3. MATERIALS AND METHODS
Tests employed for the identification of Bacillus, Staphylococcus, Vibrio and Enterobacteria.

Microscopic Morphology: Gram's staining of smears were done according to Preston and Morell's (1962) modification (Cruikshank, 1974).

Motility: This was tested by hanging drop preparation (Cruikshank, 1974).

Serological tests: Slide agglutination was carried out with specific antisera for bacteria wherever applicable. Control tests using physiological saline or acriflavine solution (if needed), in the place of antiserum were also carried out.

Oxidase test: The filter paper method described by Kovacs (1956) was followed.

Catalase test: A modification of the method described by Edwards and Ewing (1962) was followed. A small amount of culture from a fresh nutrient agar slant was rubbed on a clean glass slide followed by the addition of a small volume of hydrogen peroxide. Immediate effervescence with production of gas bubbles from the solid culture was taken to be a positive reaction.

Oxidation- Fermentation test (O-F): The method of Hugh and Leifson (1953) modified by Edwards and Ewing (1962) was followed.

Potassium Cyanide test: The method described by Braun and Guggenheim (1932) was followed.
Coagulase test:

Slide test: The method described by Cowan and Steel (1965) was followed. A thick suspension of the strain to be tested was made in normal saline on a glass slide. The bacterial suspension was stirred with a straight wire dipped in plasma. Appearance of macroscopic clumping within 5 secs. was taken to be a positive reaction.

Tube test: To 5 ml of undiluted rabbit plasma an equal volume of 18 - 20 hour broth culture of the strain to be tested was added. The mixture was incubated at 37° C and readings were taken after 1 and 4 hours. Tubes giving a negative result after 4 hours of incubation were incubated overnight and then examined.

Phosphatase test: The method described by Cowan and Steel (1965) was followed.

Gelatin liquefaction: The test described by Cowan and Steel (1965) using nutrient gelatin was employed.

Starch hydrolysis: The test was carried using starch agar. The presence of hydrolysis was detected using iodine (Lugol's) after incubation at 30° C for 3 days (Cowan and Steel, 1965).

Nitrate reduction: The test was carried out as described by Cruickshank (1974).
Indole production: The method of Kovac as described by Edward and Ewing (1962) was adopted.

Methyl Red test: The method of Clark and Lubs (1915) as described by Cruickshank (1974) was followed.

Production of Acetyl-methyl carbinol: (Voges-Proskauer reaction): The method described by Barritt (1936) was followed.

Fermentation of carbohydrates: Mannitol, Xylose, Glycerol, Maltose, sorbitol, raffinose, rhamnose, dulcitol, lactose, glucose, mannose, sucrose, and arabinose fermentation reactions were tested following the method described by Edwards and Ewing (1962). Results were recorded after overnight incubation, thereafter daily, and finally after 7 days of incubation.

3.2 Media used for the cultivation and identification of test organisms:

Liquid media:

Peptone water: This contained 0.8% peptone (Bacteriological, Oxoid), 0.5% NaCl (Analar) at pH 7.4 to 7.6. For viable counts, dilutions were made in 4.5 ml of peptone water distributed into tubes before autoclaving.

Nutrient broth: This contained 1% peptone (Bacteriological, Oxoid), 0.3% NaCl (Analar) and 1% Lab-lemco beef extract (Oxoid), pH 7.4.

Solid media:

Nutrient agar: This medium consisted of 1% agar (Oxoid, No. 3), 1% peptone (Bacteriological, Oxoid), 1% 'Lablemco' beef extract
Bromothymol blue lactose agar; This medium consisted of nutrient agar base and 1.2% bromothymol blue indicator at pH 7.4. Lactose was added after autoclaving and the medium was steamed for half an hour before the plates were poured (Sen, Chakrabarty, Sen and Ghosh, 1968).

Antibiotic media;

Determination of minimum inhibitory concentration (MIC) for selection of recipient bacteria: The MIC of different antibiotics for recipient bacteria was determined by incorporating penicillin (Pe), polymyxin (Po), gramicidin (Gr), kanamycin (Km), neomycin (Ne), streptomycin (St), tetracycline (To), singly in nutrient agar medium. The final concentrations of antibiotic per ml of medium used were 5, 10, 25, and 50 μg each individually.

Determination of the level of antibiotic resistance(s) of donor and transformant bacteria: Nutrient agar contained the antibiotics e.g., Pe, Po, Gr, Km, St or To singly, in the following concentrations: 100, 200, 400, 600, 800, and 2000 μg per ml of medium.

Selection of donor and recipient strains; The donor organisms used in this study were either antibiotic producing bacilli or highly antibiotic resistant strains of Candida albicans. The recipient bacteria used were either antibiotic producing bacilli or highly antibiotic resistant strains of Candida albicans. The recipient bacteria used were either antibiotic producing bacilli or highly antibiotic resistant strains of Candida albicans. The recipient bacteria used were either antibiotic producing bacilli or highly antibiotic resistant strains of Candida albicans. The recipient bacteria used were either antibiotic producing bacilli or highly antibiotic resistant strains of Candida albicans.
in the transformation system were sensitive to most of the antibiotics tested.

3.5 Media and tests for the identification of Candida albicans: (Emmons, Binford and Utz, 1972).

**Mounting medium:** (Lacto phenol-cotton blue): Phenol crystals-20g (melted in waterbath and weighed), lactic acid- 20g, glycerin- 40g, distilled water- 20ml, cotton blue (Poirrier's blue)-0.05g (added to the completed solution).

**Sabouraud's dextrose agar:** Dextrose (Analar) 4%, peptone (Mycological, Oxoid) 1%, pH 5.6 to 5.8.

**Beef extract agar:** Beef extract (Oxoid) 3%, NaCl (Analar) 0.5%, peptone (Mycological, Oxoid) 1%, Agar (Oxoid) 1%, pH 7.4.

**Beef extract blood agar:** Same as above with the addition of 10% sterile human blood.

**Carbohydrate broth:** Beef extract (Oxoid) 0.3g, NaCl (Analar) 0.5g, peptone (Oxoid) 1g dissolved in 90 ml distilled water and pH adjusted to 7.2; 10 ml of bromothymol blue indicator is added, filtered through cotton, distributed in 10 ml quantities and autoclaved; 0.5 ml of a 20% solution of any one of the carbohydrates (e.g. dextrose, maltose, sucrose or lactose), sterilised by keeping at 100° C for 20 minutes for three consecutive days, was then added to the former medium.

**Rice infusion agar:** Rice (2.5g) was boiled in 50ml of distilled water for one hour and filtered; the filtrate was mixed with 1%
agar previously digested in 50 ml of distilled water, pH adjusted to 5.6 and 1 ml of Tween 80 was added lastly as clearing agent before autoclaving.

Glucose-Yeast extract agar: (Waksman, 1961): Glucose 1g, Yeast extract 1g, Agar 1.5 g in 100 ml of tap water, pH 6.8.

Glucose-Asparagine agar: (Waksman, 1961): Glucose 1g, Asparagine 0.05g, K₂HPO₄ 0.05g, Agar 1.5g in 100 ml of distilled water, pH 6.8.

3.6 Transfer of polymyxin resistance from Bacillus polymyxa to Salmonella and Staphylococcus aureus by transformation in situ.

3.6.1 Source of donor: Bacillus polymyxa 2459 was received from the Dept. of Molecular biology, Madurai Kamaraj University, India which originated from Ms. Pfizer Research Laboratories, U.S.A.

3.6.2 Cultivation and maintenance of the donor organism: The strain was grown on nutrient agar and preserved as stab cultures and as freeze-dried ampoules.

3.6.3 Medium for paradoxical inhibition (MPI): This was also known as medium for in situ transformation (Chakrabarty and Dastidar, 1974; Chakrabarty, Ganguly, Chaudhuri, and Dastidar, 1977). This medium contained: beef extract 1%, peptone 1%, agar no.3 1% (all Oxoid) NaCl (Analar) 0.5% and sodium dodecyl sulphate (SDS) at a final concentration of 30 μg/ml and ethylene-diamine-tetra-acetic acid (EDTA) at a final concentration of 15 μg/ml; pH 7.4 to 7.5. Nine ml of this medium was placed in 45 mm petri dishes. This was designated as MPI. For transfer of
antibiotic resistance from the DNA of different organisms to sensitive bacteria this medium was supplemented with the respective antibiotics.

3.6.4 Basic technique for demonstration of central growth of transformation on MPI with or without antibiotic: Basic technique of Chakrabarty and Dastidar (1974) was adapted to suit the requirement of the present study. The actual technique consisted of growing the donor bacteria primary as inoculum, (*Bacillus polymyxa* 2459) as a diametrical streak on MPI medium contained in 45 mm. petridishes at 30°C. The incubation period was 48 hour, at the end of which the primary growth was removed by scraping off with the edge of a sterile glass slide, the plate was exposed to chloroform vapours for two hours and aerated for two hours.

The agar surface was turned upside down with all sterile precautions to look for transformation on the cell free surface (Chakrabarty and Dastidar, 1974).

3.6.5 Demonstration of central growth of transformation with antibiotic overlay: For this purpose the first part of the basic technique described above was carried out on MPI without added polymyxin. Each test plate in which the donor was grown earlier, and the MPI medium inverted and replaced, and was then overlayed with 1 ml of polymyxin-MPI to ensure sufficient inhibitory concentrations of the antibiotic, i.e. polymyxin ranging between 100 to 200 µg, and refrigerated overnight to allow equilibration of the antibiotic between the two layers.
Secondary bacteria, sensitive to polymyxin, which had been grown overnight in peptone water were then streaked in parallel, at right angles to the growth of the primary donor bacterium.

**Inoculation of secondary bacteria:** Routinely these were applied as five to six parallel streaks on MPI plates at right angles to the growth of the donor organism. When a continuous growth of a single bacterium was desired, 45 mm Petridishes were inoculated in one of the two ways: i) multiple overlapping streaks, 10 to 12 in number from the same culture suspension, or ii) by a sterile filter paper disc (Whatman No. 1; 40 mm in diameter) evenly moistened with $0.1 \text{ ml of } 10^{-1}$ dilution of the culture suspension, dried for 10 minutes at 37°C, placed for 1 to 2 minutes on the agar surface and removed with a pair of sterile forceps.

3.6.6 **Acquisition tests:** Secondary recipient bacteria that produced transformants manifested as central growths of paradoxical inhibition with respect to donor organism were taken up for further studies on acquisition of antibiotic-resistance markers.

Routine tests for acquisition of antibiotic-resistance were carried out on all the central growths resulting from multiple cross-streaking of secondary bacteria. Portions of central growth were suspended in peptone water and their polymyxin resistant colony count ($A$) and total colony count ($B$) were determined respectively on polymyxin agar and nutrient agar according to the technique of Miles and Misra (1938).
The incidence of cells in the population of central growth that had acquired polymyxin resistance was calculated as \(\frac{A}{B} \times 100\). In control tests, no primary inoculations were done on polymyxin-MPI and antibiotic-resistant (mutant) colonies appearing in the secondary inocula by 18 hours or later were counted for an area equal to that of the corresponding growth on test plate.

3.6.7 **Kinetics of acquisition:** Following the growth of the primary organism (donor) on MPI with or without polymyxin, 0.02 ml of a suitable dilution of an 18 hour culture suspension of the secondary organism was spread uniformly over the area of primary growth (on the cell free surface). Sterile filter paper strips (Whatman No. 1) measuring 10 x 40 mm were placed in contact with the agar surface for one to two minutes and then removed with sterility precaution into peptone water vials of 10 ml quantity, thoroughly mixed and suitably diluted for determination of colony forming units (CFU) on antibiotic and nutrient agar plates. The samples were taken at 2, 4, 6, 8, 10, 12, 24 and 48 hour intervals from parallel plates. The determination of CFU was as described by Miles and Misra (1938).

**Determination of transformation frequency:** The frequency was calculated as follows:\[ \frac{(A-B) \times X}{C \times Y} \]

where

- **A** = transformant colony count on antibiotic agar
- **B** = mutant colony count on antibiotic agar
Confirmatory acquisition tests: These were performed only when necessary. After completion of the first part of the basic technique 0.1 ml of $10^{-2}$ dilution of a secondary culture suspension was spread uniformly on polymyxin-MPI and samples of 6 hour growth from the central area were transferred by filter paper strips (10 mm x 40 mm) to 10 ml aliquots of peptone water. The incidence (%) of antibiotic resistant cells was determined as in the case of routine acquisition tests.

Determination of level of polymyxin-resistance acquired by the transformants: Polymyxin-resistant transformants of wild type sensitive recipients growing on polymyxin plates were then tested for maximum level of resistance acquired by recovering them from corresponding nutrient agar plates. The plates were incubated for 72 hours for appearance of growth.

Tests with DNase and RNase: The test conditions were as described by Schaeffer (1964); 1 ml of stock DNase solution containing 1 mg purified DNase (DNEP-1, SIGMA Laboratories, USA) with 10 mg of MgSO$_4$ in peptone water was used as the stock solution. The test with RNase was similar to the above but contained 1 mg RNase (Bovine pancreatic, BDH, India) instead.
DNase or RNase at concentrations of 25, 30, 40 and 50 μg/ml of medium was incorporated in polymyxin-MPI and the primary bacteria were inoculated according to the basic technique for demonstration of transformants (central growth of paradoxical inhibition) with and without antibiotic overlay. In this test system the top-layer also contained the corresponding enzyme in the same concentration. In the control plates, enzymes were omitted. In all these tests the plates were incubated at 37° for 24 hours, but the incubation period was prolonged if necessary.

3.6.10 Reading of results: The growth or absence of growth of the recipient bacteria (secondary inocula) on different test media were designated as follows:

a) Inhibition reaction (I): complete failure of growth of the secondary bacteria.

b) Central growth (CG): when the transformant colonies grew mostly on or around the growth of the donor bacterium, often forming a confluent band of growth in the centre of the streak of the inoculum; commonly, such growths were flanked by areas of inhibition on the two sides.

c) Resistant growth: (-): when there was confluent growth of the secondary inoculum throughout the entire length of the streak. This could happen when, either the donor or transformant or other resistant bacteria were inoculated.
"Curing" experiments with Bacillus polymyxa 2459 and the polymyxin transformant S. Uganda 101J strain.

Bacillus polymyxa 2459:

Media: Nutrient agar was used throughout the experiment. The MIC of ethidium bromide to B. polymyxa was determined in peptone water. Antibiotic-agar used was nutrient agar with polymyxin at 5, 10 or 20 μg/ml.

Methodology: (Chakrabarty and Dastidar, 1974; Bounchaud and Chabbert, 1968).

Determination of MIC of Ethidium Bromide (EB) to Bacillus polymyxa 2459: A stock solution of ethidium bromide at 100 μg/ml was used. 1 ml aliquots of peptone water were taken to which ethidium bromide was distributed to give final concentrations of 1, 2, 4, 6, 8, 10 μg/ml. One drop of an overnight growth of B. polymyxa 2459 (containing about 10^6 CFU) in peptone water was added and the tubes were incubated overnight at room temperature. The lowest concentration of EB in the series that prevented growth of B. polymyxa was taken as its minimal inhibitory concentration (MIC).

'Curing' experiments: The tube of ethidium bromide having the lowest concentration of ethidium bromide preventing growth was selected and the growth was plated out on nutrient agar (containing 1 μg of ethidium bromide) to give isolated colonies. These isolated colonies were individually stabbed onto nutrient agar and polymyxin agar plates.
containing 10 or 20 µg/ml of polymyxin. The results were read after 24 and 48 hours incubation at room temperature. Colonies failing to grow at 10 µg/ml of polymyxin were picked up from the corresponding nutrient agar plates and retested at 2 and 5 µg/ml of polymyxin.

3.7 Agarose Gel Electrophoresis.

Preparation of Lambda phage DNA: DNA from lambda phage was obtained following the procedure described by Hedgpeth, Goodman and Boyer (1972). The Escherichia coli strain C1857SuSS7 a lysogen, obtained from Dr. Boyer, Dept. of Microbiology, University of California, U.S.A., was used as a source of lambda phage. Tryptose broth cultures of lysogens (2 x 10^8 cell/ml) were heated to 42°C for 30 min and reincubated at 37°C for 3 hours. The cells were collected by low speed centrifugation and resuspended in 5 to 10 ml of 50 mM Tris-HCl (pH 7.5) containing 10 mM MgCl_2; 0.5 ml of chloroform was added to this and the suspension was incubated at 37°C for 15 minutes. After low-speed centrifugation the viscosity of the supernatant was reduced by the addition of pancreatic DNase (10 to 50 µg). The supernatant was centrifuged at 79,000 x g at 4°C for 1 hour. The pellet was resuspended in 50 mM Tris-HCl (pH 7.5) and extracted with phenol repeatedly. The aqueous phase was finally dialysed against TE buffer (50 mM Tris-HCl, pH 7.5 with 0.1 mM EDTA). This DNA was used as a control for unknown plasmid DNA molecular weight approximations.
Procedure: (Helling, Goodman and Boyer, 1974): 15 cm x 0.6 cm gels were formed in 16 cm glass tubing with a slight constriction at one end or with nylon netting across one end to retain the gel. Agarose (Sigma) in TEA - NaCl was melted by autoclaving. (TEA: 0.05 M Tris, 0.02 M Na acetate, 2 x 10^{-3} M Na$_2$EDTA, pH adjusted to 8.05 with glacial acetic acid. TEA- NaCl : TEA plus 0.018 M NaCl). A small amount was used to seal the bottom of the tube. Then additional agarose (cooled below 60°C) was added to fill the column. After hardening, the upper end of the gel was extruded and sliced evenly to form a 15 cm gel. The sample was heated for five minutes at 65°C in order to separate loosely associated DNA molecules, and quenched on ice. Bromophenol blue and sucrose were added (20% sucrose) to give a final sample volume of 15 to 100 μl. The sample was run into the gel for five minutes at 100V and thereafter at 1.5V/cm gel (22.5V). Buffer chambers contained TEA-NaCl. After about 18 hours of electrophoresis at room temperature (approximately 22°C) the dye marker was at one gel tip. With 0.7% agarose the dye mobility is equivalent to that of DNA of 1-3 x 10^5 M.W. under these conditions. All gels were stained with 1 μg/ml of ethidium bromide. After half hour the stained bands were visualized by fluorescence over long wave length ultraviolet light. Photographs were taken after staining duplicate gels with 0.2% toluidene blue as described by Morris and Smith (1977).
Transformation of polymyxin resistance from Bacillus polymyxa to Staphylococci & Enterobacteria using extracted DNA.

**Source of organism:** B. polymyxa 2459 was obtained as described in previous chapter.

**Extraction of whole DNA:** The method of Berns and Thomas (1965) modified to suit Bacillus polymyxa was used. The organism was grown overnight at 30°C for Bacillus polymyxa 2459 and at 37°C for Bacillus polymyxa NCTC 4747 in Tryptic Soy broth (Difco) at pH 7.4 to 7.5. One to two grams of bacterial deposit was suspended in 5 ml of saline-citrate (0.15 M NaCl; 0.015M Sodium citrate). The bacteria were lysed with sodium dodecyl sulphate (SDS) added to a final concentration of 0.2%, kept at 60°C for ten to twenty minutes. The resulting viscous mixture was extracted two to three times with water-saturated phenol. The aqueous phase was then extracted with ether and DNA was precipitated with twice the volume of chilled alcohol. The precipitate was dissolved in Tris-HCl buffer (10 mM Tris pH 7.5) and dialysed overnight at 4°C with Tris-EDTA buffer (Tris HCl 10 mM; EDTA 1 mM, pH 7.5).

**Extraction of DNA fractions:** Plasmid fraction: 500 ml of overnight culture of a strain of Bacillus polymyxa (or one of the transformants of polymyxin-resistance) was incubated at 30°C on shake culture and was centrifuged. The deposit was suspended in one ml of 25% sucrose in Tris-HCl buffer (0.5M, pH 8.0). The suspension was kept in an ice bath after the addition of 0.2 ml of lysozyme (5 mg/ml in 0.25 M Tris-Hcl)
pH 8.0); for 5 minutes followed by the addition of 0.4 ml of EDTA (0.25 M pH 8.0) and the mixture was kept at 0°C for another five minutes. A 25% w/v stock solution of SDS was then added to give a final concentration of 1%. The mixture was held at 60°C for complete lysis. This was followed by the addition of 5M NaCl to a final concentration of 1M. The reaction mixture was kept at 4°C overnight followed by centrifugation at 17,000 x g for 30 minutes. Deproteinisation, precipitation and dialysis were then performed as described earlier for whole DNA (Guerry, Leblanc and Falkow, 1973; Meyers, Sanchez, Elwell and Falkow, 1976).

Chromosomal fraction: The deposit obtained after centrifugation at 17,000 x g for 30 minutes at 4°C was resuspended in saline-citrate (SSC) and processed as for whole DNA extract.

Estimation of DNA:

a) Diphenylamine reaction: (Schneider, 1957)

Reagent: The diphenylamine reagent was prepared by dissolving 1g of diphenylamine in 100 ml glacial acetic acid and adding 2.75 ml of conc. H₂SO₄. This solution was stable indefinitely in cold.

Procedure: To 1 volume of the unknown containing 50-500 μg of DNA, is added 2 volumes of the reagent. The reaction mixture was heated in a water bath at 100°C for 10 min. A blue colour appears which has a maximum absorption at 595 nm. The absorbancy was checked by using a Beckman spectrophotometer.
Calf thymus DNA (Sigma Chemicals USA) was included as the standard.

b) Spectrophotometric measurement: (Marmur, 1961). DNA concentration was determined from the absorbance at 260 nm using an absorbancy per mole of nucleotide of 6000 cm$^{-1}$.

**Transformation experiments based on DNA and enzymes.**

The same medium as has been described for transformation *in situ* experiments was used here. Transformation experiments were also performed at 50 µg/ml selection levels in addition to the usual selection levels of 30 µg/ml. The transformation tests were carried out as follows:

1 cm wide troughs prepared in uninoculated MPI medium were filled up with a mixture of molten MPI medium (at 46°C) containing 100 µg of DNA in Tris-HCl buffer (pH 7.5), in the case of whole DNA fractions or chromosomal DNA fractions and 20-30 µg/ml in the case of plasmid DNA fractions. The trough medium was allowed to set and 1 ml overlay of MPI containing 50 µg of polymyxin was added. The plates were refrigerated overnight to settle. Recipient bacteria were inoculated and the results were read as described in the *in situ* techniques.

**Tests with DNase and RNase:** The DNA extract before being mixed with MPI and placed in the trough was treated with different concentrations of DNase (10, 20, 40, 50 and 100 µg) and incubated for one hour at 37°C. The stock solution of DNase was the same as used in tests for *in situ* transformation. The test with
RNase was performed in a similar manner. The mixture of DNA-DNase or DNA-RNase after appropriate incubation was finally mixed with molten MPI (48°C) and placed in the trough in the usual manner. The other procedures were the same as for transformation experiments based on purified DNA.

3.9 Transformation of Staphylococci and Vibrios to penicillin resistance using Bacillus licheniformis.

In situ transformation technique:

Source of donor bacterium: The strain of B. licheniformis 749, a constitutive producer of penicillinase was obtained from Prof. M.R. Pollock, University of Edinburg, Edinburg.

Cultivation and Maintenance: The strain was grown on nutrient agar at 37°C and preserved on nutrient agar slants as well as freeze-dried ampoules.

Transformation test: The medium for paradoxical inhibition as described for in situ transformation test with B. polymyxa was used. Here the concentrations of SDS and EDTA were 30 and 15 µg/ml respectively.

The methodology, kinetics of acquisition of penicillin-resistance and confirmatory acquisition tests also were the same as those used for transfer of polymyxin resistance, but incubation was for 24 hours in these experiments. Transformation experiments were performed on the surface opposite to that of growth of the donor bacterium; for this the block of agar medium was detached with due sterile precautions from
the petridish by means of sterile spatula and replaced in an inverted position so as to present the sterile, fresh surface to the top.

The level of selection of transformants was 300 and 500 µg/ml of penicillin.

As the recipient bacterium *Staphylococcus aureus* ML275 was found sensitive to SDS, a selection of SDS-resistance as a marker for a possible co-transfer with the penicillin resistance (Sonstein and Baldwin, 1972) was looked for by incorporating SDS at a concentration of 30 µg/ml in the medium.

**Transformation of *Staphylococci* and *Vibrios* to penicillin resistance by DNA extracted from *B. licheniformis*, 749.

**Using extracted DNA:** Extraction of whole DNA was carried out as described for *B. polymyxa*.

**Transformation experiments:** The transformation experiments on transfer of penicillin resistance were done as described for transfer of polymyxin resistance using *B. polymyxa* as donor, except for the following: the level of SDS was 20 µg/ml and that of EDTA was 10 µg/ml.

**Tests with DNase and RNase:** The methodology followed was the same as described with *Bacillus polymyxa*.

**Transformation of gramicidin resistance from *Bacillus brevis* 7096 to *Staphylococcus aureus*:**

**In situ transformation:**

**Source of donor:** *Bacillus brevis* NCTC 7096 was obtained from
the National Collection of Type Cultures, England.

Gramicidin S was obtained from Sigma Chemicals, U.S.A.

**Media:** The medium for paradoxical inhibition (MPI) as described for polymyxin resistance transfer was used.

**Methodology:** The techniques followed were the same as described for polymyxin resistance transfer except for the following: The incubation period of the primary organism was 48 hours, the secondary bacteria were streaked on the same surface as that of the primary. The transformants were selected at 100 μg/ml concentrations of Gramicidin in the medium.

The acquisition tests were performed as described earlier. The tests with DNase and RNase were performed as has been described for *Bacillus licheniformis* experiments.

3.12 **Studies on the transformation of sensitive bacteria using Candida albicans as donor.**

**Growth of Candida albicans in different sugar media:** Studies on growth of *Candida albicans* on MPI medium (Chakrabarty and Dastidar, 1974), beef extract 1%, peptone 1%, agar 1%, all oxoid NaCl (Analar) 0.5%, pH 7.4 to 7.5, containing various sugars like glucose, lactose, maltose and sucrose (each individually, at 2% level) but lacking SDS and EDTA, showed that the growth of *Candida albicans* occurred best on MPI containing sucrose. On the basis of these findings sucrose was incorporated in MPI medium in all transformation tests in which the donor
organism was *Candida albicans*, as abundant and quick growth appeared to be helpful in such tests.

Transformation of sensitive Vibrios and Enterobacteriaceae to multiple antibiotic resistance using *Candida albicans* 154 and 167 as donors.

**In situ transformation technique:**

**Source of the donors:** *Candida albicans* 154 and 167: The two strains of *Candida albicans* which were multiply resistant to streptomycin, tetracycline, kanamycin, and polymyxin were obtained from Dr. M. Sanyal, School of Tropical Medicine, Calcutta.

**Cultivation and Maintenance:** The strains were grown on Sabouraud's dextrose agar slants at 37°C, and preserved as spores in sterile distilled water, (Mcginnis, Padhya and Ajello, 1974).

**Transformation tests:** The medium for transformation employed in the transfer of multiple antibiotic resistances from *Candida albicans* consisted of MPI with nutrient agar plus 0.02% sodium dodecyl sulphate, 0.02% ethylene-diamine-tetracetic acid and supplemented with 2% sucrose; pH 7.4 to 7.5. The donor organism was incubated for 72 hours after inoculation as described in the previous chapters.

The methodology of the kinetics of acquisition of multiple antibiotic resistances were as described for others. The agar surface for transformation was the same as that on which the donor had grown. The transformants were selected by using 200 μg/ml of either of the antibiotics, i.e., streptomycin, tetracycline or...
kanamycin as an one ml overlay of the same medium.

The effects of DNase and RNase on the transfer were performed as has been described for the earlier experiments.


Extraction of DNA from Candida albicans: Extraction of DNA of *C. albicans* was carried out following the method of Marmur, (1961), Guerry, Emden and Falkow (1974).

The organism was inoculated into dextrose broth and grown with aeration for 48 hours. The packed cells were washed twice with saline-EDTA (0.15 M NaCl and 0.1 M EDTA ) suspended in 25 ml of saline-EDTA and lysed with 2 mg/ml of 2 ml lysozyme, by placing it in a waterbath at 37°C for 15 minutes. The cells were kept frozen overnight and thawed the next day. To this suspension was added 2 to 5 ml of 25% SDS and this was placed in a waterbath at 60°C for 1 to 8 hours until the lysis became apparent. The viscous lysed suspension was cooled to room temperature and sodium perchlorate was added to give a final concentration of 1 M. The entire mixture was shaken with an equal volume of chloroform : isoamyl alcohol mixture (24:1) in a ground glass-stoppered flask for 30 minutes. The resulting emulsion was separated into 3 layers by centrifugation at 5,000xg for 5 minutes. The upper aqueous phase containing DNA was carefully aspirated out in a sterile tube, precipitated by gently layering approximately twice the volume of ice-cold ethyl alcohol on the aqueous phase. The DNA was collected as a spool
on a glass rod, drained free of alcohol and dissolved in 10 to 15 ml of dilute saline citrate (0.015M NaCl and 0.0015M trisodium citrate). This solution was dialysed overnight at 4°C against saline citrate (0.15M NaCl and 0.015M trisodium citrate).

**Transformation experiments:** The methodology involved in this experiment was as had been followed in the tests on transformation of polymyxin resistance from *P. polymyxa* using extracted whole DNA, except that the medium used was as described in the *in situ* transformation experiments for *C. albicans.*

**Tests with DNase and RNase:** The technique described for the experiment with *P. polymyxa* was followed.