose (30) reported the successful demonstration of transformation in a number of experiments with a mutant of Neurospora crassa to the wild type by using a deoxyribonucleic-ribonucleic acid (DNA-RNA) complex. In addition to the DNA-RNA complex, uridine was required for transformation. Shockley and Tatum (31) believed their results provided suggestive but not convincing evidence of transformation in nutritionally deficient mutants of N. crassa. Mishra and Sen (22) reported the transformation of a mutant of Aspergillus niger.

An interspecific transformation between Allomyces arbusculus and A. macrogynus was reported (43). These

authors found several modified structures including that of the gametangia. It was their belief that the resistance of fungi to transformation may be due to the difficulty of transfer across the cell wall which was overcome by the selection of zoospores as recipient cells.

The isolation of DNA from fungi has been impaired by the absence of a method for obtaining DNA of large molecular weight. This difficulty in isolation is due to the absence of a simple and gentle method of fungal cell lysis (10). The methods usually employed are mechanical disruption, formation of spheroplasts, or use of detergent and enzymatic degradation of the cell wall (4).

Attempts to revert a morphological mutant of Penicillium chrysogenum with DNA obtained by breaking up the mycelium and treating with sodium p-aminosalicylate and phenol gave no evidence of transformation (5), while the difficulty of obtaining ruptured cells was reported. Harris and Thompson (12) also failed to produce evidence of transformation in Saccharomyces chevalieri, although Oppenoorth (23) used the same yeasts and extraction methods and reported a successful transformation experiment. The yeast cells were suspended in saline-citrate-phosphate buffer solution and snail enzymes, chloroform and lauryl sulphate were added while the mixture was stirred in a water bath at 50-60°C. The DNA from the donor conferred the ability to ferment certain sugars on a yeast deficient in this aspect. Pseudo-transformation was

described in which the acquired ability to ferment certain sugars was lost in successive generations. In a later report Oppenoorth (24) noted the biological activity of DNA preparations as being unpredictable and a factor for some negative results.

It appears that none of the authors investigating fungal transformation attempted to obtain the DNA above a minimal molecular weight or to obtain the recipient cells in a state of "competence." Spizizen, Reilly, and Evans (33) listed these two points as requirements for obtaining transformation in bacteria. Thomas (41) suggested that the term competence be applied to the capacity of cells to incorporate DNA. There are reports that competence is under genetic control (18, 28 & 49). Strains of bacteria that transform at a high level may produce mutants with low levels of competence. Divalent cations appear to be necessary for DNA uptake in some bacteria (50) as well as an energy source (18). The state of competence is considered to be unstable and not easily determined. Anagnostopoulos and Spizizen (1) noted that competence appeared in a minimal medium toward the end of the exponential period of growth. Support for a defined medium was also reported by Spencer and Herriott (32). Thomas (41) postulated that partial protoplast formed would allow for DNA penetration. Stuy and Stern (37) reported that 4-5 seconds are required for the penetration of A DNA particle of 8-9 million molecular weight in Haemophilus influenzae.

It is the purpose of this investigation to present data on the requirements for transformation in Candida pseudotropicalis from auxotrophy to prototrophy. Transformation to prototrophy in bacteria has been observed by a number of authors including Gwinn and Thorne (11) and Leonard, Corley, and Cole (19).

Candida pseudotropicalis is a yeast having cells that are shortovoid to ovoid (2.5-5) X (5-10) microns in size (21). Mutants were obtained by ultraviolet irradiation. These mutants were unable to ferment the specific sugar lactose. Purified DNA preparations of the wild type cells were obtained with a molecular weight of 20 million by a modified method of Bhargava and Halvorson (3). It is believed that this method made possible the isolation of DNA with a high level of activity needed for transformation. It would seem reasonable that if transformation could be demonstrated in C. pseudotropicalis, then the event could also prove to be common in other microorganisms that possess an eukaryotic cellular structure. It seems plausible to hypothesize that for most microorganisms the process may be the natural mechanism for genetic transfer. In the scientific field, the acceptance of this concept received its impetus with the reports previously listed on transformation in fungi.

Transformation could be of concern to medical mycology if fragments of DNA from pathogenic yeast are released in their surrounding environment and genetic recombination

occurred. The DNA may originate from cells that undergo autolysis, or release DNA, as they are growing in a patient afflicted with a yeast infection. Generally the systemic mycoses are produced by fungi that are free-living saprobes in nature (48). Infection in man may follow inhalation of the fungi from their normal saprobic habitats following genetic transfer between fungal members. If the DNA entered another yeast cell and replaced a homologous section of the recipient cell's chromosome, new genetic combinations and linkages are probable. This may give the cell some advantage, as drug resistance may be controlled by several equipotent genes similar to that of penicillin-resistance found in some bacteria (34). Resistance to various antibiotics and drugs may develop in fungi by this process. In the presence of the drug, there will be selection for the mutant which would grow under these conditions. Knowledge of this may allow the physician to avoid clinical appearance of drug-resistant strains.

CHAPTER II

MATERIALS AND METHODS

Agents Used. Candida pseudotropicalis, #9767, was obtained from the American Type Culture Collection, Rockville, Maryland. This organism required biotin, niacin, pantothenic acid, and thiamine for growth (21). The cells were grown on trypticase soy agar for six days at 27°C and then stored in the refrigerator until needed.

Auxotrophic mutants of culture #9767 were obtained by growing C. pseudotropicalis on trypticase soy agar in Petri dishes. The cells were exposed to ultraviolet irradiation for twenty minutes at a distance of 15 centimeters from a germicidal lamp source (General Electric G 4511, 4W). The mercury light source produces strong radiation at 2537 angstroms and was calibrated after use and found to provide $6.78 \cdot 10^{14}$ photons per second per cm^2 at the distance used ($5.307 \cdot 10^3$ ergs/second/cm^2). This value was obtained by the photolysis of uranyl oxalate which supplied a relation between the amount of light absorbed and the amount of chemical action produced (47). These data were obtained with the aid

of the Physics and Chemistry Departments at The University of Texas at Arlington. The cultures were next placed in the dark overnight, and then allowed to grow in the laboratory for 3 days at 27°C. Colonies were tested for their ability to ferment a specific number of carbohydrates. Each mutant was observed to breed true by subculturing with suitable media. Lactose negative cells were observed to be unable to ferment lactose during their growth. This deficiency was checked at intervals with cells from the stock culture.

Isolation of Deoxyribonucleic Acid. C. pseudotropicalis was grown in 500 milliliter (ml) Erlenmeyer flasks containing 200 ml of tryptic soy broth and yeast extract for 48 hours at 37°C. These cells had the ability to ferment lactose, glucose, galactose, sucrose, inulin, and raffinose (46). Cells were harvested after 10 minutes of centrifugation at 3500 rpm. The cells were washed twice with a solution containing 0.1M ethylenediaminetetraacetate (EDTA) and 0.15M NaCl. A 22 gram cell pellet was suspended in 100 ml of EDTA-Saline solution and frozen for 48-60 hours. The suspension was placed in a 37°C water bath until the pellet thawed and then recentrifuged. The cell pellet was placed in 5 volumes of 1M sorbitol and 20% glycerol, and this suspension was poured into a cold high pressure cylinder of a Sorvall ribi cell fractionator, model RF-1, with the fill and ribi valves removed. The high pressure cylinder was inserted into a mixture of dry ice and acetone and allowed to cool for

11 minutes. The frozen mixture was placed in the fractionator and forced through a 2 centimeter (cm) long high pressure line having an internal diameter of 0.95 millimeters (mm) with a pressure of 20,000 psi. The frozen mixture was collected in a beaker and examined microscopically. Very few cells had ruptured, so 2% sodium dodecyl sulfate was added, and after several minutes the mixture became extremely viscous. A second microscopic examination revealed 60% cell lysis. Perchlorate was added until a final concentration of 1M was reached in the viscous, lysed suspension. The mixture was shaken with an equal volume of chloroform-isoamyl alcohol by hand in a glass-stoppered flask for 30 minutes. The resulting emulsion was centrifuged for 10 minutes at 7700 rpm. The upper aqueous phase was carefully transferred to a large tube with the aid of a pipette 3.7 millimeters in diameter. Each step during the isolation procedure was designed to eliminate DNA degradation by the mechanical scission of the polymer. The nucleic acids were precipitated by slowly layering 2 volumes of ethyl alcohol on the aqueous phase. A glass stirring rod was used to "spool" the precipitate, and the precipitate transferred to 20 ml of dilute saline-citrate. Removal of the precipitate was accomplished by gently swirling the glass rod back and forth in the solution. Concentrated saline-citrate was added to adjust the solution to standard saline-citrate concentration (0.15M NaCl and 0.015M trisodium citrate). This solution was transferred to a glass-stoppered

flask, and equal volume of chloroform-isoamyl alcohol added, and the mixture shaken by hand again for 15 minutes. The supernatant was removed after centrifugation and repeatedly deproteinized with chloroform-isoamyl alcohol until no protein was observed at the interface. After the eleventh deproteinization procedure, no protein was indicated at the interface, and the supernatant was precipitated with ethyl alcohol and transferred to saline-citrate. Ribonuclease (RNase), heated in a water bath to 90°C for 10 minutes, was added to a final concentration of 50 µg/ml, and the mixture incubated for 30 minutes at 37°C. After treatment with RNase, two additional deproteinization steps of 15 minutes each were required to remove the remaining protein which had resisted earlier attempts. The supernatant, after the last treatment, was precipitated with isopropyl alcohol, and then dissolved in saline-citrate. The solution was dialized overnight at 4°C against 1000 volume saline-citrate, reprecipitated in ethyl alcohol and stored at 5°C. Four milligrams (mg) of DNA were obtained. DNA was determined by the colorimetric diphenylamine method using a sperm DNA preparation as the standard. The molecular weight of the preparation was determined to vary from 2-20 million with an average size of 10 million as determined in a 5-20% sucrose gradient. A chilled linear sucrose gradient was established by means of a density gradient mixer. The different concentrations of the gradient media were prepared immediately before use and separately

dispersed into the two gradient chambers. The vibration stirrer was started and the stopcock opened to allow the resulting concentration to move into the single outlet. The weight determination was accomplished by placing 0.05 ml sample of DNA on top of the sucrose gradient and the tube centrifuged at 64,000 rpm for one hour. The DNA sample was then moved through an ultraviolet analyzer, (Isco model UA-2 ultraviolet liquid flow analyzer). The position of the DNA sample in the gradient was noted with a recorder, and the molecular weight determined from tables containing known values.

Protocol of Transformation Experiment. The yeast cells used as the recipients were unable to ferment lactose. These cells were grown to the logarithmic phase in the basic glucose-minimal medium A on a rotary shaker at 35°C (Table 1). One milliliter aliquots of the cell suspension were transferred to 500 ml Erlenmeyer flasks each containing 100 milliliters of culture medium plus wild type DNA, suspended in 1 milliliter of saline-citrate. The culture medium was varied as indicated in Table 1, whereas the concentration of DNA was varied from 25-100 µg/ml. The media listed were selected based on previous reports listing procedures for the preparation of competent bacterial cultures (1, 18 & 49). The D-2-D glucose was used to investigate evidence that the ability to absorb DNA is correlated with the chemical composition of the cellular surface (42). If the D-2-D glucose was able to

Table 1. Composition of Culture Media for Transformation
Concentration/Liter

Ingredients	Medium A	Medium B	Medium C	Medium D	Medium E	Supplemented Culture Medium
NH_4Cl	2.5g	2.5g	2.5g	2.5g	2.5g	2.5g
KH_2PO_4	7.0g	7.0g	7.0g	7.0g	7.0g	7.0g
$NaHPO_4$	7.2g	7.2g	7.2g	7.2g	7.2g	7.2g
$MgSO_4 \cdot 7H_2O$	0.2g	0.2g	0.2g	0.2g	0.2g	0.2g
NaCl	0.1g	0.1g	0.1g	0.1g	0.1g	0.1g
Glucose	3.0g	3.0g	3.0g	3.0g	3.0g	3.0g
Distilled Water	1 liter	1 liter	1 liter	1 liter	1 liter	1 liter
Thiamin hydrochloride	150 µg	300 µg	600 µg	150 µg	150 µg	150 µg
Nicotinic acid	150 µg	300 µg	600 µg	150 µg	150 µg	150 µg
Biotin	150 µg	300 µg	600 µg	150 µg	150 µg	150 µg
Calcium pantothenate	150 µg	300 µg	600 µg	150 µg	150 µg	150 µg
D-2-D Glucose	-	-	-	0.1g	0.3g	-
Yeast extract	-	-	-	-	-	2.0g
Nutrient broth	-	-	-	-	-	2.0g

change the composition of the cell wall it may also affect the transformability of the yeast cells. Flasks containing the 102 milliliters of culture medium, cells and DNA were incubated for 24 hours on a low speed rotary shaker at 35°C. The exposure to DNA was terminated by the addition of 100 µg of crystalline pancreatic deoxyribonuclease prepared in 0.03M $MgSO_4$ at the end of the 24 hour period. The cells were immediately centrifuged at 3500 rpm for 10 minutes, washed in 10 milliliters of sterile 0.9% NaCl twice, and then suspended in 2 milliliters of 0.9% NaCl. Approximately 1 milliliter containing 10^5 cells was spread aseptically on a chemically defined agar (Table 2). The ingredients were sterilized in the autoclave for 15 minutes at 121°C while lactose was sterilized by filtration (Selas filter) and then added aseptically to the liquid agar before pouring the plates. The plates were incubated at 27°C for 4 to 5 days. One ml solution containing 50 µg donor DNA was heated in a water bath to 100°C for 10 minutes. The tube was removed from the water bath and placed in an ice chest for rapid cooling. The DNA solution was added to recipient cells, incubated in basic glucose minimal culture medium for 24 hours on the low speed rotary shaker at 35°C, and 10^5 cells plated on chemically defined agar. Donor cells were also plated to determine the period of incubation necessary for the appearance of wild-type colony formation. Other controls included utilizing a 50 µg preparation of DNA incubated with 100 µg of Mg-activated

Table 2. Chemically Defined Agar

NH_4Cl	2.5g	Distilled Water	1 liter
Lactose	3.0g	Thiamin hydrochloride	150 μg
KH_2PO_4	7.0g	Nicotinic acid	150 μg
Agar	13.5g	Biotin	150 μg
		Calcium pantothenate	150 μg

DNase solution at 37°C for 30 minutes. This preparation was then added to recipient cells and treated as above. Samples of DNA without recipient cells and recipient cells without DNA, incubated as the DNA-treated cells, were also spread on chemically defined agar. The usual precautions of sterility for solutions and equipment were observed throughout the experiment. The sterility of the media used was determined by incubating plates that were not inoculated. The recipient cells treated with DNA preparations, which showed signs of colony formation on chemically defined agar, were considered transformants. Five colonies were randomly selected from each plate and individually transferred to another plate of chemically defined agar, incubated at 27°C for 5 days, and samples transferred to 10 ml phenol red lactose broth (Difco) in 18 X 150 mm culture tubes. All samples tested were capable of fermenting lactose. Thus, the growth characteristics of three successive subcultured aliquots of transformed C. pseudotropicalis were observed. Two consecutive experiments were conducted with results of similar growth patterns. Auxotrophic cells, grown in basic glucose minimal medium A for 24 hours at 35°C on a low speed rotary shaker, were centrifuged at 3500 rpm for 10 minutes. The supernatant fluid was collected aseptically and 0.5ml incubated with different concentrations of DNA suspended in 0.5 ml saline-citrate for 30 minutes at 37°C. DNA-supernatant fluid was added to recipient cells as previously described. The purpose of this

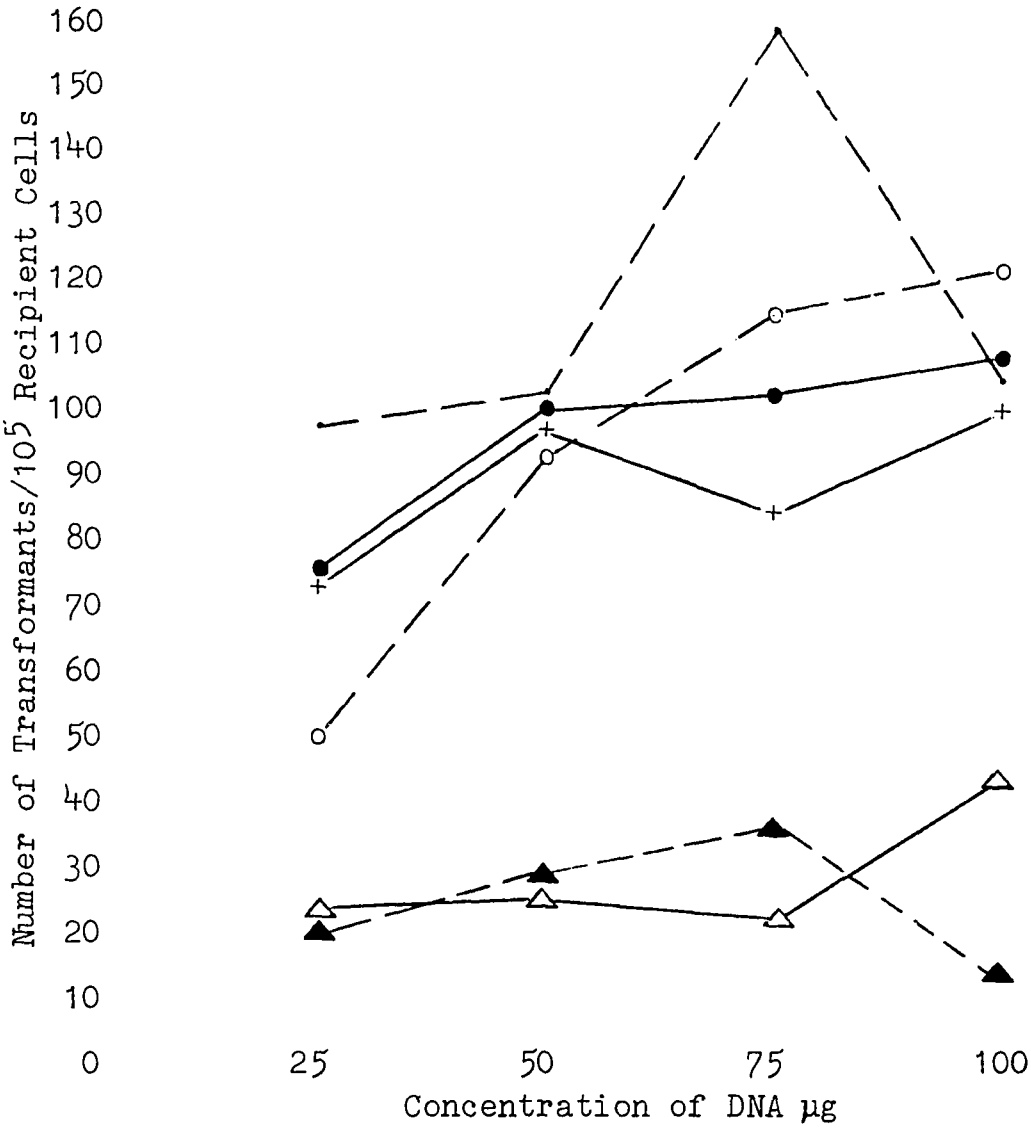
experiment was to investigate evidence for factors in the supernatant fluid that may reduce the frequency of transformation by DNA (27).

In another trial, 1 ml of auxotrophic cells in the logarithmic phase were incubated in 3 flasks containing 100 ml basic glucose minimal medium A on a rotary shaker at 35°C. DNA, 50 µg suspended in 1 ml of saline-citrate, was added to the flasks at 9, 14, and 20 hours after incubation. This was done to determine the period of time the cells were competent. The exposure to DNA was terminated as before with DNase as each culture reached the end of the 24 hour period.

CHAPTER III

RESULTS

Figure 1 presents the average results of transformation obtained in experiments 1 and 2 to prototrophic type in a number of different media. The symbols at the bottom of the figure indicate the different media utilized during incubation with the donor DNA and recipient cells. The unbroken and broken lines represent the trend of transformation in relation to increased DNA concentration along the abscissa. The ordinate shows the number of transformations observed from 10^5 recipient cells. Figure 2 provides the average number of transformants obtained in all media except those solutions containing D-2-D glucose. Although the frequency of transformation is low as compared to that of some bacteria, it is apparent that reversions had occurred in the presence of donor DNA. The levels of transformation obtained (Table 3) in this study compare with the 0.1% to 0.2% transformation observed in other fungi (30). The data show the percentage of transformants obtained in several media related to DNA concentration. The total number of prototrophic colonies



Basic Glucose Minimal Culture A ·
Basic Glucose Minimal Culture B o
Basic Glucose Minimal Culture C ●
Supplemented Medium +
Basic Glucose Minimal Culture D △
Basic Glucose Minimal Culture E ▲

Figure 1. Number of Transformants Related to DNA Concentration and Growth Medium.

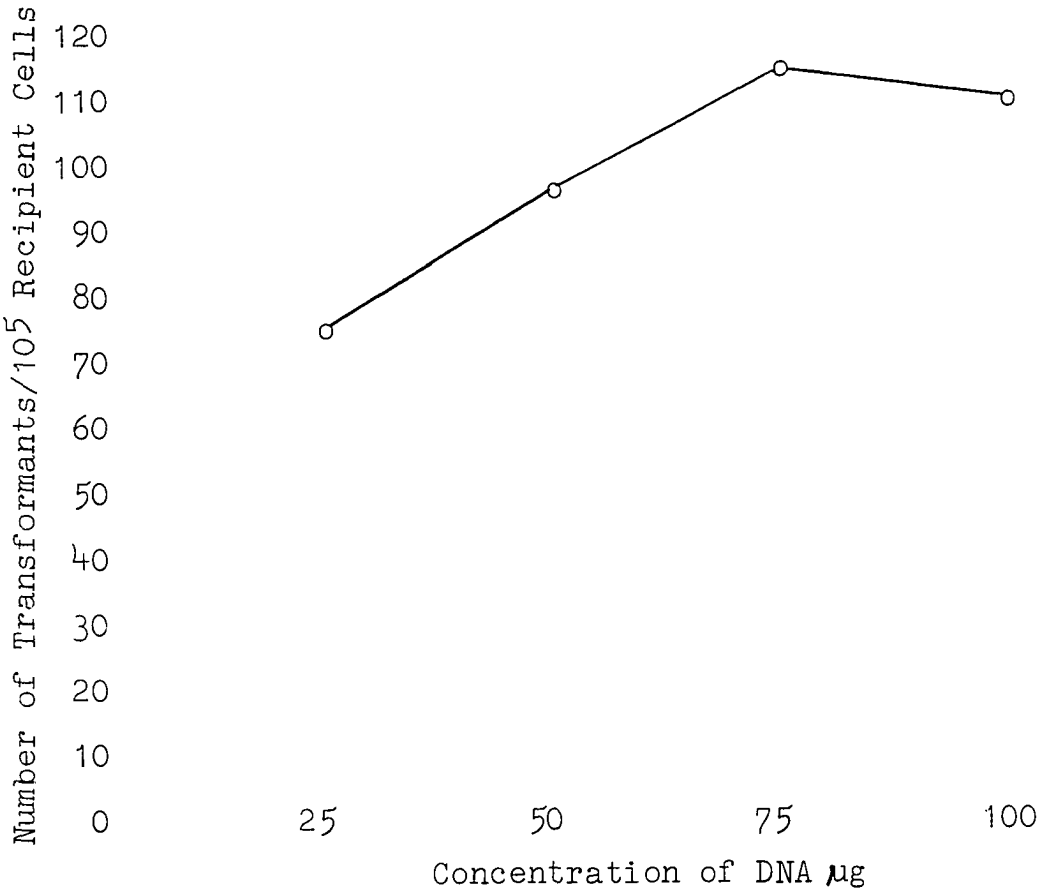


Figure 2. Average Number of Transformants on all Media Except with D-2-D Glucose.

Table 3. Percentage of Transformants Obtained With Several Media

Concentration of DNA µg	25	50	75	100
Basic Glucose A	.0975%	.0995%	0.157%	0.1065%
Basic Glucose B	.049%	.0915%	0.111%	0.119%
Basic Glucose C	.0735%	.0985%	.0995%	0.107%
Supplemented	.072%	.092%	.082%	.099%
Basic Glucose D	.023%	.026%	.0215%	.0435%
Basic Glucose E	.021%	.0265%	.0355%	.0165%

observed from experiments 1 and 2 are combined in the table. Ravin (27) reported that transformants under the most favorable conditions could be slightly above 10% in bacteria, but usually an efficiency of about 1% to 5% is expected. The average percentage value obtained in all concentrations of DNA used with basic glucose minimal culture medium A is 0.12%. If this value is compensated for by subtracting the percentage obtained with untreated recipient cells, the transformation percentage is 0.119%. Table 3 also reveals that if one omits the values obtained with 25 µg DNA the percentage transformation observed in basic glucose minimal culture media B and C is also near 0.1%. A slightly lower average percentage of .086% is obtained in the supplemental medium.

In Figure 1 the frequency of transformation appears to increase with DNA concentration from 25-75 µg in basic glucose medium A. With basic glucose media B and C a gradual increase is indicated for all values tested. The supplemental medium also supports an increase in transformation with increased DNA concentration except at the 75 µg value. It is apparent from these data (Table 3 and Figure 1) that the presence of D-2-D glucose (Schwarz and Mann) reduced the number of colonies appearing on the plate; however, there appears to be little difference if D-2-D glucose is increased relative to the glucose added.

It appears that the saturation region for DNA concentration may not have been achieved in this study as noted

for some bacteria, although 75 µg DNA produced a greater number of transformants in basic glucose A in both experiments than did 100 µg DNA (Table 4 and 5). In both experiments a gradual increase in transformation was obtained for all concentrations of DNA in basic glucose medium B.

One observes from Tables 4 and 5 the number of transformants from the two separate experiments. The information in Table 4 shows the number of colonies obtained in experiment 1. The type of culture medium is listed on the left for both experiments, while the number of colonies is listed according to the concentration of DNA at the top of the page ranging from 25 to 100 µg. Table 5 illustrates the number of colonies received from the second experiment.

The optimum concentration of DNA for transformation in C. pseudotropicalis is difficult to ascertain from this study. In previous reports (22) 25 µg DNA/ml was used with $5 \cdot 10^6$ cells of Aspergillus niger. An increase in percentage of transformation was observed in 6 µg DNA/ml with the same organism by other workers (29) using $1.4 \cdot 10^6$ to $5.5 \cdot 10^6$ cells. Turian and Ojha (43) used 165 µg DNA and 4 ml of cells with Allomyces while Shamoian, Canzanelli, and Melrose (30) used from 12-20 mg of a DNA-RNA complex in their work with $1.5\text{-}1.8 \cdot 10^6$ cells of Neurospora crassa. Shockley and Tatum (31) worked with 500 µg of DNA in their study with Neurospora crassa and $5 \cdot 10^6$ to $5 \cdot 10^8$ cells. Other authors did not mention a specific concentration of DNA in their

Table 4. Number of Transformants Related to DNA Concentration and Growth Medium Experiment 1

Concentration of DNA µg	25	50	75	100
Type of Culture Medium	Number of Transformants per 10^5 Recipient Cells			
Basic Glucose A	103	117	143	114
Basic Glucose B	45	105	107	117
Basic Glucose C	69	107	110	101
Supplemented	63	100	91	111
Basic Glucose D	29	18	24	41
Basic Glucose E	18	26	40	17

Table 5. Number of Transformants Related to DNA Concentration and Growth Medium Experiment 2

Concentration of DNA µg	25	50	75	100
Type of Culture Medium	Number of Transformants per 10^5 Recipient Cells			
Basic Glucose A	92	82	171	103
Basic Glucose B	53	78	115	121
Basic Glucose C	78	90	89	113
Supplemented	81	84	73	87
Basic Glucose D	17	34	19	26
Basic Glucose E	24	27	31	16

transformation studies with Saccharomyces chevalieri (12, 23 & 24).

Transformation activity of the donor DNA was found to diminish with heat as indicated in Tables 6 and 7, although the average number of transformants was higher (.0195%) than for recipient cells plated alone (.0095%). The information in Table 7 shows the reversions occurring in recipient cells under specific controlled conditions. In these controls, 10^5 recipient cells were incubated in the same manner as the experimental tests. No growth appeared on the plate containing only the donor DNA. The results obtained from each individual experiment are given in Table 6. The colonies appearing from recipient cells without DNA treatment are most likely due to spontaneous back mutation. For the bacterium, Escherichia coli, the spontaneous mutation rate per generation for lactose fermentation, lactose negative to lactose positive, is $2 \cdot 10^{-7}$ (20). The value of .00002% is low when compared to the percentage obtained in this study; however, one would expect the lactose reversion rate to vary in different organisms. No information on this value for C. pseudotropicalis was found in the literature. The lactose negative cells may lack the enzyme, B-D-galactoside galactohydrolase, needed for the reaction B-D-galactoside + H_2O= an alcohol + D-galactose and also in the catalyses of galactotransferase reactions (8). If this inference is correct, the character transformed represents the ability to produce this enzyme,

Table 6. Controls Utilized on Basic Glucose Minimal Culture Medium

Control	Number of Transformations on Chemically Defined Agar	
	Experiment 1	Experiment 2
10^5 Recipient cells	11	8
50 μg DNA	0	0
Heat treated DNA and 10^5 recipient cells	17	22
10^2 Donor cells	93	98
Media not inoculated	0	0
DNase treated DNA and 10^5 recipient cells	6	14

Table 7. Reversions Occurring in Controls

Control	Percentage Reversion
Untreated Recipient Cells	.0095%
Heat Treated DNA with Recipient Cells	.0195%
DNase Treated DNA with Recipient Cells	.010%
DNA Sample	.000%

as the recipient cells fail to form the protein, or form an inactive form of it, and therefore cannot ferment lactose. Additional study is needed before the point can be elucidated further.

When DNase was incubated with DNA prior to adding the DNA to recipient cells, the transforming activity was greatly reduced (Tables 6 and 7). The value (.010%) received is similar to that obtained from heat-treated DNA, but higher than the figure obtained from untreated auxotrophic cells. The information from Table 8 demonstrates that no decrease in the transforming activity of the DNA occurs in supernatant fluid. One observes from this table the number of transformants and percentage of transformation obtained from 10^5 recipient cells. The abscissa shows the respective DNA concentration from 25 to 100 µg. The percentage of prototrophic cells increased from .092% to 0.121% when the DNA concentration varied from 25 to 75 µg and then dropped to 0.117% with 100 µg DNA. This parallels the results obtained in Table 3.

The results in Table 9 furnish information on transformation in relation to age of the culture. The cultures were grown in 500ml Erlenmeyer flasks containing 100ml of basic glucose minimal medium A. All flasks were incubated at 35°C on low speed rotary shakers. DNA added to a 14 hour old culture produced the highest number of transformants; however, a smaller number of transformants was noted with a 9 hours old

Table 8. Effect on Recipient Cells Treated with Supernatant Fluid and DNA

Number of transformants	92	101	121	117
Percentage of transformation	.092%	0.101%	0.121%	0.117%
Concentration of DNA µg	25	50	75	100

Table 9. Transformants and Age of Culture in Basic Glucose Medium A and 50 μg DNA

Age in hours of culture	Number of transformants from 10^5 recipient cells
9	62
14	78
20	47

culture, and the lowest number of transformants appeared in a 20 hour culture. The results obtained from this experiment represent lower values than that obtained from either experiment 1 or 2 for the same DNA concentration and medium (Tables 4 and 5). It was not possible to repeat this investigation with expanded time periods because of the low DNA supply then available. Figure 3 reveals the number of transformants related to culture age. An increase is observed from a 9 to 14 hour old culture; however, a decrease occurred from this age to a 20 hour old culture.

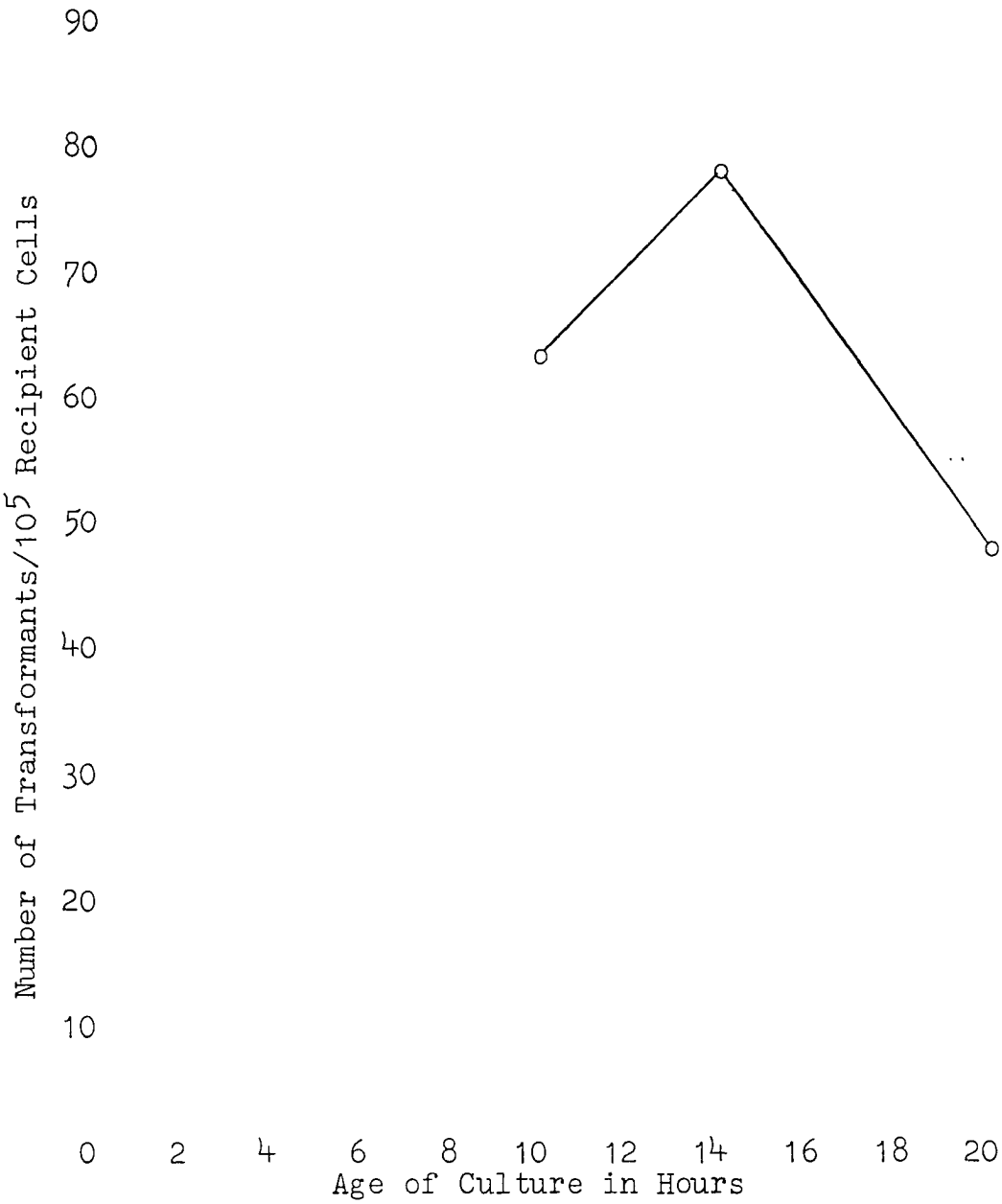


Figure 3. Number of Transformants Related to Culture Age with 50 µg DNA.

CHAPTER IV

DISCUSSION

The evidence presented in this paper shows that a transformation process occurs in C. pseudotropicalis. Although the impetus for genetic transformation was provided in 1928 by Griffith (9), the process of how a cell develops the capacity to transport DNA remains obscure. This present study adds several critical observations that should aid in determining conditions under which transformation may occur.

The failure by some previous investigations (5 & 31) to establish genetic transformation in fungi has been reported. However, most failures discussed the difficulty of extracting DNA and reported the highest yields were obtained by breaking up the cells mechanically. The preparations were often referred to as crude DNA and no mention of molecular weight was made. Investigators of bacterial genetics have considered the study of the transforming activity as increasing with the purity of the DNA preparation. With the belief that the essential features of transformation are common to all microorganisms, it would appear that the DNA

preparation used by some researchers of fungi may not have been active due to low molecular weight or lack of purity. The biological activity of a DNA preparation is extremely labile, and the activity probably was destroyed or reduced during the different stages in preparation of fungal DNA. Moreover, the activity of DNA may be dependent on age, and it is not yet possible to predict the biological activity of a preparation. In transformation the size of the transforming fragment is predetermined by the degree to which the donor DNA is broken down during extraction and purification. The molecular weight of transforming preparations of bacterial DNA has been estimated to be about $1 \cdot 10^6$ to $15 \cdot 10^6$ (20).

Current research indicates the cell does not distinguish between a molecule of DNA carrying a lactose marker and any other DNA molecule, and the transforming molecule competes with the non-transforming molecules for entry into the cell (34). Therefore, fewer transformants would be found with more numerous but smaller DNA fragments. The relatively mild extraction procedure used in this study would lessen this problem; and the results obtained here, in relation to other fungal reports, support this view.

Considerable variation exists in the use of supporting media for the production of competence. Levine (20) reported that competence is a transitory state of the recipient population and noted the duration to be restricted to a small fraction of the growth cycle. Generally, competence in

bacteria develops toward the end of the exponential phase of growth, when the susceptibility to transformation rises rapidly to a maximum. However, the physiology and biochemistry of bacteria differ markedly from that of fungi, and very little is known about the nature of competence in fungi which may depend on the structure of the recipient cell surface rather than environmental factors in the medium. The results shown in Figure 2 represent the average number of transformants obtained in all media except those containing D-2-D glucose. This figure reveals a gradual increase in transformants correlated to DNA concentration up to 75 µg.

Streips and Young (35) reported the identification of competence provoking factors in a number of bacteria. It was their conclusion that extracellular substances were involved in eliciting competence. The authors noted, however, that the phenomenon was not reproduced in many laboratories, possibly due to the instability of the factors. Streips and Welken (36) reported an incompetent cell does not release the competence-inducing factors into the culture medium, but the supernatant fluid of a competent culture contained the factors. The competence-inducing factors are believed to have different properties with different bacteria.

Tevethia and Mandel (40) reported the biological activity of double-stranded DNA was unaffected by preincubation in supernatant fluid, although an inactivating factor was noted that appeared to be a nuclease with selectivity for

single-stranded DNA. If any type of inactivating nuclease is associated with C. pseudotropicalis, no detection of its presence was made. Table 8 shows recipient cells incubated with DNA previously treated with supernatant fluid from a 24 hour old culture. The number of transformants remains nearly the same under similar conditions without prior exposure to supernatant fluid. If nucleases were present in the supernatant, incubation with DNA for 30 minutes at 37°C would reduce the number of transformants. Such results were obtained when DNA was added to commercial DNase in a controlled experiment (Tables 6 & 7). If DNases are present, these enzymes are inactive under the conditions used in this study. Pakula and Walczak (25) reported that competence in Streptococcus salivarius depends on the presence of an enzyme that renders the cell wall permeable to DNA. These authors demonstrated that the addition of this enzyme converted non-transformable cells into competent bacteria. If a nuclease is associated with the uptake of DNA, it could be located on the surface of the cell or attached to the cell so as not to be found in the supernatant. The existence of exocellular DNase, which depolymerize any DNA, would make transformation nearly impossible. In such organisms, the exocellular enzyme must be inactivated without inactivating the transforming DNA.

In this study the two strands of the double helix were separated by heating and cooling rapidly so most of the

strands would remain separate. Generally such single-stranded DNA is virtually inactive in transformation; however, several authors including Tevethia and Caudill (39) reported that under certain controlled conditions single-stranded DNA is effective in transformation. The data in Tables 6 and 7 demonstrates that heated DNA in C. pseudotropicalis may be incorporated into the cell, but the number of transformants is low. There is also the possibility that a number of complementary single strands reunite to yield the bihelical DNA molecule, and this would account for the ability of the preparation to yield several transformants. It would appear from this experiment that the cells at least preferentially incorporate the unheated double-stranded molecule.

The data in this study suggest that the yeast cells are not activated to a state of competence by factors in the medium. If this conclusion is valid, then the yeast cells may respond in a similar manner in a number of different media. This is supported in the observation that transformants developed in four different media (Table 3). Transformation was noted in a basic glucose minimal culture medium containing only the factors necessary for growth and also in a medium supplemented with nutrient broth and yeast extract. If competence in yeasts is determined by the synthesis of specific factors, one medium would be more effective in producing this factor. However, the medium that contained fewer

growth factors actually contained slightly more transformants. This is in agreement with the reported observations in bacteria that a simple medium will often produce more transformants than media that contain additional growth factors (1 & 49). This fact suggests that the presence of supplementary nutrients may reduce the sensitivity to transformation by permitting more rapid cell wall syntheses which would prevent DNA uptake. However, Horváth (14) found that more bacteria become competent in a supplemented medium.

There is evidence that the ability to absorb DNA is correlated with the chemical composition of the cell surface, and the entire apparatus for the uptake may reside in the recipient cell. DNA may exist for a period of time between the cell wall and the plasma membrane of the cell. Numerous studies have implicated the cell wall with competence in bacteria (13).

Candida pseudotropicalis cells, growing in 2-deoxyglucose, were examined under oil immersion for evidence of reduced cell wall components. Svoboda, Farkas, and Bauer (38) and Johnson (15) investigated the influence of 2-deoxyglucose on cell wall formation, and reported the inhibition of cell wall material. These authors found no rigid cell wall material on the cell's surface while the cells remained alive for over 48 hours. It was difficult to ascertain this observation in this study. When viewed under 1000 magnification, the cells walls appeared thinner when compared to

cells from a basic glucose medium using Webb's cell wall stain. The microscope used was an Olympus, model E, binocular with achromatic objectives and a Koehler illuminator. Comparison of Tables 3, 4, and 5 revealed that 2-deoxyglucose reduced the number of transformants. A complete explanation of the data is difficult at this time; however, the evidence suggests that the yeast cells allowed penetration of DNA through the cell wall, while the efficiency of passage into the cell was reduced without a complete cell wall. Lacks (17) reported that it was possible for one or more enzymes to aid in the penetration of DNA. When the end of a double-stranded DNA molecule just penetrated the cell wall, the enzyme(s) would act so as to release nucleotides from one strand and pull the other strand into the cell. If this explanation is correct, a model for the transformation process in C. pseudotropicalis may be similar. Enzymes located on the surface of the plasma membrane may aid in the penetration of DNA, and any removal of portions of the cell wall may upset the organization and relationship of these enzymes with the cell wall. This would account for the low number of transformants obtained with the cell wall inhibitor. The observation that no increase in transformation occurred with supernatant fluid (Table 8) would imply that the enzymes are not easily removed from the cell.

It is believed that in certain microorganisms, and at specific periods in their life cycles, pathways become

available for the penetration of DNA. The ability to absorb DNA in these organisms would involve structural alterations in the cell wall that occurs during the life of the cell. The alteration would permit the penetration of the macromolecules. This conclusion would exclude DNA uptake during the entire growth period. The data in Table 9 support the view that the ability of the yeast cell to absorb DNA remains throughout a 24 hour period, although increased permeability to DNA may occur at specific periods of growth. The increase may coincide with the synthesis of more enzymes located on the cell's membrane or with an alteration in the cell wall that occurs during growth.

Any uptake of DNA by diffusion would depend on the size and number of the pores in the cell wall and plasma membrane of the cell. This view would predict the smaller size DNA molecules to be more readily taken into the cell. However, most investigators have confirmed that one of the basic obstacles in effecting repeated transformation in most organisms is obtaining a high molecular weight DNA. As discussed earlier, usual preparations of competent cells do not accept small DNA fragments. Transformation is, most probably, connected with an active transport system involving enzymes that are bound to the membrane. The results of the present investigation would focus future study on the concept of cell wall involvement in transformation in C. pseudotropicalis and would infer an enzyme system to be associated with the cell wall and the plasma membrane.

CHAPTER V

SUMMARY AND CONCLUSIONS

Numerous attempts have been made to demonstrate transformation in organisms other than bacteria. Most investigators of fungi have approached the problem through use of methods that would introduce DNA degradation by the mechanical scission of the molecule. It is believed that the essential features of transformation, involving purity and size of the donor DNA, would be common to all those organisms which display it.

Evidence presented in this paper indicates that transformation occurs in C. pseudotropicalis. Data obtained from two separate experiments support this view in the conversion of lactose negative cells to lactose positive cells. When the DNA from prototrophic cells was mixed in media with auxotrophic cells, approximately 0.1% of the recipient cells were transformed to prototrophs. In view of the known complexity of factors involved in the transformation process, a critical appraisal of transformation in yeast will require a more detailed examination.

The conditions and factors responsible for the results obtained in this study indicate that the ability of C. pseudotropicalis to incorporate extracellular DNA does not depend on the acquisition of a unique physiological state. The transformation event appeared to persist throughout a 24 hour growth cycle and in a number of different media supplying various environmental factors. Data presented support the concept of an active transport mechanism involving transformation in C. pseudotropicalis, the enzymes of which appear to be membrane bound.

It is believed this information on a transformation process in C. pseudotropicalis could lead to the discovery of a similar event in other cells and microorganisms. Organisms may eventually be interbred by transferring a small genetic unit, and the simplicity of the process gives many possibilities for developing particularly desirable characteristics.

The principal contributions of these investigations have been: 1. Determination of evidence that transformation exists in C. pseudotropicalis, 2. Information on factors that may influence competency in yeast, 3. To suggest evidence that would indicate the invasion of cells by extraneous genetic material is not restricted to bacteria, and the knowledge that certain microorganisms may engage spontaneously in bilateral exchange of genes when DNA is liberated into the environment from dead cells, 4. The idea of transformation in microorganisms, in addition to bacteria, offers a promising laboratory tool to provide evidence of genetic relationships.

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