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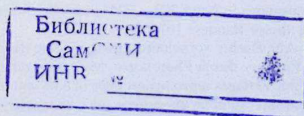
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(Institute of Vaccines and Sera, Scientific-Industrial Union "Bacteriophage", 380060 Tbilisi, Georgia, USSR)

The virulent bacteriophage IRA of *Salmonella typhimurium*: Cloning of phage genes which are potentially lethal for the host cell

REVAZ S. ADAMIA, ELVINA A. MATITASHVILI, LEILA I. KVACHADZE, VLADIMIR I. KORINTELI, DIANA A. MATOYAN, MZIA I. KUTATELADZE and TEIMURAZ G. CHANISHVILI

(Received 18 May 1990/Accepted 18 June 1990)

Morphological and biological properties of *Salmonella typhimurium* phage IRA were studied. The phage is a member of the *Styloviridae* family and exhibits a very wide spectrum of lytic activity.

Molecular cloning of phage genes whose expression is lethal for the host cell has been performed and consequences of gene expression have been investigated. Expression of recombinant plasmid pKI71 causes structural changes of the cell wall and also degradation of host DNA while plasmid DNA remains intact. Expression of pKI72 blocks normal cell division. The possibility of applying such recombinant clones in marking pathways of microbial contamination in water areas is proposed.

In spite of significant decrease of molecular studies on bacteriophages, investigation of new bacterial viruses and the mechanisms of their interaction with host cells are still of interest for several purposes. First, bacteriophages remain excellent model systems for elucidating such major problems of biology as DNA-protein and protein-protein recognition. Second, the significance of several pathogenic microorganisms oblige investigators to search for new therapeutic and diagnostic remedies. This problem seems especially difficult when analysing the spread of gastro-enteric diseases caused by pathogenic *Shigella*, *Salmonella* and *Escherichia* strains. The therapeutic use of virulent phage preparations together with antibiotics appears promising. Third, investigation of phage genomes and gene products may be useful with regard to practical applications in biotechnology as well as in some other fields of medicine.

Recently in many clinics a sharp increase of hospital outbreaks of salmonellosis was observed. It is a great problem especially in pediatric hospitals. An extremely rapid spread of multiresistant strains is limiting the effectivity of antibiotic treatment. This problem could be addressed by alternative therapeutic remedies.

Several years ago Scientific Industrial Union (SIU) "Bacteriophage" began to produce a phage preparation which is active against various strains of *Salmonella typhimurium*.

Clinical examination showed that this preparation effectively suppressed the rise and spread of salmonellosis (KIKNADZE *et al.* 1986). On the other hand the molecular properties of the phages included in this multicomponent preparation are almost unexplored. This was the main reason why we decided to investigate the basic characteristics of phage IRA. In addition we have studied some aspects of interaction of this phage with *Salmonella typhimurium* host cells. In particular, we were interested in the mechanisms by which this bacterial virus causes degradation of host cellular structures ensuring its successful replication.

Materials and methods

Phage and bacterial strains: Bacteriophage IRA (CHIRAKADZE and CHANISHVILI 1974) was kindly provided by Dr. I. G. CHIRAKADZE (SIU "Bacteriophage"). *Salmonella typhimurium* strain Q (FELIX and CALLOW 1943) obtained from the Bacterial Culture Collection of SIU "Bacteriophage" was used to study the one-step growth properties of this phage. 870 clinical strains of *Salmonella typhimurium* obtained from various hospitals of the Soviet Union were used to determine the host range of the phage. *Escherichia coli* JM105 (Δ (*lac pro*), *thi*, *strA*, *endA*, *shcB15*, *F' [lacI^qlacZ M15proAB⁺]*) (CHEZARENI *et al.* 1985) was used for all plasmid propagations. The vector pKK223-3 was kindly provided by Dr. N. A. SELIVANOV (Institute of Virology, Moscow).

The lytic activity of phage IRA was investigated by standard methods (KÜHN *et al.* 1973). Adsorption characteristics and one-step growth properties of the phage were determined as described by ADAMS (1959).

Restriction endonucleases, DNA ligase, DNAase, nuclease *Bal*31 and DNA polymerase were purchased from PHARMACIA. Buffers and media were prepared using reagents from SIGMA and MERCK. α -³²P dATP was obtained from AMERSHAM. Hybridization reactions were performed on 0.45 μ m nitrocellulose filters (MILLIPORE).

Phage IRA was grown by a standard procedure as described previously (TIKHONENKO *et al.* 1966). After tangential flow of phage suspension through a MINITAN apparatus (MILLIPORE), purification of concentrated phage particles was achieved by CsCl density gradient centrifugation.

Phage DNA was isolated by a standard alkaline-phenol procedure. Bacterial chromosomal DNA was obtained by the method of MARMUR (1961). The DNAs of pKK223-3 and of the recombinant plasmids were isolated by the method of BIRNBOIM and DOLY (1979).

Restriction and ligation of DNA, electrophoresis in 0.8% agarose slab gels, DNA transfer to nitrocellulose filters, DNA hybridization, preparation of ³²P DNA, and autoradiography were performed by standard methods (MANIATIS *et al.* 1982).

Combined hydrolysis of DNA by several restriction enzymes as well as DNA digestion by *Bal*31 endonuclease were used to construct the physical map.

Micrographs of phage particles and bacterial cells were obtained by JEM 1200EX electron microscope (JEOL). Parlodion (MALLINCKRODT) plates were overlaid by phage suspensions and contrasted by uranylacetate. Bacterial cells were first fixed by 2.5% glutaraldehyde, then by 1% OsO₄, dehydrated and poured into araldite mix. Ultrathin sections were prepared by Ultratome III (LKB). Additional contrast was obtained by the use of lead citrate (VENABLE and GOGGESHAL 1965).

Determination of the influence of cloned IRA genes on recipient bacterial cells: *E. coli* JM105 was transformed by pK171 and pK172 recombinant plasmids and grown up until OD₅₄₀ = 0.3 in LURIA-BERTANI medium (MANIATIS *et al.* 1982) containing 30 μ g/ml ampicillin. Isopropyl- β -D-thiogalactoside (IPTG) was added to a final concentration of 5 mM and growth was continued for 24 hours. Aliquots were taken at various time intervals and viable cell counts were determined. These aliquots were divided into two parts; cellular DNA was isolated from the first sample, while the cells of the second were analysed by electron microscopy. Transformed *E. coli* JM105 cells without inducer as well as nontransformed *E. coli* JM105 cells served as controls.

Results and discussion

Table 1 shows that bacteriophage IRA possesses an extremely wide spectrum of lytic activity; 844 (97%) of 870 *Salmonella typhimurium* strains were lysed. These results favour bacteriophage IRA as a component of medicine preparations.

Standard *Salmonella typhimurium* strain Q was used as a host to study biological properties of phage IRA (Table 1). The latency period, lysis time and yield of virus particles are typical for virulent phages.

Morphological features of phage IRA are presented in Fig. 1. The microphotography shows that the phage particle consists of a head with dimensions of 500 $\times$ 500 Å and a noncontractile tail with dimensions of 2800 $\times$ 150 Å. A base plate with short fibrils, possibly promoting the attachment of phage to the host cell, can be seen at the distal end of the phage tail. According to these morphological data bacteriophage IRA is a typical representative of the *Styloviridae* family and can be considered as a member of group IV by BRADLEY's classification of bacterial viruses (1967) or of the morphological type SI12 according to the taxonomical system proposed by ŠLOPEK and KRZYWY (1985).

Table 1
Biological properties of bacteriophage IRA

Lytic activity			Adsorption (37 °C)		One-step growth properties (37 °C)		
Number of strains tested	Number of strains lysed	%	% of adsorbed phage after 4 min	Adsorption constant (ml/min)	Latency period (min)	Lysis time (min)	Average burst size
870	844	97	72 ± 2	$4 \cdot 6 \times 10^{-10}$	40	20	300

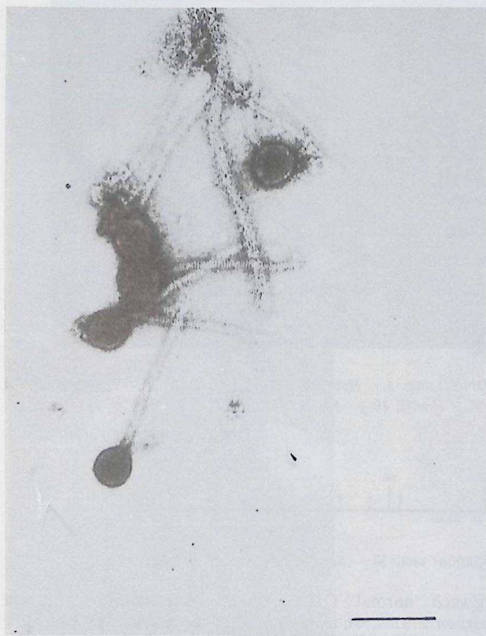


Fig. 1
Electron micrography of bacteriophage IRA.
Bar represents 100 nm

Structural parameters of bacteriophage IRA DNA were studied previously (ANDRIASHVILI *et al.* 1982, 1983). The molecular weight of the linear double-stranded polynucleotide is 48×10^6 D and the DNA does not contain any hydroxymethylated and glucosylated bases as judged by chromatographic analysis.



Fig. 2

Restriction patterns of phage IRA DNA. Lanes: 1 — *HpaII*, 2 — *BglIII*, 3 — *HindIII*, 4 — *EcoRI*, 5 — *HhaI*, 6 — *XhoI*, 7 — *TaqI*, 8 — *AvaI*, 9 — *HaeIII*, 10 — *AvaII*, 11 — *SmaI*, 12 — *EcoRV*, 13 — *SalI*

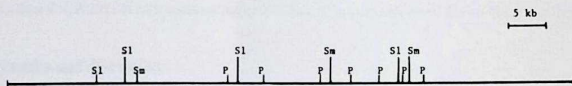


Fig. 3

Physical map of IRA DNA. Abbreviations used: S1 — *SalI*, Sm — *SmaI*, P — *PstI*

Since phage IRA containing such "normal" DNA propagates successfully on the vast majority of *Salmonella typhimurium* strains, we investigated the mechanisms by which the bacteriophage overcomes cellular defense systems.

First of all we performed restriction analysis of IRA DNA (Fig. 2). The fact that all the 13 different enzymes digested phage DNA indirectly confirmed the absence of modified bases (at least in major quantities).

These results, as well as combined DNA digestions by several restriction enzymes and *Bal31* nuclease permitted us to construct the physical map of IRA DNA (Fig. 3).

It is well known that bacteriophages possess various modes of overcoming the defense system of the host cell (KRÜGER and BICKLE 1983). For instance, in the case of T-even phages, hydroxymethylation and glycosylation of cytosine residues protect the DNA against

restriction. Several cellular exo- and endonucleases are not able to hydrolyse such modified T-even DNA. On the other hand, phages code for specific nucleases which degrade the host cell DNA (WARNER and SNUSTAD 1983).

From this point of view, it was of great interest to examine how bacteriophage IRA, while not possessing a DNA with unusual bases, overcomes defense mechanisms of host cells, induces inhibition of cellular metabolism and secures its own reproduction.

We therefore decided to clone fragments of phage IRA DNA whose expression would be lethal for the host cell. Such fragments can be cloned only in vectors which, like pKK223-3, provide a regulated expression of foreign genes. pKK223-3 is constructed in such a way that

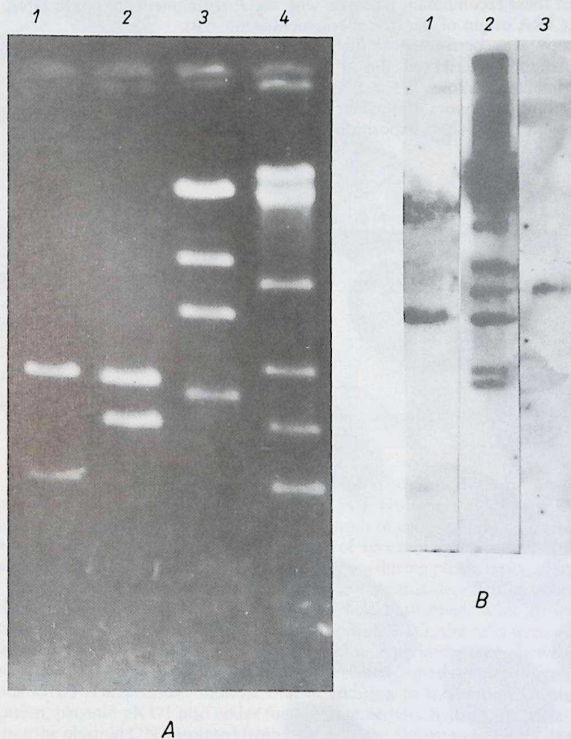


Fig. 4
Identification of phage IRA DNA fragments in pK171 and pK172 recombinant plasmids. A) Restriction patterns of *Pst*I digest: Lanes: 1 — pK172, 2 — pK171, 4 — phage IRA DNA. Lane 3 — λ + HindIII. B) Hybridization of *Pst*I-digested recombinant plasmids with α - 32 P DNA of IRA. Lanes: 1 — pK172, 2 — IRA DNA, 3 — pK171

expression of inserted fragments can be achieved only after induction by isopropyl- β -D-thiogalactoside (IPTG) (BROSIOUS and HOLY 1984).

*Pst*I fragments of IRA phage DNA were ligated to the pKK223-3 plasmid and *E. coli* JM105 cells were transformed by these constructions. Recombinant clones were identified in shotgun experiments by parallel plating in the presence and absence of inducer. This approach allowed us to isolate directly recombinant clones carrying genes lethal for the cell.

Two clones were selected which formed colonies on the plates without inducer but failed on the IPTG-containing agar. Restriction analysis of these clones is shown in Fig. 4A. The first clone contains a recombinant plasmid which we designated as pKI71. This plasmid is composed of vector DNA and the 3.6 kbp *Pst*I-E fragment of IRA DNA. The second clone carries the plasmid pKI72 which contains the 3.0 kbp *Pst*I-G fragment of phage DNA. Blot hybridization of these recombinant plasmids with the *Pst*I fragments of phage DNA confirmed the phage IRA origin of the cloned fragments (Fig. 4B).

Electron microscopy was performed to determine the character of morphological alterations that occurred in bacterial cells due to the expression of cloned phage genes. The results of these experiments are shown in Fig. 5. Expression of pKI71 (Fig. 5B) causes significant alterations in cell wall structure. Preliminary data showed that these changes are more pronounced in cells with rather short exposition to the inducer.

A



Fig. 5A

B

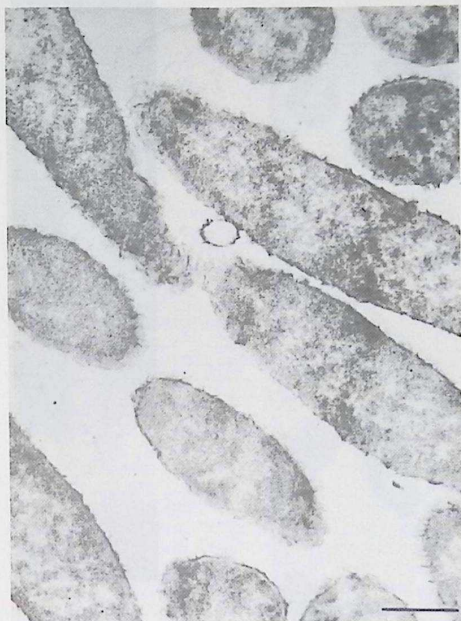


Fig. 5B

The character of cell morphology alterations provoked by expression of pKI72 is different. The cell wall appeared normal (Fig. 5C). However, in the vast majority of the cells two or more nucleoids occurred and the length of cells containing expressing pKI72 increases significantly. It seems that products of recombinant plasmids pKI72 genes disrupt normal cell division. The function of these genes during phage replication is as yet unknown.

In addition to morphological studies we investigated the effect of phage gene products on the host DNA. The following experiments were performed: after growth of bacterial cells carrying a recombinant plasmid and addition of IPTG, the cells were disrupted and the genomic DNA was isolated. The results of these experiments are shown in Fig. 6. It can be seen that in the case of pKI71 the expression of phage genes causes degradation of chromosomal DNA. These results indicate that in addition to the activity causing cell wall modification, plasmid pKI71 also codes for nuclease activity hydrolyzing host DNA. At the same time the plasmid DNA isolated from such cells was shown to be intact. It seems that a mechanism still unknown provides selective degradation of genomic DNA. In the case of pKI72 no nucleolytic activity was found.

The experiments described here suggested the use of the recombinant clones for an additional purpose: epidemiological marking of microbial contamination pathways of natural and artificial water reservoirs. Such experiments are necessary to identify zones of sanitary

C

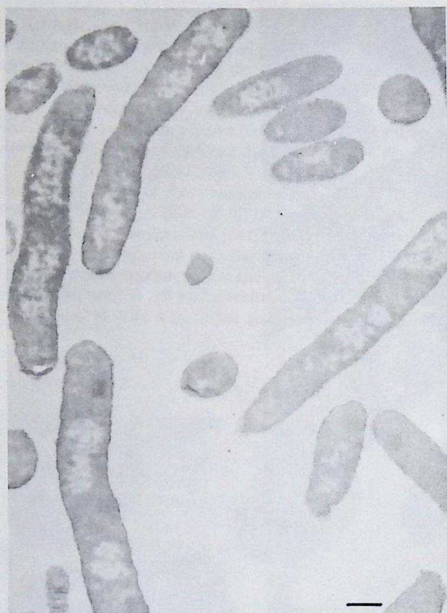


Fig. 5C

Fig. 5

Effect of recombinant gene expression on *E. coli* JM105 cells. A) Normal *E. coli* JM105 cells. B) *E. coli* JM105 cells transformed by pK171 plasmid and grown in presence of IPTG. C) *E. coli* JM105 cells transformed by pK172 plasmid and grown in the presence of IPTG. Bars represent 500 nm

control and to determine the effectivity of water purification, sewage systems, etc. The major problem in such investigation is the reliable indication of the marker strains. Application of recombinant strains as markers would guarantee the reliability of results — such strains do not occur in nature. Use of clones with inducible plasmids excludes expression of inserted genes under natural conditions. Besides, application of phage genes for insertion is probably the least dangerous to the natural environment.

Moreover, the use of clones carrying recombinant plasmids with lethal activity should add to safety because spontaneous expression of inserted genes causes cell death.

Model experiments which showed that clones carrying the recombinant plasmids pK171 and pK172 are easily identified under natural conditions have been performed. Therefore, the clones carrying these plasmids are suitable for marking the microbial contamination pathways of water areas.

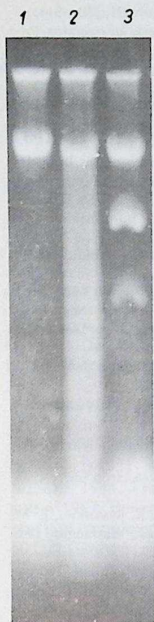


Fig. 6

Determination of nuclease activity in the cells carrying recombinant plasmids. Lane 1 — DNA isolated from nontransformed *E. coli* JM105 cells; Lane 2 — DNA from *E. coli* JM105 transformed by pKI71 and grown in the presence of IPTG; Lane 3 — DNA from *E. coli* JM105 transformed by pKI72 and grown in the presence of IPTG

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Purification and properties of α -amylase from *Micrococcus varians*

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An extracellular α -amylase from *Micrococcus varians* was partially purified (63 fold) by ammonium sulphate precipitation followed by dialysis and separated by molecular exclusion into three components with molecular weights ranging from approximately 14,000 to 56,000. The amylase had a pH optimum of 7.0 and apparent K_m of approximately 0.5 mg ml^{-1} for starch. The enzyme activity was stimulated by Ca^{2+} and Mg^{2+} ions. Optimum temperature was 45°C while there was a complete absence of activity at 70°C in 20 min. Ethylene diaminetetra acetic acid (EDTA) and iodoacetic acid (IA) inhibited the activity of the enzyme.

The occurrence of microbial amylases with varied catalytic properties has been extensively reviewed by FOGARTY and KELLY (1980). In general, depending on the source, α -amylases are known to exhibit the following properties: pH range of stability between 4 and 11 (BERNFELD 1951, BUONOCORE *et al.* 1976, FOGARTY and KELLY 1980, HOPKINS 1946, SIMOES-MENDES 1984, TAKAGI *et al.* 1971) with optimum between 5.3 and 7 (AMUND and OGUNSHINA 1987, BERNFELD 1951, FOGARTY and KELLY 1980), MICHAELIS constant for enzyme-amylase complex of 1.8×10^{-4} – 6.3×10^{-4} (BERNFELD 1951), molecular weight of 45,000–60,000 (BERNFELD 1951, KRISHNAN and CHANDRA 1983, SIMOES-MENDES 1984, TAKAGI *et al.* 1971), ion-dependent (BERNFELD 1951), heat labile at above 60°C (HOPKINS 1946), and susceptible to enzyme inhibitors (BERNFELD 1951). Among the bacterial species producing high levels of amylase, those of *Bacillus* species have been widely reported (AMUND and OGUNSHINA 1987, BUONOCORE *et al.* 1976, KRISHNAN and CHANDRA 1983, SRIVASTARA and BARUAH 1986, TAKAGI *et al.* 1971, WELKER and CAMPBELL 1967). A preliminary study has been undertaken on the production and control of α -amylase of *Micrococcus varians* (ADELEYE 1990). Because of the immense potential of this enzyme for industrial applications, the current report deals with the purification, catalytic and molecular properties of the *M. varians* α -amylase. The effects of divalent cations as well as enzyme inhibitors were also examined.

Materials and methods

Organism and culture conditions: The isolate, *Micrococcus varians* (IFU 79) was isolated from the soil at the Obafemi Awolowo University, Ife Campus, Nigeria. Stock cultures were maintained on nutrient agar at 2°C in an incubator. The isolate was kept fresh by repeated transfers into nutrient agar slants. Young cultures were used in all experiments by subculturing the organism 24 hours before inoculation.

Organisms were cultivated in a basal medium containing NaCl, 3g; K_2HPO_4 , 1g; glycine, 2g; $\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$, 0.05 g; CaCl_2 , 0.01 g and Yeast Extract, 0.5 g in 1 litre of distilled water. The basal medium was autoclaved at 1.1 kg/cm^2 . Soluble starch or any other carbon source employed was autoclaved separately and added to the basal medium to give a final concentration of 1% (w/v). Each 250 ml ERLLENMEYER flask contained 100 ml of growth medium. A suspension of the organism was prepared in sterile 0.85% saline, 0.25 ml of the suspension was inoculated in each experimental flask. The flasks were incubated at 30°C in a rotary shaker (GALLENKAMP) at 120 r.p.m.

Partial purification of enzyme: Preparation of culture filtrate concentrate: The culture filtrate of the organism, harvested after 30-hour growth, showed maximum enzyme activity. It was centrifuged at 10,000 g

for 1 hour at 4 °C. The supernatant was subsequently concentrated to about 20% of the original volume in a vacuum rotary evaporator (QUICKFIT, Rotavapor-R-, Buchi, Switzerland) set at 30 °C. Frotting was avoided.

Ammonium sulphate precipitation and dialysis: The concentrate (150 ml) was precipitated with ammonium sulphate (analytical grade) to 85% (WHITAKER *et al.* 1963, WOOD 1968). The solution was centrifuged at 10,000 g at 4 °C for 1 hour. The supernatant was discarded. The precipitate was dissolved in 0.1 M citrate phosphate buffer (pH 6.0). The solution was dialysed against 10 litres of 0.1 M citrate phosphate buffer for 24 hours at 4 °C. Dialysis was carried out in cellophane tubing (GALLENKAMP). The resulting partially purified enzyme was employed in the determination of some properties of the enzyme as follows:

Effect of temperature on enzyme activity: The effect of temperature on the partially purified enzyme was examined. The reaction mixture containing 2.5 ml of 0.03% starch and 0.1 ml enzyme in test tubes was incubated at 20, 25, 30, 35, 40, 45, 50 and 55 °C respectively for 1 hour before enzyme activity was assayed.

Enzyme assays: Activity of α -amylase was determined by a modification of dextrinogenic assay of PFUELLER and ELLIOT (1969). One unit of α -amylase activity (I.D.P.) was defined in arbitrary unit as the amount of enzyme in 1 ml of reaction mixture which produces 0.01% reduction per minute in the intensity of blue black colouration of starch iodine complex. Enzyme activity was calculated using the following equation:

$$\frac{C - E}{C} \times 100 \text{ of } 0.01\% \text{ per ml/min.}$$

Effect of substrate concentration on enzyme activity: The substrate concentration at which maximum enzyme activity took place was determined. Enzyme solution (0.1 ml) was incubated with different concentrations of starch (0.01, 0.015, 0.02, 0.025, 0.03, 0.035, 0.04 and 0.045% w/v, respectively at 35 °C for 1 hour and analysed for α -amylase activity.

Effect of cations on enzyme activity: Different concentrations (10, 20, 30, 40 and 50 mM) of CaCl_2 and MgCl_2 respectively were added to 0.03% starch and employed as substrate in enzyme assays.

Effect of chemicals on enzyme activity: Starch solution (0.03%) containing different concentrations of iodoacetic acid or ethylenediamine tetraacetate were prepared. The starch solution was allowed to react with the chemical for 1 hour before being employed as substrate in enzyme assays.

Effect of heat on enzyme activity: Samples of partially purified enzyme were heated at 60 and 70 °C respectively for 0 to 60 min. Appropriate quantity (0.1 ml) of the enzyme preparation was added to starch substrate incubated at 35 °C for 1 hour and α -amylase activity analyzed.

Effect of pH on enzyme activity: Starch solution (0.03% w/v) was adjusted to different pH levels (pH 3–11) and employed as substrate in enzyme assays.

Enzyme fractionation on Sephadex G-75: Preparation of Sephadex G-75 for column chromatography: The column (2.5 cm $\times$ 70 cm) of Sephadex G-75, surrounded by a water jacket maintained at 20 °C was prepared and calibrated according to the methods of OLUTIOLA and AKINTUNDE (1979). The Sephadex was suspended in excess distilled water and stirred gently to expel trapped air bubbles. It was allowed to swell for three days after which the finest particles were removed by decanting. The gel suspension was de-aerated at low pressure. The particles were swollen further by suspension for three days in the eluting buffer of pH 6. The buffer was composed of 0.1 M citric acid, 0.2 M Na_2HPO_4 and 5 M NaN_3 to prevent microbial contamination. The finest particles remaining were removed by decanting. The column was then partially filled with the buffer and the gel was poured into the column to bring the liquid to the top of the glass tube, simultaneously allowing the buffer to flow through the growing bed of gel. More gel was added until the column was connected to the reservoir and the buffer allowed to run through the bed of the gel. After two days, a constant bed height was obtained. A sample applicator (2.3 $\times$ 5.0 cm, PHARMACIA Ltd.), was carefully placed on top of the gel to prevent distortion of its top during sample application and continuous flow of buffer through it. The column was checked for uniform packing before and after each experiment by observing the passage of Ferritin, a coloured protein, through it.

Calibration of the column: The column was calibrated with substances of known molecular weight listed as follows: Sephadex G-75 (particle size 40–140 μm , supplied by PHARMACIA, Sweden); ferritin (mol. wt. 480,000); calf catalase (mol. wt. 67,000); egg albumin (mol. wt. 45,000); chymotrypsin A (mol. wt. 25,000); myoglobin (mol. wt. 17,800); cytochrome C (mol. wt. 12,000); and DNP-alanine (mol. wt. 255.2) obtained from *Serrafinbio* Chemical, Heidelberg, Germany.

The substances (5 mg each) were dissolved in 10 ml of the buffer (citrate phosphate, pH 6.0) and the solution was applied to the gel bed. The protein content of the eluent was recorded by continuous measurement of the extinction at 280 nm and fractions were collected on L.K.B. automated fraction collector (700 A ultra Rac). The relationship between the elution volume of each reference material and the logarithm of its molecular weight was close to a straight line over the molecular weight range of 12,000–67,000.

Application of partially purified (ammonium sulphate precipitation) enzyme to sephadex G-75 column: The partially purified enzyme concentrate (10 ml) was applied to the sample collector placed on top of the gel. It was eluted by addition of 5 ml of buffer periodically for a while. (The buffer was 0.1 M citric phosphate pH 6.0, containing 5 mM NaN_3). Later the top of the column was connected to the reservoir and the buffer allowed to run continuously through the bed of the gel. The elute (5 ml fraction) was automatically collected in an L.K.B. 2089 UNICORD III fraction collector. The eluted fractions, were analysed for protein content (LOWRY et al. 1951) and α -amylase activity (PFUELLER and ELLIOT 1969).

Results

Effect of pH on enzyme activity

M. varians α -amylase exhibited maximal activity at pH 7.0. There was a steady decrease in enzyme activity with further increase in pH.

Effect of temperature on enzyme activity

The α -amylase activity of *M. varians* was affected by temperature. There was a steady increase in activity from 20 °C to 45 °C. Thereafter, there was a steady decrease in activity. Activity was higher at 55 °C than at 20 °C.

Effect of heat on enzyme activity

Treatment of the enzyme at 60 °C for 10 min resulted in approximately 40% decrease in α -amylase activity. Similar treatment at 70 °C for 10 min resulted in approximately 98% reduction in activity. Heat applied for 20 and 30 min led to 100% decrease in enzyme activity at 70 and 60 °C, respectively.

Effect of substrate concentration

There was a gradual increase in α -amylase activity as the concentration of starch increased. It reached a maximum at 0.3 mg/ml⁻¹. Further increase in substrate concentration was accompanied by a fall in amylase activity. From the LINEWEAVER-BURK plot, the apparent K_m value for the hydrolysis of starch by amylase was 0.5 mg/ml⁻¹ (Fig. 1).

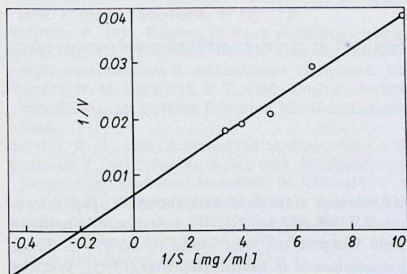


Fig. 1

LINEWEAVER-BURK plot for the hydrolysis of starch by a partially purified (ammonium sulphate precipitation) α -amylase enzyme obtained from a 30-h-old culture filtrate of *M. varians*

Effect of cations on enzyme activity

The divalent ions, Ca^{2+} and Mg^{2+} increased α -amylase activity. Calcium did so for all concentrations employed whereas the optimum concentration for magnesium was found to be 20 mM.

Effect of chemical inhibitors

Activity of *M. varians* amylase was inhibited by both ethylenediamine tetra acetic acid (EDTA) and iodoacetic acid (IA). However, low concentration (1–4 mM) of IA did not inhibit amylase activity.

Fractionation on Sephadex G-75 column

Fractionation of the enzyme concentrate on Sephadex G-75 produced three absorption peaks designated A, B and C, respectively (Fig. 2). The molecular weights of the components estimated from their elution volumes were approximately 56,000 (peak A), 20,000 (peak B), and 14,000 (peak C). Components of peak A exhibited α -amylase activity (Table 1) while those of peaks B and C did not.

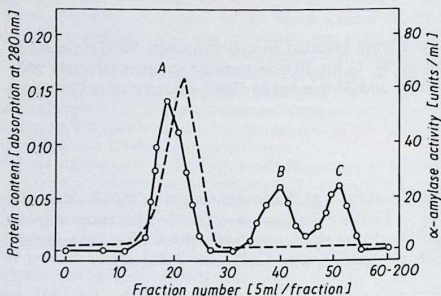


Fig. 2

Separation of proteins in concentrated culture filtrates of *M. varians* by gelfiltration and enzymic activity of the fractions towards starch.

○—○ protein (E_{280}), ---- α -amylase activity

Discussion

In comparing some of the properties of *M. varians* α -amylase with those of other micro-organisms a number of similarities were noted. High enzyme activity was recorded between pH 5–8 with an optimum at 7.0. Optimum temperature was found to be 45 °C. These properties are similar to those expressed by α -amylase of *B. subtilis* (BERNFELD 1951, WELKER and CAMPBELL 1967) and *Schwanniomyces alluis* (SIMOES-MENDES 1984).

Amylase of *M. varians* was found to be relatively less thermostable than those of some bacterial species reported in the literature. BUONOCORE *et al.* (1976) reported a stable,

Table 1
Partial purification of α -amylase of *M. varians*

Purification step	Volume (ml)	Individual activity (units/ml)	Total activity (Units)	Individual protein (mg/ml)	Total protein (mg)	Specific activity (units/mg protein)	Yield %	Purification fold
Crude culture filtrate	800	9.9	7920	0.165	132	60.0	100	1
Concentration by rotary evaporator (30 °C)	100	50.6	5060	0.175	17.5	289.1	95.8	4.8
(NH ₄) ₂ SO ₄ precipitate	48	101.2	4859	0.13	6.2	783.7	61.3	13.1
Peak A	5	64.5	322.5	0.017	0.085	3794.1	4.1	63.2

inducible, thermoacidophilic α -amylase from *B. acidocaldarius* which has an optimum at 80 °C.

Several structural and kinetic properties (molecular weight, number of polypeptide chains, dependence upon divalent ions, affinity constants, substrate specificity) are common to most α -amylases (TAKAGI *et al.* 1971). In this regard, *M. varians* enzyme behaved as a typical α -amylase with a single polypeptide chain, having a molecular weight of 56,000. Its activity was stimulated by calcium and magnesium ions. The affinity constants for the substrates were of similar order of magnitude as those usually reported for other α -amylases (BERNFELD 1951). Moreover, the enzyme was inhibited by ethylenediamine tetraacetic acid (EDTA) and iodoacetic acid (IA).

In conclusion, *Micrococcus varians* could be exploited on a large scale as source of amylase.

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Production and control of extracellular α -amylase in *Micrococcus varians*

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An extracellular α -amylase was induced in cultures of *Micrococcus varians* during growth in a liquid medium containing starch as sole carbon source. Synthesis of this enzyme was repressed by the addition of glucose or fructose to starch metabolizing cells and was induced in a glucose or fructose metabolizing culture by the addition of starch.

Glycine was found to enhance growth and production of α -amylase while the least growth and enzyme activity was recorded in medium containing ammonium sulphate as nitrogen source.

The use of microorganisms as commercial source of enzyme production in industry has been a common practice for the past few decades. Many bacteria have been reported to produce α -amylase (COLEMAN and ELLIOT 1962, INGLE and ERICKSON 1978, MEERS 1972, SAITO and YAMAMOTO 1975, WELKER and CAMPBELL 1963, WINDISH and MHATRE 1965). α -Amylase attacks the α 1-4 linkages of both amylose and amylopectin (of starch) at random, yielding, at first, linear oligosaccharides and dextrins. Further hydrolysis produces maltose, glucose and shorter chain dextrins. Only the branched points (α 1–6 linkages) of amylopectin remain intact (HOPKINS 1946).

There is strong evidence to show that the rate of bacterial amylase biosynthesis is controlled by both substrate induction and catabolite repression. The evidence of substrate induction with either starch or α 1,4-linked oligosaccharides has been obtained in studies with *Bacillus stearothermophilus* (WELKER and CAMPBELL 1963), and *B. licheniformis* (SAITO and YAMAMOTO 1975). The studies with *B. stearothermophilus* demonstrated that although enzyme synthesis in the strain was partially constitutive, there was an instantaneous increase in the rate of amylase synthesis after the addition of a series of oligosaccharides (e.g. maltose).

The experimental evidence regarding catabolite repression is no less equivocal and has been demonstrated in many species studied to date. Studies with *B. stearothermophilus* (WELKER and CAMPBELL 1963) indicated that carbohydrate source at concentrations that tend to increase the growth rate will inhibit the formation of amylase. Other studies indicate the *Bacillus* amylase biosynthetic system is under catabolite repression (MEERS 1972, SCHAEFFER 1969) and that glycerol, glucose or acetate at a level of 0.5% (w/v) can completely repress amylase synthesis (SAITO and YAMAMOTO 1975).

Most work on bacterial alpha amylase has concentrated on *Bacillus* spp. and a few related organisms. In this study, the ability of *Micrococcus varians* to produce α -amylase was examined with a view to exploiting the organism as a potential enzyme source.

Materials and methods

Organism, culture conditions and growth studies: The isolate, *Micrococcus varians* (IFU 79) was isolated from the soil at the Obafemi Awolowo University, Ife Campus, Nigeria. Stock cultures were maintained on nutrient agar at 2 °C in an incubator. The isolate was kept fresh by repeated transfers into nutrient agar slants. Young cultures were used in all experiments by subculturing the organism 24 hours before inoculation.



Organisms were cultivated in a basal medium containing NaCl, 3 g; K_2HPO_4 , 1 g; glycine, 2 g; $MgSO_4 \times 7 H_2O$, 0.05 g; $CaCl_2$, 0.01 g and Yeast Extract, 0.5 g in 1 litre of distilled water. The basal medium was autoclaved at 1.1 kg/cm². Soluble starch or any other carbon source employed was autoclaved separately and added to the basal medium to give a final concentration of 1% (w/v). Each 250 ml ERLLENMEYER flask contained 100 ml of growth medium. A suspension of the organism was prepared in sterile 0.85% saline, 0.25 ml of the suspension was inoculated into each experimental flask. The flasks were incubated at 30 °C on a rotary shaker (GALLENKAMP) at 120 r.p.m. At various intervals, 6, 12, 24, 30 and 48 h, respectively (unless otherwise stated), samples were taken to determine growth, protein concentration and α -amylase activity. Growth was determined by aseptically taking 5 ml of the bacterial suspension and measuring its absorbance at 470 nm.

Protein assay: Protein concentration was determined by the method of LOWRY *et al.* (1951) with egg albumin as standard.

Enzyme assay: Activity of α -amylase was determined by a modification of dextrinogenic assay of PFUELLER and ELLIOT (1969). One unit of α -amylase activity (I.D.P.) was defined in arbitrary unit as the amount of enzyme in 1 ml of reaction mixture which produces 0.01% reduction per minute of the intensity of blue colour of starch iodine complex.

Enzyme activity was calculated using the following equation:

$$\frac{C - E}{C} \times 100 \text{ of } 0.01\% \text{ per ml/min,}$$

where C is the absorbance of the control and E , the absorbance of the experimental samples.

Effect of carbon sources on growth and amylase production: The effects of eight carbon sources, starch, lactose, sucrose, mannitol, mannose, maltose, fructose or glucose as sole carbon sources were investigated. Each carbon source was employed at a final concentration of 1% (w/v).

Effect of nitrogen sources on growth and amylase production: The effect of seven nitrogen sources, peptone, ammonium sulphate, glycine, sodium nitrate, ammonium tartrate, leucine and potassium nitrate were investigated. Each nitrogen source was employed at a final concentration of 0.2% (w/v).

Growth conditions for catabolite repression and induction of α -amylase activity: *M. varians* was grown in synthetic medium containing starch (0.5% or 1.0%), starch plus glucose (0.5% + 0.5%) or starch plus fructose (0.5% + 0.5%). Experiments were also set up in which glucose (0.5%) or fructose (0.5%) was added to 0.5% starch medium after incubation for 6 h and 12 h respectively. Induction experiments were also set up as follows: The organism was grown in medium containing glucose (0.5%), or fructose (0.5%); glucose (0.5%) plus starch (0.5%), or fructose (0.5%) plus starch (0.5%) at the onset of experiment. After incubation for 6 and 12 hours respectively, starch (0.5%) was added to some of the experimental flasks containing 0.5% glucose or 0.5% fructose alone.

Those without added starch served as control. All cultures were incubated for a maximum of 30 hours. Experimental flasks were treated as before and supernatants were employed in enzyme assay procedure.

Results

Micrococcus varians grew in liquid medium containing starch as a sole carbon source (Fig. 1). There was good growth as from the sixth hour of incubation and maximum cell density was obtained after 30 hours of incubation. During the period of growth, proteins were released into the medium. Increase in quantity of protein was directly proportional to increase in growth. The proteins exhibited α -amylase activity in that they caused a reduction in the intensity of the blue black colour of starch-iodine complex. Production of amylase increased with period of incubation achieving a maximum level after 30 hours. There was a rapid decrease in enzyme activity thereafter.

Growth and production of amylase on different carbon sources

When supplied with different carbon sources, *M. varians* grew and secreted protein into the medium. The highest growth was recorded on mannose while sucrose supported the least growth (Table 1). However, production of α -amylase was detected where starch was present in the medium and no amylase activity occurred wherever lactose, sucrose, mannitol, mannose, maltose, fructose or glucose was the sole carbon source.

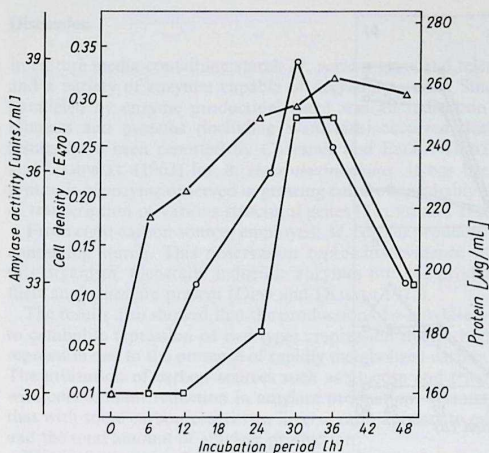


Fig. 1

Growth of *M. varians* (as cell density) in liquid medium containing 1% starch, showing protein content and α -amylase activity of culture filtrates

Table 1

Growth of *M. varians* on different carbon sources and enzymic activity of culture filtrates. Each value is a mean of three replicates.

C-sources	Growth (E ⁺ 470)	Protein (µg/ml)	Amylase activity (Units/ml)
Lactose	0.52	255	0.0
Sucrose	0.26	175	0.0
Mannitol	0.37	160	0.0
Mannose	0.60	180	0.0
Maltose	0.52	215	0.0
Starch	0.50	195	50.5
Fructose	0.55	345	0.0
Glucose	0.57	200	0.0

Growth and production of amylase on different sources of nitrogen

M. varians grew on each of the nitrogen sources employed in this investigation (Table 2). The best growth was obtained on peptone while potassium nitrate supported the least growth. Highest α -amylase activity was recorded on glycine while the least activity occurred in ammonium sulphate medium. There appeared to be a fairly close correlation between the extent of growth and the amount of α -amylase produced on each nitrogen source.

Repression and induction of α -amylase activity

It was observed that there was a decrease in amylase activity when glucose was added to growing cultures of *M. varians* (Fig. 2). Repression was higher when glucose or fructose

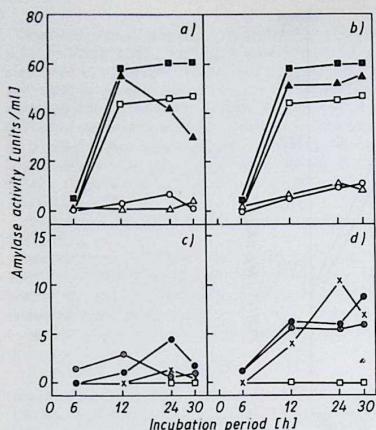


Fig. 2

Repression and induction of α -amylase activity of *M. varians* grown on single and mixed sources of carbon for 30 hours.

□ 1% starch (a, b), 1% glucose (c), 1% fructose (d), ■ 0.5% starch (a, b), ○ 1% starch + glucose at 0 h (a), 1% starch + fructose at 0 h (b), △ 1% starch + glucose at 6 h (a), 1% starch + fructose at 6 h (b), ▲ 1% starch + glucose at 12 h (a, b), 1% starch + fructose at 12 h (b), × glucose + starch at 0 h (c), fructose + starch at 0 h (d), ⊗ glucose + starch at 6 h (c), fructose + starch at 6 h (d), ● glucose + starch at 12 h (c), fructose + starch at 12 h (d)

Table 2

Growth of *M. varians* on different nitrogen sources and enzymic activity of culture filtrates. Each value is a mean of three replicates.

Effect of nitrogen sources on growth and enzyme production

N-sources	Growth (E ⁺ 470)	Amylase activity (Units/ml)
Peptone	0.80	62.0
Ammonium sulphate	0.22	19.78
Glycine	0.72	63.50
Sodium nitrate	0.20	29.0
Ammonium tartrate	0.40	35.18
Leucine	0.34	32.9
Potassium nitrate	0.18	30.1

was added (to starch metabolizing cells) at the onset of incubation (0 hour) than after 12 hours of incubation. No repression occurred if fructose was added after 12 hours of incubation. High starch concentration appeared inhibitory to α -amylase activity as activity on 1% starch was lower than activity on 0.5%.

Alpha amylase activity was induced when starch was added to glucose or fructose metabolizing cultures of *M. varians* (Fig. 2). Induction was higher when starch was added to fructose metabolizing cells at the onset of the experiment.

Discussion

In culture media containing starch *M. varians* grew and released into the medium proteins and a variety of enzymes capable of degrading starch. Since the increase in growth was paralleled by enzyme production, there was an indication that the production of both biomass and proteins (including α -amylase) occurred during logarithm phase. Similar results have been reported by COLEMAN and ELLIOT (1962) for *B. subtilis* and WELKER and CAMPBELL (1963) for *B. stearothermophilus*. It has been suggested that the periodic synthesis of enzyme observed in growing culture is probably the result of an ordered process of transcription of various structural genes (TAURO and HALVORSON 1966).

From eight carbon sources employed, *M. varians* produced α -amylase only in the medium containing starch. This observation represents evidence of inducibility of α -amylase in this organism. Generally inducible enzymes are not produced unless their substrates or their analogues are present (DEW and DEMAIN 1977).

The results also showed that the production of α -amylase by *M. varians* was also sensitive to catabolite repression of two types: repression due to high concentration of starch and repression due to the presence of rapidly metabolized carbon source (fructose and glucose). The utilization of carbon sources such as glucose and fructose gave rise to good growth with concomitant reduction in amylase production. WELKER and CAMPBELL (1963) found that with some carbon sources an inverse ratio appears to exist between the rate of growth and the total amount of amylase production.

The introduction of microbial amylases for commercial uses some seventy years ago represents a milestone in industrial enzymology. Since that time great strides have been made in the knowledge of these enzymes and in the production of different types — fungal amylase, bacterial amylases — for important commercial applications. *Micrococcus varians* studied in this work could also be exploited on a large scale as a source of amylase. Further work is in progress on the purification and characterization of *M. varians* α -amylase.

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Book Reviews

Concise Encyclopedia Biochemistry (Second Edition, revised and expanded by THOMAS SCOTT and MARY EAGLESON). 649 S., zahlr. Abb. Berlin—New York. Walter de Gruyter. DM 148,00. ISBN: 0-899-25-457-8.

Seit dem Erscheinen der 1. Auflage, die im wesentlichen der ins Englische übersetzten, nur wenig aktualisierten 2. Auflage des „Brockhaus ABC Biochemie“ (1981) entsprach, sind 5 Jahre vergangen, in denen vieles „Lexikonreife“ erreicht hat. So unterscheidet sich die 2. Auflage von ihrem Vorläufer nicht nur durch das größere Format, sondern auch durch die gestiegene Seitenzahl (649 anstelle von 519 Seiten). Aktualisiert bzw. neu aufgenommen wurden insbesondere Begriffe, die dem Intermediärstoffwechsel und dessen Regulation sowie der Molekularbiologie zuzuordnen sind. Beim Durchblättern fällt die umfangreiche Darstellung der Puffer (ca. 8 Seiten) und erblichen Stoffwechselstörungen (16 Seiten) ins Auge, beides Stichworte, die in der 1. Auflage überhaupt nicht auftauchten. Neu ist, daß sich am Ende einiger aktueller Begriffe ein Hinweis zur weiterführenden Literatur befindet. Etwas zu kurz kommt die mikrobielle Biochemie. So wird man nicht nur vergebens nach z. B. Xanthan, Pullulan, Biotensiden bzw. „biosurfactants“ sondern auch nach den Coenzymen methanogener Bakterien suchen. Entsprechendes gilt u. a. auch für Pyrrolochinolinchinon und HPLC. Zum letztgenannten Begriff findet man allerdings Ausführungen unter dem Stichwort „protein“. Bei der zu erwartenden nächsten Auflage sollten biographische Daten zu berühmten Biochemikern (z. B. auf S. 328 bei den Stichworten „KORNBERG enzyme“, „KOSHLAND model“, „KREBS cycle“ oder unter „WARBURG's Atmungsferment“) nicht fehlen. Die Verwendung von Singular und Plural ist nicht einheitlich (z. B. „fats“ aber „protein“). Das führt z. B. dazu, daß den „enzymes“ 13 „enzyme . . .“ Begriffe vorgelagert sind. Der unter „microbiological industry“ aufgeführte Verweis „industrial microbiology“ fehlt als Stichwort.

Insgesamt kann das durch übersichtliche Abbildungen sowie instruktive Tabellen illustrierte Buch auf Grund der Fülle von Informationen und der Tatsache, daß die Biochemie einen festen Platz als Grundlagendisziplin der Biowissenschaften hat, einem breiten Leserkreis empfohlen werden.

H.-P. KLEBER (Leipzig)

J. M. CRUSE and R. E. LEWIS, JR. (Editors), Conjugate Vaccines (Contributions to Microbiology and Immunology Vol. 10). VI + 198 S., 24 Abb., 17 Tab. Basel-München-Paris-London-New York-New Delhi-Singapore-Tokyo-Sydney 1989. S. Karger AG. DM 198,00. ISBN: 3-8055-4932-6.

Historisch bekannt sind LANDSTEINERS Versuche über Träger-Hapten-Konjugate. Sie wurden 1929 von AVERY und GOEBEL dahingehend erweitert, um bakterielle Konjugat-Impfstoffe herstellen zu können. Hierbei hat sich eindeutig herausgestellt, daß die Konjugation von z. B. Polysaccharid-Antigenen mit Protein-Carriern deren Immunogenität deutlich erhöht. Die ursprünglich insbesondere bei gramnegativen Bakterien (*H. influenzae* — PRP-vaccine = Polyribosyl-Ribitol-Phosphat-Impfstoff) entwickelten Vorstellungen werden heute bei Neisserien und *Pseudomonas* sp., aber auch bei Protozoen (Malaria, Leishmanien), entwickelt und erprobt. Dieser modernen Impfstoffentwicklung für ausgewählte Zielgebiete trägt die vorliegende Monographie Rechnung. 8 Beiträge werden von 20 Autoren vorgestellt; es lassen sich in etwa 3 Themenkomplexe formulieren: Herstellung und Design, immunologische Grundlagen sowie Indikationen. Es wird bei der prinzipiell günstigen kovalenten Bindung zwischen Kohlenhydrat-Antigenen und ausgewählten Proteinen die Erwartung formuliert, Morbidität und Mortalität ausgewählter Erkrankungen senken zu können. Damit ist eine Zukunftserwartung angesprochen worden, die es letztlich noch zu beweisen gilt.

Die vorliegende Monographie eröffnet hierzu gedankliche und praktische Freiräume. Sie kann und muß hierfür allen an der Impfstoffentwicklung Interessierten als Lektüre empfohlen werden. Die Ausstattung ist solide, die Literaturangaben zahlreich (zwischen etwa 40 und 274), der Preis ist jedoch recht beachtlich (198,— DM).

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The *Streptomyces aureofaciens* plasmid pIMB R8 and its use for shuttle vector construction

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The cryptic low copy plasmid designated as pIMB R8 was isolated from an industrial strain of the chlortetracycline producer *Streptomyces aureofaciens* R8/26. Using restriction endonucleases the pIMB R8 plasmid was characterized to have 15 kb. Subcloning of the *Bam* HI fragments of pIMB R8 into replication probe vector resulted in the identification of the replication part. The 0.7 kb *Bcl*I — *Bgl*II fragment is sufficient for normal replication, but produces about forty times high plasmid copy numbers than the original pIMB R8. Using the replication part of pIMB R8 we constructed several shuttle cloning vectors for the *E. coli*-streptomycete system.

Streptomyces aureofaciens is the producer of medically and commercially important antibiotics, tetracycline and chlortetracycline.

To study the biosynthetic pathway in our strain we decided to construct an *E. coli*-streptomycete shuttle vector system based on the pIMB R8 plasmid replicon, since some procedures can be performed much more easier in *E. coli* as in *Streptomyces*. Advantages of procedures available in *E. coli* have been reported by LARSON and HERSHBERGER (1984, 1986), STANCZAK *et al.* (1986) and PARADISO *et al.* (1987).

The essential DNA region for stable replication is often relatively small (SHINDOH *et al.* 1987, LARSON and HERSHBERGER 1986, KIESER *et al.* 1982, MACNEIL and GIBBONS 1986, MUTH *et al.* 1988) and is suitable for vector constructions.

In this paper we present the identification of the minimal replicon of the pIMB R8 plasmid using replicon probe vector and construction of series of *E. coli*-*Streptomyces* shuttle cloning vectors.

Materials and methods

Media and culture cultivation: *E. coli* strains of HB 101 (*recA*, *Res*⁻, *Mod*⁻, *Str*^r) (BOYER and ROULLAND-DUSOIX 1969), respectively ED 8767 (*recA56*, *supE*, *supF*, *hsdS*⁻*R*⁻*M*⁺, *met*⁻) (MURRAY *et al.* 1977) were grown at 37 °C in LB medium (MANIATIS *et al.* 1982) containing, according to need, 100 µg of ampicillin/ml or 30 µg of chloramphenicol/ml. For protoplasting, *Streptomyces aureofaciens* strains of 2201 (GODÁNY *et al.* 1986), 13/386E (MUCHOVÁ *et al.* 1989) were cultivated in S medium (OKANISHI *et al.* 1974) and for plasmid isolation in YEME medium (HOPWOOD *et al.* 1985). *Streptomyces lividans* 66 and *Streptomyces parvulus* ATCC 12434 were cultivated for protoplast and plasmid preparation in YEME medium.

Plasmid isolation: *E. coli* plasmids were isolated by the rapid alkaline method according to MANIATIS *et al.* (1982). *Streptomyces* plasmids were isolated by alkaline lysis (KIESER 1984), or by neutralization using PEG 6000 precipitation and following CsCl-EtBr buoyant density centrifugation (HOPWOOD *et al.* 1985).

Transformation of microorganisms: *E. coli* strains were made competent and transformed according to HOPWOOD *et al.* (1985). Frozen competent cells were used for most experiments. Transformation of *S. aureofaciens* strains was carried out according to MUCHOVÁ *et al.* (1989) and *S. lividans* and *S. parvulus* according to THOMPSON *et al.* (1982). Protoplasts were regenerated on R2YE agar medium for 16–20 hrs and overlaid with 1 ml of a suspension of 300 µg of thiostrepton/ml of water.

DNA manipulations: Digestion with restriction enzymes and ligations were carried out as recommended by the suppliers (Institute of Molecular Biology, Slovak Academy of Sciences and ÚSOL Praha, Czechoslovakia).

Replication probe vectors: For streptomycete plasmid replicon identification replicon probe vectors were used that carry streptomycete resistance markers and an *E. coli* vector. These vectors are unable to replicate in streptomycete.

Determination of copy numbers of plasmid DNA: The copy number of plasmid DNA in strains of *S. aureofaciens*, *S. lividans* and *S. parvulus* were estimated according to KIESER *et al.* (1982).

Results and discussion

The chlortetracycline producing strain of *S. aureofaciens* R8/26 has been investigated for the presence of plasmid DNA. From this strain plasmid DNA could be isolated only by the procedure of KIESER (1984). Other alkaline or neutral procedures for plasmid isolation were unsuccessful.

The restriction endonuclease map of pIMB R8 was determined by digestion with various restriction endonucleases (Fig. 1A). Restriction endonucleases *EcoRI* and *HindIII* did not cleave pIMB R8 plasmid DNA. Five fragments were generated by *Bam*HI, *Sal*I and *Pst*II; *Sph*I and *Kpn*I cleaved pIMB R8 at three sites and *Bgl*II, *Sau*I and *Bcl*I at two sites. Restriction endonucleases *Sma*I, *Sac*II, *Pvu*II and *Bgl*II have single sites on the pIMB R8.

To localize the essential replication region of pIMB R8 replicon probe vectors were constructed by cloning of *Streptomyces* resistance markers into the *E. coli* plasmids pACYC 184 (CHANG and COHEN 1978) and pUC 18 (NORRANDER *et al.* 1983). Replicon probe vector pIMB 01 (4.2 kb) was constructed by ligation of the *Hind*III-*Sal*I part of pUC 18 (Amp^r) with the 1.5 kb *Hind*III-*Sal*I fragment of the Tn5 kanamycin gene resistance (BECK *et al.* 1982).

By replacing the 0.34 kb *Hind*III-*Bam*HI fragment of pACYC 184 (Cm^r, Tet^r) by the 1.5 kb *Hind*III-*Bam*HI fragment of the Tn5 kanamycin resistance gene or by the 2.2 kb *Hind*III-*Bam*HI fragment containing thiostrepton resistance gene (HOPWOOD *et al.* 1985) vectors pIMB 02 (5.1 kb) and pIMB 03 (5.8 kb) were constructed.

The vector pIMB 04 (6.9 kb) was constructed by replacing of the *Hind*III-*Xho*I fragment of pIMB 03 with the 1.5 kb *Hind*III-*Sal*I fragment of the Tn5 kanamycin gene resistance.

During characterization of replication part of pIMB R8 plasmid DNA only the pIMB 03 replicon probe vector with available single cloning sites for *Xba*I, *Hind*III, *Bgl*II, *Xho*I, *Bam*HI and *Sph*I was used. The usefulness of the pIMB 03 replication probe vector is demonstrated (Fig. 1B, 2) without detectable deletions from constructed plasmid. In several cases shuttles based on the replication probe vectors pIMB 01 pIM 02 and pIMB 04 transformed from streptomycete into *E. coli*, deletions spanning different region of Tn5 gene were observed.

In order to characterize the replication function *Bam*HI fragments resulting from digestion of the pIMB R8 plasmid were cloned into replication probe vector pIMB 03. All cloning experiments were done in *E. coli*. Fig. 1B summarizes constructed hybrid plasmids which were tested for replication in *S. lividans*. From constructed plasmids only two, pIMB 801 with the 1.8 kb *Bam*HI fragment and pIMB 802 with two *Bam*HI fragments, created probably from the partial digest of pIMB R8, were able to replicate in *S. lividans*. Thus plasmid pIMB 801 is a shuttle plasmid and all information for normal plasmid replication in *Streptomyces* is located on the 1.8 kb *Bam*HI fragment. The strain of *Streptomyces aureofaciens* R8/26 contained about 1–2 copies of pIMB R8 plasmid DNA per host genome. But all of transformants (*S. lividans*, *S. parvulus*, *S. aureofaciens*) with shuttles containing the 1.8 kb *Bam*HI fragment of pIMB R8 carried about 40 copies of plasmid DNA per host genome. Increased copy number of constructed vectors is caused by removing of sequences responsible for control of plasmid copy number.

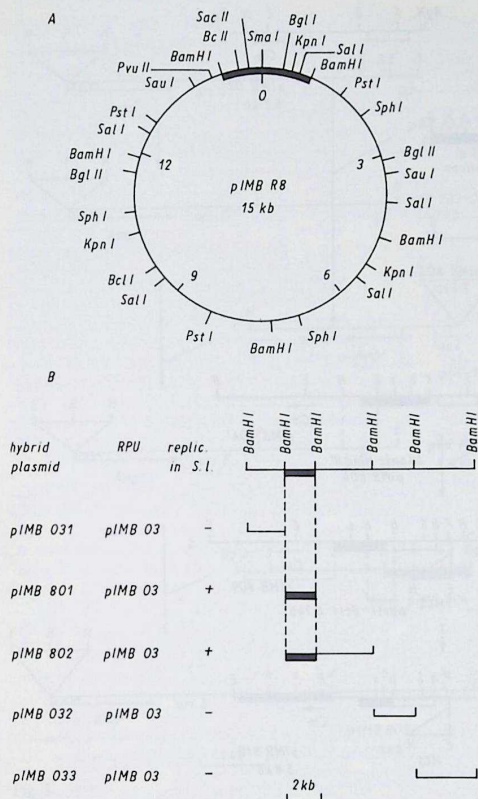


Fig. 1

A. Restriction endonuclease cleavage map of the plasmid pIMB R8. The single *Sma*I site was chosen as the beginning of the map.

B. Localization of the pIMB R8 replication region by subcloning into replication probe vector. The *Bam*HI restriction fragments of pIMB R8 subcloned into replication probe vector pIMB 03 are shown. Replication of these plasmids in *S. lividans* is indicated: +, stable replication

+ stable replication
— no replication

Black bar: The replication region of the pIMB R8 plasmid DNA

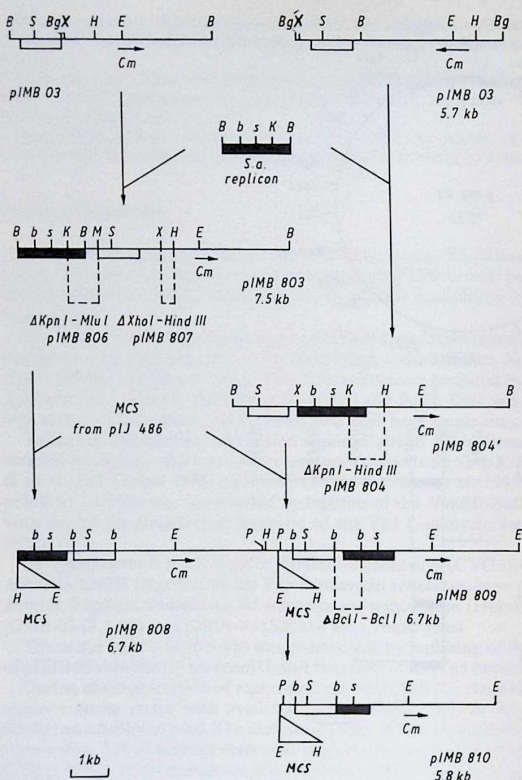


Fig. 2

Construction of shuttle cloning vectors from replicon probe vector pIMB R8 plasmid DNA.

Black bar: The replication part of *S. aureofaciens* plasmid pIMB R8.

Open bar: The thiostrepton resistance gene from *S. azureus*.

Cm: Chloramphenicol resistance gene.

MCS (multicloning site): *HindIII*, *SphI*, *PstI*, *SalI*, *XbaI*, *BamHI*, *SmaI*, *KpnI*, *SstI*, *EcoRI*.

B, *BamHI*; S, *SalI*; Bg, *BglII*; H, *HindIII*; E, *EcoRI*; K, *KpnI*; X, *XhoI*; b, *BclI*; s, *SmaI*.

The bifunctional plasmid pIMB 801 was used for further identification of the minimal replicon and construction of shuttle cloning vectors. The 1.8 kb *BamHI* fragment containing the streptomycete replicon was ligated to the pIMB 03 replicon probe vector in the *BamHI* and *BglII* site (Fig. 2) to produce pIMB 803 and 804' vectors. These plasmids transform *S. lividans*, *S. aureofaciens* and *S. parvulus* to thiostrepton resistance and can be shuttled between *E. coli* and the streptomycete host strain without any detectable deletions or rearrangements.

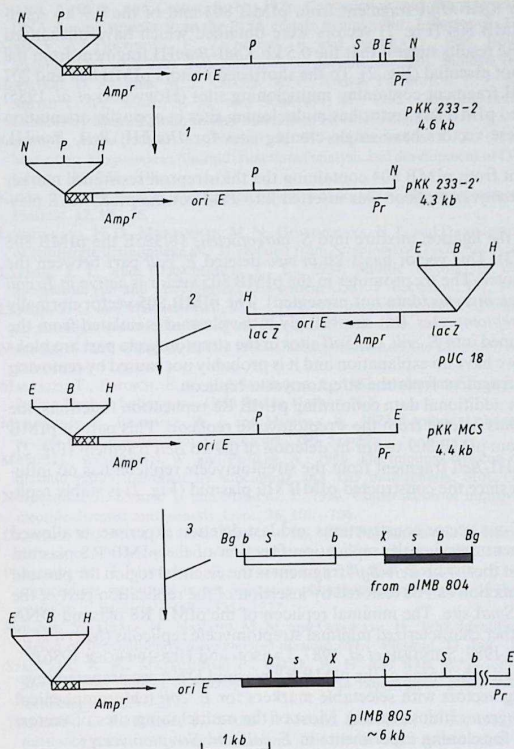


Fig. 3

Construction of the pIMB 805 shuttle vector.

1. pKK 233-2 plasmid (AMANN *et al.* 1983) was digested with *EcoRI* + *BamHI*. Sticky ends were made blunt with KLENOW fragment and ligated.
2. Deleted plasmid pKK 233-2' was linearized with *NcoI*, sticky ends were filled with KLENOW fragment and plasmid was digested with *HindIII*.
The pUC 18 plasmid was linearized with *EcoRI*, sticky ends were filled with KLENOW fragment and plasmid was digested with *HindIII*.
The digests were mixed, ligated and then used for transformation of *E. coli*. From transformants plasmid pKK MCS was screened.
3. The pKK MCS was linearized with *PvuII* and ligated with 2.5 kb *BglII* fragment of pIMB 804. Sticky ends of this fragment were filled with KLENOW fragment.

Black bar: The replication part of *Streptomyces aureofaciens* plasmid pIMB R8.

Open bar: The thiostrepton resistance gene from *S. azureus*.

Cross-hatched bar: DNA segment containing the strong *rrnB* ribosomal RNA transcription terminators.

Pr: *trc* promoter.

N, *NcoI*; P, *PstI*; S, *SalI*; B, *BamHI*; E, *EcoRI*; X, *XhoI*; H, *HindIII*; Bg, *BglII*; b, *BclI*; p, *PvuII*; s, *SmaI*

By deletions of the 0.6 kb *KpnI-MluI* fragment from pIMB 803 and of the 0.9 kb *KpnI-HindIII* fragment from pIMB 804 (Fig. 2) vectors were obtained which have unchanged replication properties. These results suggest that the 0.5 kb *KpnI-BamHI* fragment from the streptomycete replicon is not essential (Fig. 2). To the shortened vectors pIMB 804 and 807 in the *BamHI* site the *BglII* fragment containing multicloning sites (HOPWOOD *et al.* 1985) was ligated. The constructed pIMB 808 vector has multicloning sites in opposite orientation as pIMB 809 (Fig. 2). These vectors have single cloning sites for *HindIII*, *PstI*, *BamHI*, *XbaI* and *KpnI*.

The 2.5 kb *BglII* fragment from pIMB 804 containing the thiostrepton resistance marker and again shortened *Streptomyces* replicon was inserted into *PvuII* of the pKK MCS plasmid.

After transformation of the ligation mixture into *S. aureofaciens* 18/385E the pIMB 805 vector was obtained (Fig. 3). This vector has 1 kb *in vivo* deleted *E. coli* part between the *PvuII* site and the *trc* promoter. The *trc* promoter in the pIMB 805 vector is active in *E. coli* and does not work in *Streptomyces* (data not presented). The pIMB 805 vector normally replicates in *E. coli* and *Streptomyces*. But surprisingly if the plasmid is isolated from the streptomycete and transformed into *E. coli*, the *SalI* sites in the streptomycete part are blocked. For this phenomenon we have no explanation and it is probably not caused by removing of the 0.6 kb *BglII-BamHI* fragment from the streptomycete replicon.

To confirm and to obtain additional data concerning pIMB R8 replication functions, the 0.5 kb *Bam-BclI* fragment was deleted from the streptomycete replicon. This part of pIMB R8 plasmid was removed from pIMB 809 vector by deletion of 0.9 kb *BclI* fragment (Fig. 2). Deletion of the 0.5 kb *BamHI-BclI* fragment from the streptomycete replicon has no influence on plasmid replication since the constructed pIMB 810 plasmid (Fig. 2) is stably replicated in all used strains.

Combining the results of our vector constructions and last deletion experiments allowed us to define minimal fragment carrying the replication function of the pIMB R8 plasmid DNA. It has been shown that the 0.7 kb *BclI-BglII* fragment is the essential region for plasmid maintenance. Replication function can be restored by insertion of the replication part of the pIMB R8 plasmid into the *SmaI* site. The minimal replicon of the pIMB R8 plasmid DNA is comparable in size with other characterized minimal streptomycete replicons (MUTH *et al.* 1988, MACNEIL and GIBBONS 1987, SHINDOH *et al.* 1987, LARSON and HERSHBERGER 1986).

During characterization and subcloning of the pIMB R8 plasmid DNA we prepared several high copy shuttle cloning vectors with selectable markers for *E. coli* (chloramphenicol and ampicillin) and *Streptomyces* (thiostrepton). Most of the multicloning sites of vectors have been successfully used for cloning experiments in *E. coli* and *Streptomyces*.

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Book Reviews

KARL DRLICA and MONICA RILEY (Editors), *The Bacterial Chromosome*. XVI + 469 S., 136 Abb., 82 Tab. Washington, D. C. 1990. American Society for Microbiology. \$ 87.00 ISBN: 1-55581-018-7.

Die Herausgabe des „The Bacterial Chromosome“ wurde angeregt im Ergebnis einer gleichnamigen Konferenz, die von den Autoren unter der Schirmherrschaft der ASM und mit Unterstützung der National Science Foundation verantwortlich organisiert worden war. Da das Buch mehr als ein Symposiums-Band sein sollte, wurden die Autoren gebeten, instruktives Material in die Einleitungen ihrer Darstellungen einzufügen und Ausblicke über die künftigen Entwicklungen ihres Themenkreises zu geben. Beginnend mit einer historischen Einführung in die bakterielle Chromosomenforschung durch die Herausgeber wurden in 37 Beiträgen der namhaftesten Fachleute die aktuellen Kenntnisse über die primärstruktur-genetische und physikalische Karte-, die Konfiguration der DNA, die Chromosomen-Replikation und Segregation, die Rekombination und die wesentlichen genetischen Veränderungen, die generelle Reaktion auf Streß und schließlich die cryptische genetische Information vorgestellt und perspektivisch sowie verallgemeinernd interpretiert. Abgeschlossen wird das Buch durch einen Epilog von ALLAN CAMPBELL, der die genannten Schwerpunkte noch einmal zusammenfaßt und auf drei Besonderheiten hinweist: Bakterien haben ein (und nur ein) Chromosom, die Reihenfolge der Gene ist konservativ, und die bakteriellen Species resultieren aus einer genetischen Isolierung. Diese Besonderheiten seien lediglich das Ergebnis eines Selektionsdruckes. Das Buch ist eine Fundgrube, die nicht so schnell veralten wird und die auch dem fortgeschrittenen Studenten das Auffinden der gesuchten Informationen leicht macht. D. NOACK (Jena)

HENRY A. ERLICH (Editor), *PCR Technology: Principles and Applications for DNA Amplification*. X + 246 S. New York-Tokyo-Melbourne-Hong Kong 1989. McMillan Publishers (Stockton Press). £ 15.00. ISBN: 0-333-48948-9.

Nach der Publikation der ersten PCR-Arbeiten im Jahre 1985 ist diese Technik in kurzer Zeit in vielerlei Hinsicht optimiert worden. Ihr breites Anwendungsgebiet hat zu einer Explosion von Arbeiten geführt, die zeigen, wie Sequenzinformation, die in nur einer Kopie vorzuliegen braucht, amplifiziert und damit identifizierbar und analysierbar gemacht werden kann. Die Beiträge im vorliegenden Buch sind 3 Teilen zugeordnet, die sich auf die grundlegende Methodologie und deren Anwendung in molekularbiologischer Forschung und medizinischer Praxis beziehen. Der Herausgeber hat jedem Teil eine kurze Einleitung vorangestellt, in der er auf relevante Literatur und Gebiete hinweist, die in den Einzelbeiträgen nicht berührt werden. Letztere sind unterschiedlich angelegt; einige geben einen Überblick über ein bestimmtes Gebiet (z. B. Automatisierung, Mutationsnachweis, Amplifizierung spezifischer Sequenzen in cDNA-Banken, Alu-PCR, Evolutionsgenetik, Diagnose von Infektionskrankheiten), und andere liefern Ergebnisse und detaillierte Laborprotokolle für spezifische Zwecke (z. B. Probengewinnung, Sequenzierung und Mutagenese amplifizierter DNA, Diagnose monofaktorieller Erbkrankheiten, Aufbereitung forensischen Materials). Das Buch spricht breite Kreise in Biologie und Medizin an und ist geeignet, Interessenten bei der Formulierung eigener spezifischer PCR-Strategien zu helfen. HORST MALKE (Jena)

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Leghämoglobin aus Wurzelknöllchenextrakten von Luzerne bei genotypischer Variation der Symbiose

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The host plant genotype significantly influenced the nodule Lb content in all of the combinations examined between 5 alfalfa (*Medicago sativa*) cultivars and 5 *Rhizobium meliloti* strains inoculated, on the contrary not the bacterial genotype. The inoculation with the strains exhibiting an increased activity of uptake hydrogenase caused an elevation of the nodule Lb content. By IEF 4 major Lb components were found. Their proportions were changed throughout the nodule development.

Leghämoglobin, das in den Wurzel- sowie Stammknöllchen der Leguminosen vorkommt, beeinflusst wesentlich die Effektivität der N₂-Fixierung (APPLEBY 1974, 1984). Lb¹) ist für die Sauerstoffversorgung der Bakterioide in den Knöllchenzellen verantwortlich (BERGERSEN *et al.* 1973, 1979, WITTENBERG *et al.* 1974, APPLEBY 1984). Es ist auch in nichtinfizierten Zellen der Knöllchen gefunden worden, allerdings in geringerer Konzentration als in infizierten Zellen (VAN DEN BOSCH und NEWCOMB 1988).

Mit Hilfe von IEF und HPLC wurden in Wurzelknöllchenextrakten von Sojabohnenpflanzen verschiedene Lb-Formen getrennt und isoliert. Die Nomenklatur der mittels IEP getrennten Lb-Komponenten ist an absteigenden IEP-Werten orientiert. Vier Lb-Hauptkomponenten, Lba, Lbc₁, Lbc₂ und Lbc₃, wurden identifiziert und mit Lbb, Lbd₁, Lbd₂ und Lbd₃ die gering vorkommenden, jeweils am N-Terminus acetylierten Lb-Formen bezeichnet (FUCHSMAN und APPLEBY 1979, WHITTAKER *et al.* 1981, PUPPO und RIGAUD 1987). In Abhängigkeit vom Knöllchenalter sind zwei proteolytisch modifizierte Lb-Formen nachgewiesen worden, Lba' und Lbc' (SARATH *et al.* 1986). Während der Entwicklung der Sojabohnenknöllchen ändern sich die Anteile der einzelnen Lb-Isoproteine, weshalb eine entwicklungs-kontrollierte Expression angenommen wird. Die funktionale Bedeutung der Lb-Heterogenität ist unklar (POWELL und GANNON 1988). Beim Symbiosesystem *Medicago sativa*/*Rhizobium meliloti* wurden durch JING *et al.* (1982) die Hauptkomponenten LbII und LbIII und die Nebekomponenten LbI, LbIV und LbV elektrophoretisch fraktioniert sowie als mRNA-*in vitro*-Translationsprodukte nachgewiesen. Die Komponenten zeigten immunologische Kreuzreaktion, jedoch unterschiedliche Aminosäurezusammensetzung. LULLIEN *et al.* (1987) fanden bei Luzerne nach *in vitro*-Translation der Knöllchen-mRNA insgesamt 8 Polypeptide (vier starke und vier schwache Signale), die gegen Antiseren der Lb von Luzerne und *Sesbania rostrata* immunpräzipitierten. Bei dieser tropischen Leguminose wurden von DE LAJUDIE und HUGUET (1988) in 32 Tage alten Wurzel- und Stammknöllchen nach zweidimensionaler Polyacrylamidelektrophorese der Poly(A)⁺-RNA-Translationsprodukte 6 Lb-Formen gefunden, von denen eine die höchste bisher gemessene O₂-Affinität aufweist

¹) Abkürzungen: HPLC = High performance liquid chromatography; Hup, *hup* = Hydrogen uptake, uptake hydrogenase (Aufnahmehydrogenase); IEF = Isoelektrische Fokussierung; IEP = Isoelektrischer Punkt; Lb = Leghämoglobin.

(KORTT *et al.* 1986). Bei der mikrobiellen N_2 -Fixierung zwangsgebildeter molekularer Wasserstoff kann durch Oxidation zu Protonen unter der Mitwirkung von Aufnahmegydrogenasen energetisch rezykliert werden. Daraus ist theoretisch ein zusätzlicher Bedarf an O_2 zur ATP-Synthese bei gleichzeitiger Einsparung von Photosynthaten abzuleiten (STAM 1986). Die gentechnische Übertragung von Genen, die für die Aufnahmegydrogenase kodieren, in *hup*-defiziente Stämme von *R. meliloti* (LENTZSCH und MIKSCH 1989) ist nur dann sinnvoll, wenn in den Knöllchen mindestens zeitweilig ein Mangel an energiereichen Kohlenhydraten für die Atmung vorliegt und die Sauerstoffversorgung der Knöllchen durch die Aufnahmegydrogenaseaktivität nicht limitiert wird (STAM 1986).

Das Ziel der Untersuchungen bestand deshalb darin, durch Einbeziehung mehrerer Luzernesorten und *R. meliloti*-Stämme Hinweise auf eventuelle Variabilität und Begrenzung des Lb-Gehaltes in Abhängigkeit vom Wirtspflanzengenotyp zu erhalten. Es sollte ermittelt werden, ob sich der Anteil von Lb-Komponenten in Knöllchenextrakten von Luzerne verändert, die mit auf hohe Nitrogenaseaktivität selektierten Stämmen und davon durch Transfer klonierter *hup*-Gene abgeleiteten Stämmen von *R. meliloti* beimpft worden war.

Material und Methoden

Saatgut von *Medicago sativa* der Sorten 'Hunterfield' (Australien), 'Kara' und 'Europe' (Frankreich), 'Tokmaksaja mestnaja' (UdSSR) sowie 'Verko' (DDR) wurde vor der Aussaat oberflächensterilisiert (80%iges Ethanol, 30 min). Die Anzucht erfolgte in Plastikgefäßen ($8 \times 8 \times 8$ cm). Als Substrat wurde unsteriler Krumenboden eines Müncheberger D3a-Standortes verwendet, dem pro Kilogramm 3,5 g Superphosphat (P-Gehalt 8,2%) und 15 ml Nährlösung (je Liter 0,2 g KCl, 0,04 g $MgSO_4 \times 7 H_2O$, 0,14 ml 5%ige $FeCl_3$ -Lösung, 3 mg H_3BO_3 , 3 mg $MnSO_4$, 0,9 mg $(NH_4)_2MoO_4$) zugesetzt worden war. Es wurden 3 Pflanzen pro Gefäß belassen. Während des Keimblattstadiums erfolgte zweimal im Abstand von 4 Tagen die Beimpfung mit je 3 ml Bakteriensuspension (späte log-Phase) der *R. meliloti*-Stämme (Tab. 1). Die Bakteriensuspension wurde gewonnen aus einer Übernachtskultur im ERLÉNMEYER-Kolben mit TA-Medium (TY-Medium ohne $CaCl_2$); letztere wurde zentrifugiert und das Sediment in Gibson-Medium (Menge wie TA-Medium) resuspendiert (CANNON 1980).

In verschiedenen Phasen der Pflanzenentwicklung wurden Knöllchen mit Wasser gewaschen und geerntet und anschließend in flüssigem Stickstoff eingefroren. Die genannten Zeitpunkte geben den Abstand der Knöllchenenernte der Beimpfung an.

Die Knöllchenextrakte wurden durch Zerreiben der tiefgefrorenen Knöllchen im Mörser mit 5 mm Hepes-Puffer (mit 1 mM EDTA), pH 7,4, gewonnen. Die Suspension wurde bei $5500 \times g$ und Raumtemperatur 20 min zentrifugiert, der rötliche Überstand mit 2 mg/ml Dithionit reduziert (APPLEBY 1974, APPLEBY *et al.* 1975, DILWORTH 1980, APPLEBY und BERGERSEN 1984). In dem Überstand erfolgte eine Proteinbestimmung (LOWRY *et al.* 1951). Die Proben einer Serie wurden auf die gleiche Proteinkonzentration eingestellt.

Für die IEF wurden folgende Bedingungen gewählt:

Gel: 0,5 mm dünnes Polyacrylamidgel auf Gel-Bond-Trägerfolie (PHARMACIA-LKB) mit 2% Trägerampholyten (Ampholine, PHARMACIA-LKB), $4 < pH < 6$.

Elektrolytlösungen: Anode 0,5 M H_3PO_4 , Kathode 0,2 M L-Histidin. Probenauftrag: 4 µl des rötlichen Überstands in Schablonenfenster mit den Maßen 7 (in Laufrichtung) $\times$ 2 mm.

Elektrophoresekammer: „Multiphor II“ (PHARMACIA-LKB).

Vorfokussierung: 600 Vh bei konstanter Leistung, Anfangsspannung 300 V; Fokussierung: 4600 Vh bei konstanter Leistung, Endspannung 2500 V.

Unmittelbar nach der IEF wurden die Positionen der visuell erkennbaren roten Lb-Banden auf dem Gel gekennzeichnet. Das komplette Bandenmuster wurde mit Coomassie-Blau-Färbung (Gesamtproteinkonzentration 6–50 mg/ml) bzw. mit Silberfärbung (Gesamtproteinkonzentration < 6 mg/ml) sichtbar gemacht. Zur Bestimmung der IEP der Lb-Komponenten dienten Markerproteine (Proteingemische Nr. 4 und 9 von Serva). Die IEP wurden mittels GSXL-Software berechnet.

Die densitometrische Auswertung der Proteinbandenmuster erfolgte an einem Laser-Densitometer („Ultroscean XL“, PHARMACIA-LKB). Die Daten stellen die Absorption des jeweiligen Gelortes (Absorptions-einheiten/mm²), somit für die Lb-Proteinbanden einen quantitativen Ausdruck des Lb-Gehalts dar. Das Verhältnis der Absorption von 2 Lb-Banden entspricht der Mengenrelation der entsprechenden Lb-Kompo-

nenten und gestattet vergleichende Aussagen. Der Lb-Gesamtgehalt ist als Summe der Flächen der vier Hauptpeaks ermittelt worden, die in allen Kombinationen von Pflanzensorte und Impfstamm und in jedem Knöllchenalter in zentrifugierten Extrakten nachweisbar waren.

Tabelle 1
Herkunft und Charakteristik der verwendeten *Rhizobium*-Stämme

Stamm	Herkunft und Merkmale	Autoren
ZB555	Derivat des Wildstammes <i>Rm41</i> ; <i>cys 46</i> , <i>phe 15</i> , <i>Rif^r</i> , <i>Sm^r</i>	BANFALVI, Szeged
ZB555Hup ⁺	ZB555 selektiert auf hohe Hydrogenase- aktivität; <i>Nod⁺</i> , <i>Fix⁺</i> , <i>Hup⁺</i>	LENTZSCH und MIKSCH, Müncheberg
L321, <i>me17</i>	Wildstämme; <i>Nod⁺</i> , <i>Fix⁺</i>	HÖFLICH, Müncheberg
<i>me14</i> , <i>me18</i>	Wildstämme; <i>Nod⁺</i> , <i>Fix⁺</i>	HICKISCH, Halle
CXM1	Wildstamm; <i>Nod⁺</i> , <i>Fix⁺</i>	TICHONOVICH, Leningrad
<i>me17</i> (pPL1)	<i>me17</i> mit pPL1 (pLAFR1 mit <i>hup</i> -Genen von <i>R. meliloti</i>); <i>Nod⁺</i> , <i>Fix⁺</i> , <i>Hup⁺</i>	LENTZSCH und MIKSCH, Müncheberg
<i>me17</i> (pLT47)	<i>me17</i> mit pLT47 (pGS72 mit <i>hup</i> -Genen von <i>Azorhizobium caulinodans</i> ORS571); <i>Nod⁺</i> , <i>Fix⁺</i> , <i>Hup⁺</i>	LENTZSCH und TAUSCHKE, Müncheberg

Ergebnisse

Der Gesamt-Lb-Gehalt und seine Veränderung mit dem Knöllchenalter erwiesen sich als sortenbeeinflusst (Abb. 1a und 2). Ein Vergleich der Lb-Gesamtgehalte der Knöllchen von fünf Luzernesorten verschiedenen genetischen Hintergrundes jeweils als Mittel der Impfung mit fünf unterschiedlichen Impfstämmen (Abb. 1) zeigte, daß der Lb-Gehalt in den Wurzelknöllchen der Sorte 'Europe' signifikant höher als bei den anderen getesteten Sorten lag. Die mittleren Lb-Gehalte der Sorten 'Kara', 'Verko', 'Tokmakskaja mestnaja' und 'Hunterfield' differenzierten nicht signifikant.

Die Sorte 'Verko' besaß die größte Variationsbreite: In Abhängigkeit vom Impfstamm war der Lb-Gehalt sehr unterschiedlich. Die geringste Veränderung des Lb-Gehaltes infolge Änderung des Impfstammes wurde bei der Sorte 'Hunterfield' festgestellt. Bei der Sorte 'Europe' war der Lb-Gehalt dann am niedrigsten, wenn mit dem Stamm *me17* beimpft wurde. Nur bei zwei ('Verko'/CXM1 und 'Tokmakskaja mestnaja'/ZB555) von insgesamt 20 Sorten/Stamm-Kombinationen lag der Lb-Gehalt im Variationsbereich der Sorte 'Europe'. Die Kombinationen mit dem höchsten und dem niedrigsten Lb-Gehalt der Knöllchen waren 'Europe'/*me18* bzw. 'Kara'/*me14*.

Im Gegensatz dazu unterschieden sich die Mittelwerte für jeden der Stämme, kombiniert mit jeder Sorte, nicht signifikant; *me14* fiel tendenziell ab (Abb. 1b). Die sortenbedingten Streuungen waren überwiegend sehr groß.

Im weiteren sollten die Lb-Gehalte in Wurzelknöllchen der Luzerne nach Beimpfung mit selektierten *R. meliloti*-Wildstämmen und deren Hup⁺-Derivaten verglichen werden. Dazu wurden Pflanzen verschiedener Sorten mit den Stämmen ZB555 und ZB555Hup⁺ (Abb. 2), in einem weiteren Versuch Pflanzen nur der Sorte 'Verko' mit dem Wildstamm *me17* und dessen Hup⁺-Derivaten *me17*(pPL1) und *me17*(pLT47) beimpft (Tab. 2).

Der unter Selektionsdruck ausgelesene Substamm ZB555Hup⁺ bewirkte bei mehreren Sorten nach 3 Wochen zunächst eine Senkung des Lb-Gehalts, nach 5 Wochen aber in Symbiose mit 'Europe', 'Tokmakskaja mestnaja' und 'Verko' eine Erhöhung des Lb-Gehalts im Vergleich mit dem Ausgangsstamm (Abb. 2). Die Differenzierung durch das Hup⁺-Derivat in Abhängigkeit vom Knöllchenalter zeigte der Ausgangsstamm nicht. Auch die Beimpfung mit den gentechnisch konstruierten Hup⁺-Derivaten des Stamms *me17* führte nach 5, 8 und

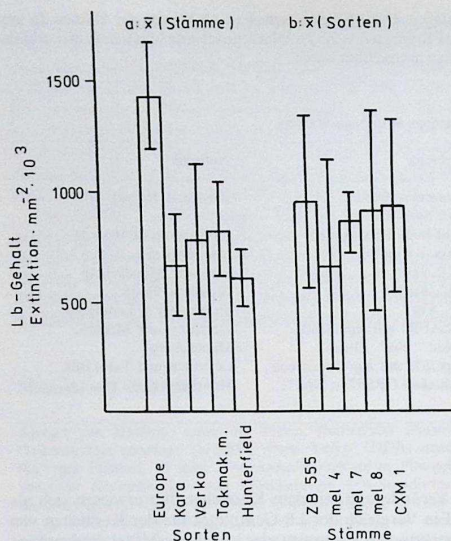


Abb. 1

Mittlerer Lb-Gehalt in Wurzelknöllchen 5 verschiedener Luzernesorten nach Beimpfung mit 5 verschiedenen *R. meliloti*-Stämmen; Standardabweichung (Balken) bei $P < 0,05$

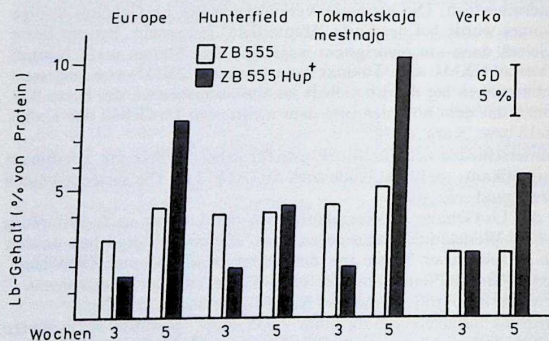


Abb. 2

Lb-Gehalt in Wurzelknöllchen verschiedener Luzernesorten nach Beimpfung mit dem *R. meliloti*-Stamm ZB 555 und dessen Hup⁺-Derivat im unsterilen Gefäßversuch mit Boden eines Müncheberger D3a-Standes in der Klimakammer

9 Wochen mit nur einer Ausnahme zu erhöhten Lb-Gehalten in den Knöllchen im Vergleich zum Ausgangsstamm (Tab. 2). Während der Lb-Gehalt in den Knöllchen mit dem Ausgangsstamm auf etwa die Hälfte während der Blüte (beim Übergang von der 8. zur 9. Woche) abgefallen war, sank er in den Knöllchen mit beiden Derivaten nur auf etwa 80% im Vergleich zum Niveau nach 5 Wochen.

Tabelle 2
Lb-Gehalt in Wurzelknöllchen von Luzerne 'Verko' nach Beimpfung mit dem *R. meliloti*-Stamm *me17* und zwei Hup⁺-Derivaten in Abhängigkeit vom Pflanzenalter im unsterilen Gefäßversuch

Stamm	Lb-Gehalt (Extinktion · mm ⁻² · 10 ³)		
	nach 5 Wochen ¹⁾	8 Wochen ¹⁾	9 Wochen ¹⁾
<i>me17</i>	929 ± 93a	838 ± 84a	504 ± 50a
<i>me17</i> (pPL1)	983 ± 98a	1103 ± 110b	783 ± 78b
<i>me17</i> (pLT47)	1262 ± 126b	1038 ± 104b	993 ± 99c

¹⁾ nach Beimpfung
a, b, c: verschiedene Buchstaben bedeuten, die Differenz der Mittelwerte ist nach NEWMAN-KEULS-Test signifikant (P < 0,05)

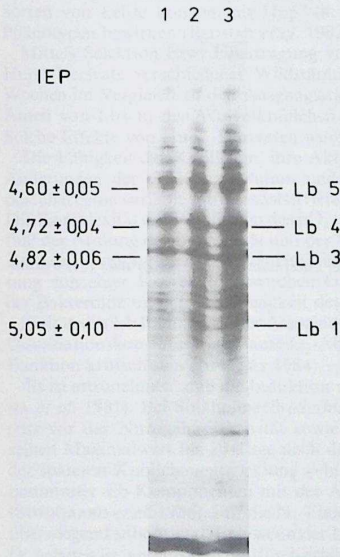


Abb. 3
IEF-Trennung der Lb-Komponenten aus Wurzelknöllchen der Luzernesorte 'Verko' 9 Wochen nach Beimpfung mit einem selektierten *R. meliloti*-Stamm *me17* (1) und seinen Derivaten *me17*(pPL1) (2) und *me17*(pLT47) (3); die IEP sind Mittelwerte mit Standardfehlern der Ergebnisse aus 3 unabhängigen Versuchen

Im Verlauf der Untersuchung qualitativer Veränderungen mittels IEF wurden vier Hauptformen des Lb der Kombination „Verko“/mel7 (bzw. *mel7*-Derivate) ermittelt. Unter den gewählten methodischen Bedingungen betrugen ihre IEP 5,05, 4,82, 4,72 und 4,60 (Abb. 3). Neben diesen sind bei mancher Sorte/Stamm-Kombination weitere zwei Banden festgestellt worden (hier nicht gezeigt), deren Anteil am Lb-Gesamtgehalt sich jedoch als gering erwies und quantitativ vernachlässigt wurde. Die Bestandteile des Luzerneknöllchen-Lb wurden hier in der Reihenfolge der Anordnung im pH-Gradient, beginnend mit dem höchsten IEP mit arabischen Ziffern bezeichnet (Lb1 bis Lb6).

Der Anteil von Lb1 an Gesamt-Lb erwies sich als deutlich abhängig vom Impfstamm, bei verschiedenem Knöllchenalter in ähnlicher Weise (Abb. 4). Er erhöhte sich mit dem Knöllchenalter bei den untersuchten Impfstämmen in ähnlichem Maße. Der selektierte Substamm ZB555Hup⁺ erhöhte bei der Sorte 'Verko' im Vergleich zu seinem Ausgangsstamm zum

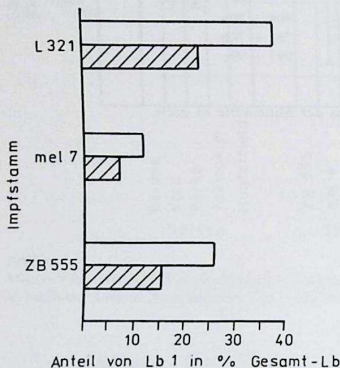


Abb. 4

Anteil von Lb1 am Gesamt-Lb-Gehalt in Wurzelknöllchen der Luzernesorte 'Verko' 5 (▨) und 8 (□) Wochen nach Beimpfung mit drei *R. meliloti*-Stämmen

Tabelle 3

Mengenverhältnis der Lb-Formen in Wurzelknöllchen von Luzerne 'Verko' nach Beimpfung mit den *R. meliloti*-Stämmen ZB555 und ZB555Hup⁺ (unsteriler Pflanzentest, 5 Wochen nach Beimpfung)

Lb-Form	Lb-Gehalt (Extinktion · mm ⁻² · 10 ³)			
	ZB555		ZB555Hup ⁺	
	abs.	rel. (%)	abs.	rel. (%)
Lb gesamt	1497	100,0	1999	100,0
Lb1	234	15,6	380	19,0
Lb3	401	26,8	534	26,7
Lb4	396	26,5	505	25,3
Lb5	466	31,1	580	29,0

Zeitpunkt 5 Wochen nach Beimpfung sowohl den Gesamt-Lb-Gehalt, absolut wie relativ (als Anteil von Protein), als auch den Gehalt jeder der 4 Lb-Hauptformen (Tab. 3, auch Abb. 2). Der Zuwachs von Lb1 war doppelt so hoch wie der von Lb3, Lb4 und Lb5. Das Verhältnis Lb1:Lb5 wurde von etwa 1:2 auf 2:3 verändert.

Diskussion

Legt man zugrunde, daß Lb das Produkt pflanzlicher (Globin-Komponente) und bakterieller Gene (Häm-Gruppe) ist, dann könnten sowohl der Impfstamm als auch die Luzerne-sorte einen Einfluß auf den Lb-Gehalt haben. Die signifikanten Unterschiede beim Vergleich der Lb-Gehalte der Knöllchen zwischen der Sorte 'Europe' und jeder anderen trotz Mittelung über die 5 einbezogenen Bakterienstämme zeigen, daß der Wirtspflanzengenotyp einen sehr wesentlichen Einfluß haben kann. Ein solch starker Effekt ist im Fall der gewählten Wildstämme für den Genotyp des Mikrosymbionten in Anbetracht der Mittelung über die 5 einbezogenen Sorten nicht feststellbar. Dieser Unterschied im genotypischen Einfluß des Makro- und des Mikrosymbionten auf den Lb-Gehalt kann in einem Unterschied der genetischen Variation des einbezogenen biologischen Materials begründet sein. In dem Zusammenhang ist interessant, daß die Sorte 'Europe', bei der der Lb-Gehalt signifikant über dem der anderen lag, speziell zur Eignung für *Rhizobium*-Impfung gezüchtet wurde. Auch der symbiontische Hup-Phänotyp wird durch die Pflanze beeinflusst: Verschiedene Sorten von Erbse können mit Hup⁺-*R. leguminosarum*-Genotypen Hup⁺- oder Hup⁻-Phänotypen bewirken (BEDMAR *et al.* 1983).

Mittels Selektion bzw. Übertragung von *hup*-spezifischen DNA-Sequenzen gewonnene Hup⁺-Derivate verschiedener Wildstämme von *R. meliloti* steigerten nach 5 und mehr Wochen im Vergleich zu den Ausgangsstämmen die Produktion von Lb, insbesondere den Anteil von Lb1 in den Wurzelknöllchen der Luzernesorte 'Verko', auch anderer Sorten. Solche Effekte von Hup⁺-Derivaten wurden bisher nicht beschrieben.

Die Fähigkeit der Knöllchen, ihre Aktivität zu erhalten, ist groß und selbst bei starken Änderungen der O₂-Konzentration und der Lb-Oxygenierung ausreichend; ein Modell postuliert eine variable Diffusionsbarriere (SHEEHY und BERGERSEN 1986, HUNT *et al.* 1988). Die Komplexität der Regulation des pO₂ und des O₂-Transports kann sich noch um den Einfluß der Atmung der Bakterioide und der Wirtszellen erweitern. Außerdem ist eine mögliche Rolle des Hup-Systems zu beachten (DIXON 1972). Mehr und mehr bildete sich die Auffassung von einer Korrelation zwischen O₂-Affinität von Lb, Atmungsenzymausschüttung der Bakterioide und O₂-Abhängigkeit der N₂-Fixierungsaktivität heraus. Extrem hohe O₂-Affinität von Lb folgt aus hoher Assoziationskonstante (schnelle O₂-Bindung) und niedriger Dissoziationskonstante (langsame O₂-Abgabe) von LbO₂ und kann für seine biologische Funktion kritisch sein (APPLEBY 1984).

Es ist anzunehmen, daß die Induktion von Lb unabhängig von der N₂-Fixierung ist (VERMA *et al.* 1981). Bei Sojabohne/*Bradyrhizobium japonicum* erscheint Lb im Knöllchen bereits vor der Nitrogenaseaktivität sowie auch in nichtfixierenden Knöllchen; es erreicht seinen Maximalwert bis 20 Tage nach dem Maximum der Nitrogenaseaktivität. Während der späteren Knöllchenentwicklung geht die drastisch gesteigerte Transkription der Gene bestimmter Lb-Komponenten mit der Aktivierung mehrerer anderer Nodulingene einher (STOUGAARD *et al.* 1986). Für die N₂-Fixierung ist der pO₂ im fixierenden Knöllchen zeitlich überwiegend suboptimal. Erst wenn der Lb-Gehalt kritisch niedrig, somit Lb vollständig mit O₂ beladen ist, verliert sich die durch erhöhten externen pO₂ ansonsten bedingte Verstärkung der N₂-Fixierung (WERNER 1987). Bei Bakteroiden aus Sojabohnenknöllchen bewirkt erhöhter externer pO₂ eine schnelle Oxygenierung des Lb und anschließend — trotz anhaltend erhöhtem externen pO₂ — einen ebenso schnellen Rückgang auf den ursprünglichen Lb-Beladungszustand; dabei sind Atmung und H₂-Bildung unverändert (KING *et al.* 1988).

Bakterioide von *Azorhizobium caulinodans* ORS571 aus Stammknöllchen von *S. rostrata* fixieren N_2 zu NH_3 mit maximaler Geschwindigkeit bei 10 mM freiem, gelösten O_2 , wobei die O_2 -Aufnahme nur etwa halbmaximal ist (BERGERSEN *et al.* 1988). Diese Ergebnisse unterstreichen die Bedeutung der regulierten, aktiven Diffusion. Bei solchen Konzentrationen sind die Knöllchen-Lb's von *S. rostrata* effektiver als das Lb von *Glycine max.* wegen ihrer höheren O_2 -Affinität; dagegen ist die Nitrogenaseaktivität der Bakterioide von *Glycine max.* O_2 -effizienter als die von *S. rostrata* (BERGERSEN *et al.* 1986).

Die durch erhöhten externen pO_2 bedingte Verstärkung der N_2 -Fixierung bedeutet, daß der Gehalt von O_2 -beladbarem Lb ausreichend ist, somit eine solche pO_2 -Limitierung der N_2 -Fixierung nicht von Lb abhängig, sondern anders verursacht sein wird. Sofern die Nitrogenaseexpression nicht limitiert ist, sollte gelten: Liegt keine pO_2 -bedingte Verstärkung der N_2 -Fixierung vor, dann kann der Lb-Gehalt begrenzt sein, muß es aber nicht, da weitere Lb-Gehalt begrenzt ist, dann könnte eine infolge genetischer Bakterienveränderung gesteigerte Lb-Produktion eine Veränderung der Symbiosequalität bewirken: von einer durch Mechanismen der Regulation von internem pO_2 und O_2 -Fluß wirksam werden. Wenn der erhöhten externen pO_2 unbeeinflusst zu einer dadurch verstärkten N_2 -Fixierung. Ein solcher Fall wäre in weiteren Arbeiten systematisch zu suchen.

Die Nitrogenaseaktivität nimmt infolge bis auf 80% erhöhten Gehalts an O_2 in der experimentellen Atmosphäre bei Klee- und Luzernesymbiosen zu, dagegen oberhalb 40% bei Sojabohne und Erbse ab (WITTY 1984). In der Symbiose von Luzerne mit Wildstämmen von *R. meliloti* kann in 30% O_2 enthaltender Atmosphäre die Nitrogenaseaktivität höher als in Normalatmosphäre sein (MIKSCH 1989). Das Auftreten einer solchen O_2 -Limitierung scheint von *R. meliloti*-Stamm und Luzernesorte abhängig zu sein. Insbesondere L321 und 'Tokmaksaja mestnaja' fielen positiv auf. Die O_2 -Limitierung kann im Falle von Hup⁺-Derivaten aufgehoben sein (MIKSCH 1989).

Diese Aufhebung des Einflusses des externen pO_2 auf die N_2 -Fixierung durch Hup⁺-Derivatisierung hat vermutlich keine Beziehung zum Lb- bzw. Lb¹-Gehalt. Sie kann sowohl durch erhöhten O_2 -Verbrauch bzw. durch Energierezyklisierung infolge gesteigerter Hup-Aktivität (LENTZSCH und MIKSCH 1989) verursacht sein. Sorten/Stamm-Kombinationen, die im Vergleich zu anderen — darunter Hup⁺-Derivate im Vergleich zu Ausgangsstämmen —, einen erhöhten Lb-Gehalt aufweisen (Abb. 1 und 2), müßten ein erhöhtes Potential für O_2 -Versorgung der Nitrogenase, auch für die Zunahme der Nitrogenaseaktivität mit wachsendem pO_2 aufweisen. Diese Hypothese wäre durch weitere Untersuchungen zu überprüfen. In den Fällen, in denen die O_2 -Versorgung der Symbiose tatsächlich limitiert ist, müßte gesteigerte Hup-Aktivität einen energetischen Nachteil bringen (STAM 1986).

Symbiotische Hup⁺-Phänotypen von *R. meliloti* könnten infolge verstärkter H_2 -Oxidation zu einer Verringerung des internen pO_2 beitragen (DIXON 1972). Die bakterielle Häm-Synthese wird durch niedrigen pO_2 im Knöllchengewebe gefördert, der anfangs infolge *Rhizobium*-Vermehrung entsteht (AVISSAR und NADLER 1978, KEITLHEY und NADLER 1983). Seit kurzem gelten die bakterielle Häm-Bildung und der Häm-Transport in das Wirtszytosolplasma als essentiell für die symbiotische Lb-Synthese in den Knöllchen (O'BRIEN *et al.* 1987, DE BRUIJN *et al.* 1988), zumindest für schnell wachsende *Rhizobium*-Arten (STANLEY *et al.* 1988). Hierin kann eine Erklärung für die Auslösung erhöhter Lb-Produktion durch Hup⁺-Bakterienderivate liegen. Auch der Lb-Abbau, dessen Folge eine Erhöhung des O_2 -Diffusionswiderstands ist, setzt an der Häm-Gruppe an, die durch Nitrit, das aus Nitrat gebildet wurde, oxidiert wird (SPRENT *et al.* 1987, DREVON *et al.* 1988). Nitrat kann in Knöllchen mehrerer Leguminosenarten außer Gelblupine den Gesamtgehalt um 13–61% reduzieren und das Verhältnis der Lb-Komponenten einer Art drastisch verändern (BECANA und SPRENT 1989).

In weiteren Untersuchungen sollten die Merkmale N_2 -Fixierung, O_2 -Limitierung, Lb-Gehalt, Hup-Aktivität und Photosynthese bzw. Atmung parallel und möglichst über den gesamten Symbiosezeitraum verfolgt werden.

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Some factors affecting bioconversion of whole bagasse into fungal biomass

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Two isolated basidiomycetes mould cultures *Polyporus* BH₁ and *Polyporus* BW₁ and two standard white rot fungal cultures *Pleurotus ostreatus* and *Polyporus versicolor* were cultivated under submerged conditions on whole (untreated) bagasse for protein production. Some factors promoting better bioconversion were studied and selected. NORKRAN's medium at pH 5.0 with 1.0% bagasse of 80 mesh particle size using an appropriate quantity of inoculum, supplementing medium with an amino acid, without addition of any vitamin, etc. gave best results.

In the sugar industry, the large amount of cellulosic waste — bagasse is mainly used as fuel, even though some remains unutilized, creating disposal problems. Hence, it may be used as a substrate for biomass and energy production. Attempts have been made to convert the cellulosic residues into protein by microbial methods (LINKO 1977). In some processes, the cellulolytic microorganisms are directly cultivated on cellulosic substrates (BELLAMY 1975, PEITERSEN 1975). Many microorganisms belonging to *Basidiomycetes* are able to convert lignocellulosic substrates directly into fungal protein suitable for human and animal consumption (HAN 1978, KIRK and HARKIN 1973).

The present work deals with the study of selected mould cultures capable of utilizing whole bagasse substrate. Some parameters have been studied with a view to optimizing bioconversion of bagasse into biomass.

Materials and methods

Microorganisms: In the present investigation, the work was carried out using two isolated cultures BH₁ and BW₁ (NIGAM and PRABHU, 1985). The characteristics of these cultures were studied in detail and they were found to belong to order *Polyporales*, family *Polyporaceae* under class *Basidiomycetes*. The properties of fruiting bodies show these cultures to be genus *Polyporus* spp. (ALEXOPOULOS and MIMS 1983).

Two standard cultures — *Pleurotus ostreatus* (IARI, New Delhi) and *Polyporus versicolor* (NCL, Pune) were also taken.

Substrate: The substrate bagasse obtained from the Sugar factory, was thoroughly washed, dried and powdered.

Preparation of inoculum: As previously described (NIGAM and PRABHU 1985).

Culture cultivation: ERLENMEYER flasks (250 ml) containing 50 ml medium, 1% w/v substrate and inoculum (10–12 mg protein) were incubated at 30 °C for 7 days on a rotary shaker (150 rpm).

Study of factors: a. Effect of cultivation medium: Three media reported for mould growth in the utilization of lignocellulosic substrates were used. These include KIRK and KELMAN's (1965), HOFSTEN and RYDEN's (1975) and modified NORKRAN's medium (ERIKSSON and LARSON, 1975). b. Effect of pH of medium: Initial pH of one selected medium (NORKRAN's) was adjusted to different pH values of 4.0 to 6.0. c. Effect of inoculum size: Inoculum was prepared as per procedure with the only difference being in number of mycelial pieces (1–3 discs) taken from plates. d. Effect of substrate concentration: Bagasse powder of 35–80 mesh was added to medium at three concentrations of 0.5, 1.0 and 1.5%. e. Effect of substrate particle size: Bagasse powder was sieved to 35, 48, 65 and 80 mesh particle sizes and taken 1% as substrate. f. Effect of amino acids: Some amino acids were added to growth medium (0.1% w/v), like 1 — aspartic acid, 2 — aspara-

gine, 3 — methionine, 4 — lysine, 5 — serine, 6 — valine, 7 — tyrosine, 8 — cystine, 9 — iso-leucine. g. Effect of vitamins: Different vitamins viz 1 — ascorbic acid, 2 — biotin, 3 — cyanocobalamine, 4 — niacin, 5 — thiaminehydrochloride, 6 — riboflavin, and 7 — para amino benzoic acid were added in medium at 100 µg/l concentration.

Analytical methods: After 7 days fermentation, the contents of the flasks were analysed for biomass-protein formed, by the BIURET method (HERBERT *et al.* 1971) and total carbohydrate content by phenol sulphuric acid method (DUBOIS *et al.* 1956).

Results and discussion

The three media selected for study, are those which are specifically reported for fungal cultivation on lignocellulosic substrates. Results recorded in Table 1 (a) show that maximum bioconversion of bagasse can be achieved by using the modified NORKRAN's medium. Though growth was good in all three media, cultures were clear mycelial. And KIRK and KELMAN's medium was comparable to NORKRAN's medium but being more economic due to absence of DL-valine, and adenosine like components, was later preferred for further studies.

Table 1
Selection of medium and pH

a. Media		Protein formed mg/g bagasse			% TCD		
Cultures	A	B	C	A	B	C	
1	76.8	65.0	78.2	55.8	53.2	57.9	
2	70.5	58.5	74.8	58.0	52.0	58.3	
3	68.7	70.4	72.0	53.6	59.6	56.4	
4	69.8	69.4	68.5	50.5	45.0	51.5	
b. pH of medium		4.0	4.5	5.0	5.5	6.0	
Protein formed mg/g							
1		69.5	81.5	83.0	76.9	63.6	
2		64.0	72.9	76.8	72.8	58.9	
3		62.8	69.5	76.0	70.9	56.0	
4		59.0	70.3	72.5	66.5	52.8	
% TCD							
1		53.2	58.0	60.5	57.9	49.6	
2		55.0	62.5	65.2	58.3	50.6	
3		52.5	59.5	63.8	56.0	47.3	
4		48.0	53.8	59.8	51.5	43.2	

A — KIRK and KELMAN's, B — HOEFEN and RYDEN's, C — NORKRAN's

1 — *Polyporus BH₁*, 2 — *Polyporus BW₁*, 3 — *Pleurotus ostreatus*, 4 — *Polyporus versicolor* TCD, — Total carbohydrate degradation

The initial pH of NORKRAN's medium was adjusted in the range of 4 to 6 as these fungal cultures were found to grow in this wide range. Results (Table 1b) show that at pH 5.0 maximum carbohydrate degradation occurred, also yielding maximum protein, hence for further experiments pH of the medium was always adjusted to 5.0. Though ERIKSSON and LARSON (1975) used this medium with pH 5.5, but for the cultivation of *S. pulverulentum*. For inoculum preparation mycelial pieces of 10 mm diameter cut into hollow

Table 2

Selection of inoculum size, substrate concentration and particle size (mesh)

c. Inoculum- No. of discs		Protein formed mg/g			% TCD			
		1	2	3	1	2	3	
1		42.5	76.0	79.5	27.4	58.0	59.2	
2		38.9	73.5	74.8	32.8	59.5	61.0	
3		36.2	71.2	73.5	31.0	57.3	60.0	
4		35.5	68.0	70.2	28.2	52.0	54.0	
d. Substrate- %, w/v		0.5	1.0	2.0	0.5	1.0	2.0	
1		76.9	92.5	73.8	57.9	77.5	51.5	
2		72.8	86.3	68.9	58.3	72.8	55.0	
3		70.9	79.5	65.0	56.0	68.8	48.0	
4		66.5	72.3	63.0	51.5	65.0	47.8	
e. Mesh	35	48	65	80	35	48	65	80
1	56.0	60.2	71.5	76.1	36.0	42.5	54.2	57.9
2	48.3	56.8	64.6	72.8	38.4	48.6	55.0	58.3
3	45.1	55.3	62.8	70.9	39.0	43.8	52.0	56.0
4	43.0	48.9	56.0	66.5	35.2	41.6	47.0	51.5

Abbr. as in Table 1

discs were taken from malt agar plates. 1, 2 and 3 such discs were used to make the inoculum for each 50 ml medium. It is obvious from the results given in Table 2(c) that one disc inoculum was insufficient, giving poor substrate degradation, 2 and 3 discs inoculum gave about 60% bagasse-carbohydrate degradation, also producing equal protein. Hence 2 discs of mycelium were found to be sufficient.

Results in Table 2(d) indicate that at 1.0% concentration of bagasse in the medium, utilization of substrate and protein formation were found to be at a maximum in all four cultures. No further increase in bioconversion was noticed on increasing the substrate concentration beyond 1.0%. ENRIQUEZ (1981) also reported that as he increased bagasse concentration the growth rate of bacteria increased but lower bagasse concentration favoured conversion into biomass. It was noticed that as the mesh of particles increased i.e. the size reduced, the yield of protein also increased (Table 2(e)), the reason may be attributed to the fact that the rate of carbohydrate degradation increased with increase in mesh size, providing more free sugars for fungal growth.

It is obvious from the data in Table 3 that all the amino acids enhanced the protein production and carbohydrate degradation in *Polyporus* BW₁ culture. Except methionine, lysine and serine, all amino acids enhanced protein formation in *Polyporus* BH₁ culture, whereas carbohydrate utilization was stimulated by all the amino acids tested. Tyrosine and aspartic acid were the best stimulant for both cultures. The other two media used for lignocellulosic bioconversion (reported in methods) also contained amino acid whereas NORKRAN's medium has no amino acid as constituent, hence aspartic acid may be used in NORKRAN's medium as stimulator.

Addition of any vitamin to the medium did not show any capability of improving protein production in either culture, even removal of vitamin thiamine (control), a constituent of NORKRAN's medium, did not inhibit cultures' growth. Vit B-12 enhanced substrate

Table 3
Effects of amino acids and Vitamins

Amino acids	Protein mg/g		% TCD		Vitamins	Protein mg/g		% TCD	
	BH ₁	BW ₁	BH ₁	BW ₁		BH ₁	BW ₁	BH ₁	BW ₁
C	108.0	92.4	51.2	55.2	C	106.4	86.0	52.6	57.8
1	134.0	159.6	58.6	59.8	1	111.2	74.2	51.3	54.6
2	124.0	108.8	56.3	59.3	2	113.6	95.6	53.0	59.0
3	93.5	100.8	53.9	58.2	3	106.0	91.8	58.9	63.8
4	104.0	140.0	56.0	55.0	4	107.6	95.0	57.7	59.6
5	93.5	140.2	52.7	56.2	5	104.0	85.2	53.5	58.2
6	120.0	107.8	57.9	56.0	6	99.5	81.3	47.5	54.8
7	155.8	149.8	52.5	58.7	7	102.8	84.0	49.2	55.8
8	132.2	120.8	54.8	57.3	—	—	—	—	—
9	127.8	115.0	53.5	56.8	—	—	—	—	—

C — Control, 1–9 amino acids and 1–7 vitamins, as described in Methods.

utilization but PABA, riboflavin and ascorbic acid inhibited substrate degradation in both cultures.

Though research work has been done on SCP production from bagasse, using a number of microorganisms such as *Trichoderma* (SIDHU and SANDHU 1980), *Aspergillus* (WANG *et al.* 1981), *Cellulomonas* (ENRIQUEZ 1981) etc., all used only treated (delignified) bagasse due to incapability of these cultures to utilize the cellulosic part of bagasse in the presence of lignin. The cultures used in the present study demonstrated the capability of using ligno-cellulosic substrate needing no delignification (NIGAM and PRABHU 1985). Hence the present work was undertaken with a view to bioconverting bagasse in a single step of main fermentation, which required the study of some factors, enabling the selection of an optimized process, that is the modified NORKRAN's medium at initial pH value of 5.0 with 1.0% bagasse powder of 80 mesh, 2 pieces of mycelium/flask (10 mm dia) for inoculum, supplementation of an amino acid (aspartic) at 0.1% concentration and needing no vitamin. The performance of our isolated cultures was found to be comparable to the standard cultures, therefore any culture may be employed for the process.

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Book Reviews

RICHARD M. HYDE, *Microbiology and Immunology* (Second Edition; Oklahoma Notes). 229 S., 53 Abb. Berlin-Heidelberg-New York-London-Paris-Tokyo-Hong Kong 1989. Springer-Verlag. DM 32,00. ISBN: 3-540-97008-8.

Es handelt sich bei der vorliegenden Schrift um einen in 2. Auflage vorliegenden „Basic-Sciences Review“ der Universität von Oklahoma, USA. Mikrobiologie und Immunologie stellen die Topics dar. In 4 Sektionen werden 6 verschiedene Bereiche behandelt: Mikrobielle Physiologie, mikrobielle Genetik, Immunologie, spezielle Mikrobiologie, Virologie, Prüfungsvorbereitung. Die Gliederung im einzelnen ist recht interessant: Definitionen, spezielle Darstellungen (verbunden mit Grafiken), Statements, Leistungstest. Die Anordnung ist didaktisch außerordentlich geschickt und für das Selbststudium gut geeignet. Auch in kleineren Gruppen sind Frage-Antwort-Spiele möglich, die Domäne liegt zweifellos im individuellen Arbeitsstil.

Bei insgesamt vorliegenden 10 Reviews über medizinische Basiswissenschaften bleibt abschließend eigentlich die Frage offen, ob nicht evtl. eine etwas andere Gliederung zweckmäßiger sein könnte: Verselbständigung der Immunologie (bzw. Reduktion auf Infektionsimmunologie) und Einbindung der Mykologie und Parasitologie in die Mikrobiologie.

Insgesamt eine empfehlenswerte, preiswerte Schrift für Studenten, Promovenden und Kollegen, die sich selbst einer kritischen mikrobiologischen Überprüfung stellen.

A. STELZNER (Jena)

ABNER LOUIS NOTKINS and MICHAEL B. A. OLDSTONE (Editors), *Concepts in Viral Pathogenesis III*. XIX + 389 S., 48 Abb., 12 Tab. New York-Berlin-Heidelberg-London-Paris-Tokyo 1989. Springer-Verlag. DM 148,00. ISBN: 3-540-96974-8.

Die bisher in zwei Bänden vorliegenden „Konzepte der viralen Pathogenese“ haben mit vorliegender Monographie eine deutliche Erweiterung erfahren, liegen doch jetzt insgesamt 145 Kapitel zur gestellten Thematik vor. 82 Autoren zeichnen im 3. Buch für insgesamt 43 Beiträge verantwortlich. Sieben Kapitel wurden hierbei berücksichtigt: Struktur-Funktionsbeziehungen, Genregulation, Zellbiologie, transgene Mäuse, Retroviren, Konzeptentwicklung, Therapie und Impfstoffe. Die Autorenliste enthält klangvolle Namen, die Gewähr dafür bieten, daß führende Virologen schlüssige Statements formulierten. Die Texte sind hierbei im einzelnen klar verfaßt, manchmal vielleicht auch ein wenig knapp. Zusammenfassungen sind meist vorhanden und ergeben ein „Komprimat 1989“. Bis zu 20 (40) Literaturzitate verweisen auf wichtige aktuelle Berichte der letzten 3–8 Jahre. Es fällt schwer, für die vorliegende Monographie differenzierende Wertungen vorzunehmen — drei Besonderheiten verdienen wohl aber doch besonders hervorgehoben zu werden: die Analyse der Zellbiologie ausgewählter viraler Infektionen (Rezeptoren, Immunsuppression, IR), die Darstellung der Expression viraler Gene in transgenen Mäusen (SV 40, Hepadna-Viren, HTLV-1) und die Entwicklung von neuen bzw. modifizierten Konzepten (Onkogenese, Genfunktionen, Immuntoleranz, Autoimmunität, Ätiologie). Es bedarf insgesamt keiner Frage, daß letztlich alle drei Bände der neuen „Konzeptreihe“ in die Hand jedes Virologen, Zellbiologen und Infektionsimmunologen gehören.

A. STELZNER (Jena)

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Effect of organic soil amendments on the incidence of stalk rot of maize caused by *Macrophomina phaseolina* and *Fusarium moniliforme*

S. O. OSUNLAJA

(Received 27 September 1989/Accepted 5 February 1990)

Five organic materials, *Calopogonium* sp., rice straw, wood sawdust, fresh guinea grass and poultry manure, were added to soil in a field with a recent history of high stalk rot in maize caused by *Macrophomina phaseolina* and *Fusarium moniliforme*. The two stalk rot organisms reacted differently to the organic amendments. All the amendments significantly reduced the incidence of *Fusarium* stalk rot disease. Amendment of soil with fresh *Calopogonium* leaves had almost no effect in controlling the incidence of *Macrophomina phaseolina*. Organic amendment with poultry manure stimulated the disease. The other amendments appeared to be equally effective in checking the disease. Suppression of the disease appeared to be related in increased microbial activities.

Stalk rots are serious and widespread diseases which attack maize wherever the crop is grown (HOOKER and BRITTON 1962, HOOKER and WHITE 1976, PETERSON 1961). Several fungi have been associated with this disease in different parts of the world (KOEHLER and BOEWE 1957, MACKIE 1932, MCKEEN 1953, YOUNG 1944). In Nigeria the major causal organisms are *Fusarium moniliforme*, *Macrophomina phaseolina* and *Botryodiplodia theobromae*, the former two fungi being more prevalent. These organisms can occur separately or together in the same stalk. Stalk rots not only reduce the yield and quality of maize but also cause premature death and lodging of the plant thereby making harvesting difficult (GRISENKO 1967, WILCOXSON, 1962, WYSONG and KEER 1969). The primary source of inoculum for this disease complex in the field is the soil or crop residue (DHINGRA and SINCLAIR 1978). Disease incidence is known to increase if susceptible crops are grown annually. One of the ways of reducing disease inoculum in the soil is by organic matter amendment (ADAMS *et al.* 1968, ECK 1978, GHAFAR *et al.* 1969, SINGH *et al.* 1981). The present study was therefore undertaken to determine the role and the effectiveness of certain organic amendments in the biological control of root and stalk rot of maize in Nigeria.

Materials and methods

The field experiment was carried out at Moor Plantation, Ibadan, latitude 7° 25' 25"N longitude 3° 52' 25"E on sandy loam soil in the late cropping season of 1987 (August–November). The field has been under continuous maize cultivation for five consecutive years and had a recent history of high stalk rot incidence.

The three maize varieties used in the experiment were a hybrid 8505-3W, a local maize composite NLC and an improved maize variety TUSR-W.

The organic materials used in the amendment were a green legume cover crop *Galopogonium* sp., rice straw, mixed wood saw dust of *Terminalia* sp., *Triplochyton* sp. and *Kaya* sp., fresh green leaves of guinea grass and poultry manure.

The land was ploughed and harrowed once. Trials were laid out as a complete randomised block design with three replications. Soil was amended at the rate of 5 kg per plot. Planting was done on the day of amendment at a planting distance of 90×25 cm equivalent to 44,444 plants per hectare. Fertilizer was applied uniformly to all the plots at the recommended rate of 120 kg N, 20 kg P, 30 kg K per hectare. Two weeks after mid silk the stalks were split lengthwise and incidence of the disease was taken.

Soil samples were collected randomly from each plot at 3, 6, 9 and 12 weeks of amendment. Microbial isolations were made by the dilution plate method. Fungal isolation was performed at a soil dilution of 10^3 on PDA to which antibacterial supplement chloramphenicol had been added (ANONYM, 1968). Bacterial isolation was also made performed at a soil dilution of 10^6 on nutrient agar. Five replications of each set were prepared for the dilutions tested. The fungal colonies were counted 5 days after incubation at 25°C and bacteria at 30°C . Sub samples of the soil were dried at 105°C for 24 h and the microbial population was estimated from the dry weight of the soil.

Results

The effect of organic amendment on the control of stalk rot in maize caused by *Macrophomina phaseolina* and *Fusarium moniliforme* is shown in Fig. 1. The two stalk rot organisms reacted differently to the organic amendments.

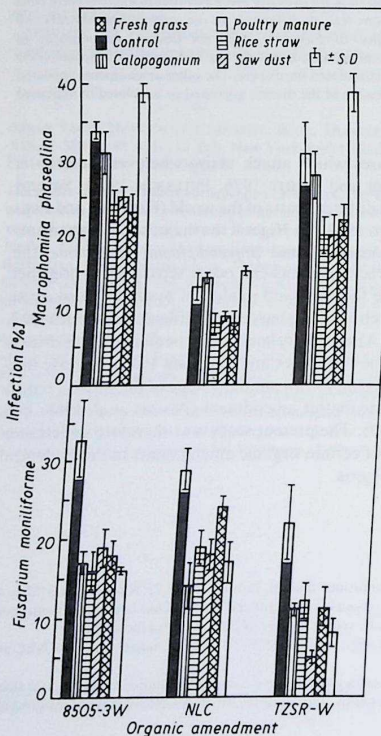


Fig. 1
Effect of organic amendments on stalk rot of 3 maize varieties as indicated by percent infection

In comparison with the unamended control, addition of *Calopogonium* leaves, rice straw, wood sawdust, fresh guinea grass and poultry manure to soil significantly reduced infection of *Fusarium* stalk rot in the three maize varieties by fifty percent. On the average *Calopogonium* leaves, poultry manure and wood sawdust appeared to be equally effective in checking the disease while rice straw and fresh guinea grass were of lesser effect.

Response of *Macrophomina phaseolina* to the various amendments was slightly different from that of *Fusarium moniliforme*. Addition of rice straw, wood sawdust and fresh, matured guinea grass resulted in a significant decline in *Macrophomina* stalk rot incidence. The three amendments appeared to be equally effective. However, amendment with *Calopogonium* leaves (a legume) and poultry manure proved ineffective in checking the disease. Organic amendment of soil with poultry manure significantly increased the disease incidence.

Comparison of data from the control showed that the three varieties differ significantly in their reaction to stalk rot disease. The hybrid variety 8505-3W was the most susceptible to the stalk rot pathogens while TZSR-W and NLC were the more tolerant to *Fusarium* and *Macrophomina* stalk rot, respectively.

Microorganism count in Table 1 showed that except for saw dust and guinea grass there was a significant increase in the population of bacteria when the soil was amended. Highest numbers were obtained with rice straw and *Calopogonium* amendment. Except for poultry manure, the stimulation was highest within three weeks of amendment and then declined thereafter.

Table 1
Effect of organic amendment of soil on total numbers of bacteria

Amendment	Colonies recovered per g soil $\times$ millions 3, 6, 9 and 12 weeks after amendment			
	3	6	9	12
Control	11.8	11.4	8.3	6.2
Poultry manure	13.1	28.8	19.4	9.4
Guinea grass	15.2	13.4	8.7	7.0
Sawdust	18.3	12.2	8.6	7.1
<i>Calopogonium</i>	29.9	20.2	14.8	11.3
Rice straw	31.2	21.1	16.9	9.6
LSD ($P = 0.05$)	6.6	4.6	3.1	2.0

Trends with fungal population were also similar to those of bacteria (Table 2). The organic amendment of soil resulted in a manifold increase in the population of fungi. Amendment of soil with sawdust was found to be the most stimulatory, followed by guinea grass amendment. Poultry manure, when added to soil did not show a significant change in the population of fungi during the first six weeks. Thereafter, a significant increase in total number of fungi was observed.

Discussion

The effectiveness of some organic amendment in decreasing the severity of stalk rot of maize in the field was evident in this study. The suppressive effect of organic amendment of soil on *Fusarium* sp. and *Macrophomina phaseolina* has been demonstrated by many

Table 2
Effect of organic amendment of soil on total numbers of fungi

Amendment	Colonies recovered per g of soil $\times$ thousands 3, 6, 9 and 12 weeks after amendment			
	3	6	9	12
Control	96	104	29	19
Poultry manure	102	108	37	38
<i>Calopogonium</i>	683	133	35	20
Rice straw	860	141	37	39
Guinea grass	893	116	41	23
Sawdust	2033	115	43	20
LSD ($P = 0.05$)	500	10	4.8	7.1

workers (BUXTON *et al.* 1965, ECK 1978, FILHO and DHINGRA 1980, GHAFAR *et al.* 1969, SEQUEIRA 1962, SNYDER *et al.* 1959, VASUDEVA *et al.* 1962). The reason for suppression has been attributed to antibiosis, lysis and exploitation by the stimulated antagonistic micro-organisms. Increased population of bacteria and fungi in the amended soil as observed in this investigation was probably responsible for the decline in the incidence of the stalk rot disease.

A significantly high correlation between increased microbial population in the amended soil and decrease in the population of pathogens is well documented in literature (BUXTON *et al.* 1965, GHAFAR *et al.* 1969, SINGH and SINGH 1981). Although all the organic amendments applied to soil reduced the incidence of *Fusarium* stalk rot, only rice straw, sawdust and fresh guinea grass were effective in checking the incidence of *Macrophomia phaseolina*. *Calopogonium* sp. (a legume) and poultry manure were ineffective and actually led to an increase in the disease. This result suggests that perhaps a comparatively high C/N ratio of the organic amendment controls the *Macrophomina* stalk rot disease of maize. Similar results have been reported by several workers (DAVEY and PAPAIVAS 1959, DAVEY and PAPAIVAS 1960, SINGH *et al.* 1981, SNYDER *et al.* 1959).

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Book Review

ETTORE OLMO (Editor), *Cytogenetics of Amphibians and Reptiles* (Advances in Life Sciences). XI + 270 S., 87 Abb., 25 Tab. Basel-Boston-Berlin 1990. Birkhäuser Verlag. DM 78,00. ISBN: 3-7643-2358-2.

Das handliche Buch entstammt einer Sammlung von Beiträgen eines Symposiums gleichen Titels, das im Rahmen des ersten Weltkongresses der Herpetologie am 17. September 1989 in Canterbury in Großbritannien stattfand. In insgesamt 19 Artikeln, 10 über Amphibien und 9 über Reptilien werden aber auch weiter interessierende, über das Symposium hinausgehende Themen, meist in Übersichten, dargeboten. Dabei werden zahlreiche mögliche Zusammenhänge cytogenetischer, taxonomischer, evolutionärer und auch immunologischer Art diskutiert. Die modernen Techniken der Chromosomen- und Karyotypanalyse spielen eine entscheidende Rolle bei der Schaffung der experimentellen Grundlagen für derartige Untersuchungen. Sie kommen entsprechend dem Übersichtscharakter der Arbeiten etwas zu kurz. Bandingtechnik, DNS-Sequenzanalyse, Durchflußcytometrie, experimentelle Hybridisierung, Quetsch- und Farbe-Methoden werden erwähnt und nur in einzelnen Beiträgen ausführlicher dargestellt. Spezielle Probleme der Beziehungen zwischen Gattungen und Arten sowie allgemeinere Fragen der Chromosomen- und DNS-Variation in makro- und mikroevolutionären Prozessen und die Evolution bestimmter DNS-Fraktionen werden diskutiert. Die Autoren stammen aus den verschiedenen Teilen der Welt — Japan, Korea, China, Australien, Südamerika, USA, Europa — und berichten aus ihrer Sicht über die Gattungen und Arten der entsprechenden Regionen.

Das Buch dürfte zahlreiche Interessenten unter Biologen, Genetikern und Cytologen finden, die sich mit Chromosomenanalyse und mit Amphibien und Reptilien in taxonomischer, cytogenetischer und phylogenetischer Hinsicht beschäftigen.

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Einsatz der Fermenter-Dialysekultur zur Kultivierung medizinisch relevanter Erreger

III. *Candida albicans*

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(Received 24 January 1990/Accepted 18 June 1990)

For mass cultivation of *Candida albicans* (serotyp A), a fermenter dialysis culture technique is described and compared with shaking culture and fermenter batch culture techniques.

Important growth parameters such as yeast dry weight and viable cell counts demonstrate the advantage of the fermenter dialysis culture.

Mannan, the major antigen from *Candida albicans* prepared by phenol-water extraction followed by gel chromatography was tested with the monoclonal IgM antibody H5.

Candida albicans kann beim Menschen häufig im Respirations-, Digestions- und Urogenitaltrakt nachgewiesen werden. Während die kommensale Kolonisation beim gesunden Menschen in der Regel nur zu einer inapparenten Immunisierung führt, ist bei Risikopatienten mit entsprechender Prädisposition (Immundefekte; neoplastische Erkrankungen; Stoffwechselerkrankungen; Gabe von Chemotherapeutica insbesondere Cytostatica, Immunsuppressiva und Breitbandantibiotica; und andere Faktoren) ein gehäuftes Auftreten insbesondere von Systemmykosen zu beobachten. Etwa 1 % der Ergebnisse großer Sektionsstatistiken weisen Systemmykosen aus. Weltweit ist eine Zunahme der Systemmykosen zu beobachten (BERNHARDT *et al.* 1986).

Die sichere Diagnostik einer Systemmykose ist zur Zeit noch nicht möglich. Die klinische Symptomatik, ein quantitativ geführter kultureller Nachweis von Hefepilzen und die Ergebnisse serologischer Tests können auf eine systemische Mykose hinweisen. Es muß aber mit falsch positiven bzw. falsch negativen Ergebnissen, insbesondere bei der serologischen Diagnostik gerechnet werden.

Verstärkte Aufmerksamkeit wird derzeit dem Nachweis von Pilzantigenen im Patientenserum gewidmet (HOPWOOD u. WARNOCK 1986). Zirkulierendes Antigen weist immer auf eine aktuelle Infektion hin und ist auch bei anergisch reagierenden Patienten erfaßbar. Notwendig sind dafür sehr empfindliche und spezifische Testsysteme (z. B. ELISA u. Latex-Tests), die die Rate falsch negativer Ergebnisse gering halten. Bei der Entwicklung und Anwendung solcher Testsysteme sind monoklonale Antikörper heute ein nicht mehr wegzudenkender Faktor mit großer Perspektive (BORKHARDT u. MÜLLER 1989).

Bei der Herstellung monoklonaler Antikörper werden größere Mengen an *Candida albicans*-Antigen zum Screening und für die chemische Charakterisierung benötigt. Das Hauptantigen dieses und zahlreicher anderer Hefepilze ist das proteingebundene Polysaccharid Mannan.

Ziel der Arbeit ist es, die Fermenter-Dialysekultur (FDK) als effektive Anzuchtmethode für *Candida albicans* vorzustellen und mit der Schüttelkultur (SK) und der Fermenter-Batch-

kultur (FBK) zu vergleichen, sowie über die Ergebnisse der Polysaccharid-Präparation zu berichten.

Material und Methoden

Untersuchungsstamm: *Candida albicans*, Serotyp A, Stamm Leuschke, Dresden.

Verwendete Nährmedien: Für die Submerskultur der Hefen wurde SABOURAUD-Glucose-4%-Bouillon und für die Keimzahlbestimmung SABOURAUD-Glucose-2%-Agar (SIFIN) verwendet.

Kulturverfahren: SK, FBK und FDK wurden mit 4 l Kulturmedium im Laborfermenter BIOTEC 1601 Ultraform (LKB) bei konstanter Temperatur (35 °C) und ständiger Durchmischung des Inhaltes (Rührgeschwindigkeit = 400–600 U/min) durchgeführt. Die Regelgrößen pH-Wert und Sauerstoffpartialdruck (pO₂), sowie die optische Dichte (OD 650/1 cm) wurden stündlich gemessen und protokolliert. Die Regulation des pH-Wertes (7.3) erfolgte durch Zugabe von 9 M NaOH und die Regulierung des pO₂ (> 1%) durch Preßluftzufuhr.

Bei der SK wurden pH-Wert und pO₂ nicht geregelt. Die beiden anderen Fermenter-Kulturverfahren erfolgten im geregelten Fermenter. Zur Durchführung der FDK wurde der Laborfermenter durch eine künstliche Niere (MLW, Typ 2, Dialyseaustauschfläche 1,8 m², Molekulargewichtsaustauschgrenze 1000 Dalton) mit einem Kreislaufsystem verbunden, das gegenläufig zum Dialyseflüssigkeitskreislauf (40 l SABOURAUD-Glucose-4%-Bouillon) angeordnet ist. Fermenter- und Dialyseflüssigkeitskreislauf werden durch eine Peristaltikpumpe (10 l/h, IP22, MLW) kontinuierlich aufrechterhalten. Als Feedinglösung diente 10fach konzentrierte Glucose-Nährbouillon.

Inokulation: Die Inokula (OD = 0,9–1,4) betragen 10% des Gesamtvolumens.

Berechnung wachstumskinetischer Parameter:

$$\text{spezifische Wachstumsrate: } \mu = \frac{\ln D_1 - \ln D_2}{t_2 - t_1}$$

$$\text{Teilungsrate: } v = \frac{\log N_t - \log N_0}{\log 2 (t - t_0)}$$

Trübungsmessung, Lebendkeimzahl- und Trockengewichtsbestimmung wurden nach STARKE *et al.* (1987) durchgeführt. Der nach Abzentrifugation der Hefezellen für die Trockengewichtsbestimmung verbleibende Kulturüberstand wird nach Steril-Filtration (Filter EKSI Größe 14, SEITZ-Filter-Werke, BRD) bis auf 200 ml eingengt (Ultrafiltration, Filter YU5, AMICON Corp., USA) und lyophilisiert (LGA 05 JANEZKI, DDR).

Präparation des Polysaccharid-Antigens: Die Präparation der Polysaccharide erfolgte durch Phenol-Wasser-Extraktion bei 60 °C (WESTPHAL *et al.* 1952). Die gelchromatographische Auftrennung und Molekulargewichtsbestimmung wurde an Sepharose 4B (PHARMACIA) vorgenommen. Die Äquilibration wurde mit aqua dest. (pH = 7,0) bei einer Elutionsgeschwindigkeit von 5,2 ml/h durchgeführt. Zur Eichung der Säule (60 cm × 2 cm, Laborglas Ilmenau) wurden Dextranblau und T-Dextrane (PHARMACIA) verwendet (T2.000 = 2 Mio D, T500 = 500000 D, T400 = 400000 D, T250 = 250000 D, T110 = 110000 D).

Immunologische Spezifität: Zur Erfassung der immunologischen Spezifität wurden die Eluate der Säulentrennung mit Hilfe des monoklonalen Anti-*Candida albicans*-IgM-Antikörpers H5 in einem Enzymimmunoassay (direkter Zweiseitenbindungassay) überprüft (Publikation in Vorbereitung).

Enzymimmunoassay: Die Beladung der Scopyrol-Mikrotestplatten (MLW, Polyplast Halberstadt) mit 50 µl gereinigtem H5-Antikörper (5 mg/l) in 0,1 M Carbonatpuffer, pH = 9,5) erfolgte für 21 h bei 4 °C. Nach Waschen mit phosphatgepufferter (0,01 M) Natriumchloridlösung (0,15 m/l) pH = 7,2 unter Zusatz von 0,5 g/l Tween 20 (PBS-T) schloß sich eine zweistündige Inkubation der Untersuchungsproben an. Als Verdünnungspuffer diente PBS-T mit 20 g/l Polyethylenglykol 6000 (SERVA) und 50 ml/l Gelafusal (Serumwerk, Bernburg, DDR). Nach zweitem Waschschritt erfolgte eine zweistündige Inkubation mit H5-Antikörper-POD-Konjugat. Die Substrateaktion wurde mit H₂O₂/o-Phenylendiamin durchgeführt. Die Messung der Extinktionen erfolgte bei 492 nm (SUMAL PE2, CARL ZEISS, Jena, DDR).

Proteinbestimmung: Methode nach LOWRY *et al.* (1951).

RNA-Bestimmung: Photometrische Messung bei 260 nm, nach Proteinfällung mit 1 M Perchlorsäure.

Kohlenhydratbestimmung: Phenol-Schwefelsäure-Methode nach DUBOIS *et al.* (1956).

Ergebnisse

Die wesentlichen wachstumskinetischen Parameter der drei Fermentationen sind in Tabelle 1 dargestellt. Sowohl Biotrockenmasseertrag (X) und spezifische Wachstumsrate (μ) als auch maximale Lebendkeimzahl (KbE = Kolonie bildende Einheit) und Teilungsrate (v) erhöhen sich mit zunehmendem Regulationsaufwand der Fermentation. Dabei vergrößern sich Biotrockenmasseertrag und maximale Lebendkeimzahl bei der FBK im Vergleich zur SK um etwa das 20fache. Die entsprechenden Parameter der FDK erhöhen sich um etwa das 30fache (Abb.1 und 2).

Den Abbildungen 2 und 3 kann entnommen werden, daß die logarithmische Wachstumsphase bei der SK nur sehr kurz ist (etwa 2 h). Um etwa das Doppelte länger sind die logarithmischen Phasen bei FBK und FDK. Die Verzögerungsphase ist bei der FDK deutlich verlängert.

Die absoluten Ausbeuten an Polysacchariden (Tabelle 2) nach Phenol-Wasser-Extraktion der Hefetrockenmasse sind größer als die nach Extraktion der Trockenmasse des Kulturüberstandes. Die Ausnahme bildet das Pellet der SK.

Berücksichtigt man die prozentuale Ausbeute, so widerspiegelt sie den deutlich höheren Polysaccharidgehalt des Kulturüberstandes. Vergleicht man alle drei Kulturverfahren untereinander, wird sichtbar, daß sowohl absolut (g/l) als auch relativ (%/l) aus der FBK-Biomasse die größte Antigenmenge extrahiert wurde. Hinsichtlich der Reinheit der Phenol-Wasser-Extrakte gibt es deutliche Unterschiede zwischen Pellet- und Überstandspräparation. Während der Proteingehalt des Pelletextraktes sehr gering und relativ einheitlich bei den drei Kultivierungsarten ist (4,5% bei SK, 2,7% bei FBK, 3,4% bei FDK), gibt es

Tabelle 1
Vergleich der Ergebnisse der Kultivierungsverfahren von *C. albicans*

Kulturverfahren	Biomasse			Zellzahl		
	X (g/l)	μ (1/h)	lg-Phase (h)	max. LKZ (KbE/ml)	v (1/h)	lg-Phase (h)
SK	0,38	0,21	2	$1,2 \times 10^7$	0,56	2
FBK	8,73	0,33	4	$2,7 \times 10^8$	0,86	5
FDK	12,16	0,43	5	$3,8 \times 10^8$	1,39	3

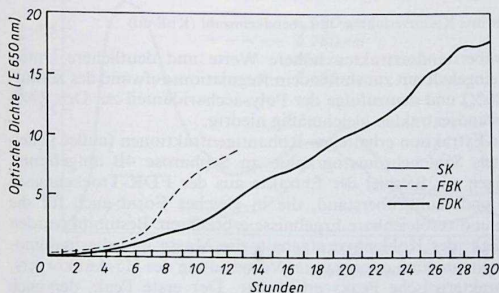


Abb. 1
Vergleich der Wachstumskurven der drei Kulturverfahren: Optische Dichte (E^{650})

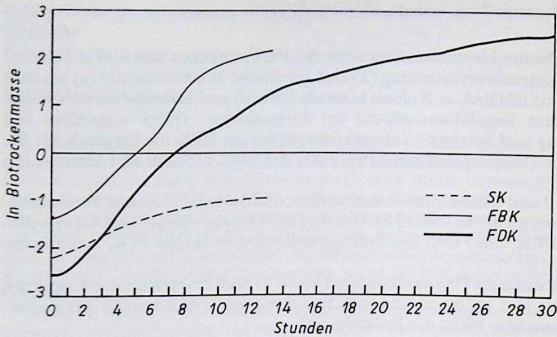


Abb. 2

Vergleich der Wachstumskurven der drei Kulturverfahren: In Biotrockenmasse (g/l)

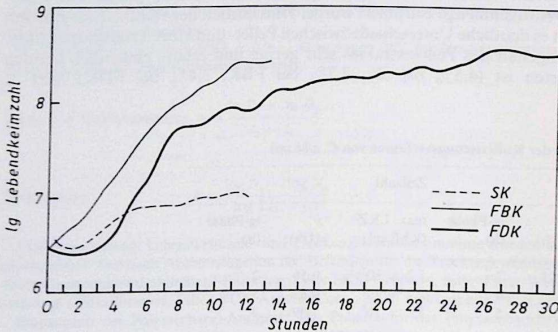


Abb. 3

Vergleich der Wachstumskurven der drei Kulturverfahren: lg Lebendkeimzahl (KbE/ml)

diesbezüglich bei den Kulturüberstandsextrakten höhere Werte und deutlichere Unterschiede. Dabei nimmt der Proteingehalt mit zunehmendem Regulationsaufwand des Kulturverfahrens ab (50%, 37,6%, 26%) und demzufolge der Polysaccharidanteil zu. Der RNS-Gehalt ist bei den Kulturüberstandsextrakten gleichmäßig niedrig.

Die nach der Phenol-Wasser-Extraktion erhaltenen Rohantigenfraktionen (außer Pellet-extrakt der SK) wurden mittels Säulenchromatographie an Sepharose 4B aufgetrennt. Die Abbildungen 4 und 5 zeigen am Beispiel der Extrakte aus der FDK-Trockenmasse die Elutionsprofile für Pellet und Kulturüberstand, die in gleicher Form auch für die anderen Trennungen vorliegen und verbleichbare Ergebnisse erbrachten. Bestimmt wurden in den Eluatn der Proteingehalt, der Kohlenhydratgehalt, die Masse und die immunologische Aktivität mittels Zweiseitenbindungsassay unter Verwendung des H5-Antikörpers. In Abbildung 4 sind drei charakteristische Peaks erkennbar. Der erste Peak, der nach Elution des Ausschußvolumens auftritt, wird einheitlich durch die Massekurve und alle drei Extinktionskurven repräsentiert. Er enthält das immunologisch aktivste Antigen, vermutlich

Tabelle 2

Vergleich der unterschiedlichen Kulturverfahren hinsichtlich der Polysaccharid-Antigen-Ausbeute nach Phenol-Wasser-Extraktion

	SK	FBK	FDK
Ausgangsmaterial für Präparation (g/l Kulturmedium)			
Pellet	0,375	8,725	12,165
Überstand	0,525	0,709	1,443
Ausbeute nach Phenol-Wasser-Extraktion (g/l)			
Pellet	0,005	0,353	0,158
Überstand	0,076	0,200	0,137
Ausbeute bezogen auf Ausgangsmenge (%)			
Pellet	1,3	4,1	1,3
Überstand	14,5	28,2	9,5
Ausbeute an immunol. aktivem Antigen nach Gelfiltration (g/l)			
Pellet	n.b.	0,2	0,059
Überstand	0,012	0,074	0,062
Ausbeute bezogen auf Ausgangsmenge (%)			
Pellet	n.b.	2,3	0,5
Überstand	2,3	10,4	4,3

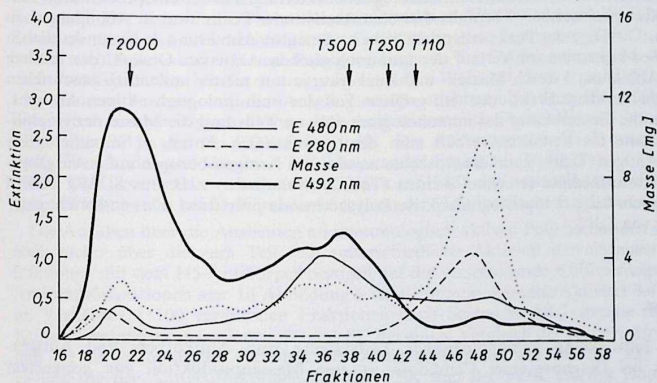


Abb. 4
Sepharose 4B-Trennung: Elutionsprofil des Phenol-Wasser-Extraktes aus dem Fermenter-Dialysekultur-überstand

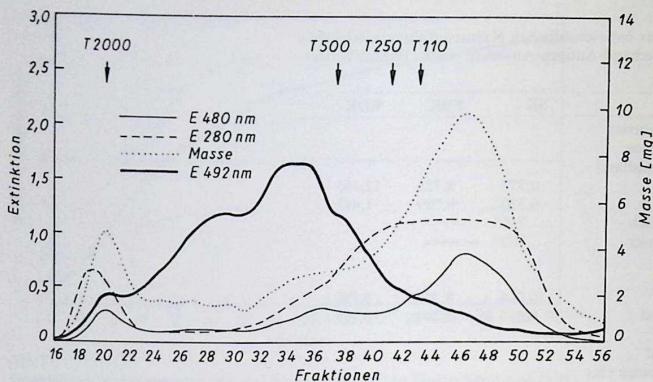


Abb. 5

Sephacrose 4B-Trennung: Elutionsprofil des Phenol-Wasser-Extraktes aus dem Fermenter-Dialysekultur-pellet

hochmolekulare Mannoproteine. Der zweite Peak wird lediglich durch die UV-Kurve nicht erfaßt, was darauf hindeutet, daß es sich hierbei um relativ proteinfreies Polysaccharidantigen mit einem Molekulargewicht von 1,1 Mio D bis 220.000 D handelt. Immunologische Aktivität ist deutlich nachweisbar, wenn auch in geringerem Maße als in den Fraktionen des ersten Peaks. Der dritte Peak stellt sich hauptsächlich in zwei Kurven dar, in der Massekurve und UV-Absorptionskurve. Durch die Kurven die den Zuckeranteil und die immunologische Aktivität beschreiben, wird er nur angedeutet. Dieser Peak, der mit Elution des Totvolumens erscheint, enthält hauptsächlich Protein und in geringem Maße niedermolekulares Polysaccharid. Immunologische Aktivität ist in den entsprechenden Fraktionen kaum nachweisbar. Ebenfalls drei charakteristische Peaks sind in Abbildung 5 zu erkennen. Die Lage der Peaks entspricht nahezu denen in Abbildung 4. Einen deutlichen Unterschied sieht man im Verlauf der immunologischen Aktivität. Obwohl der mittlere Peak in Abbildung 5 durch Massen- und Zuckerkurve nur relativ undeutlich beschrieben wird, enthalten diese Fraktionen den größten Teil des immunologisch aktiven Antigens. Setzt man die Gesamtmasse des immunologisch aktiven Teils und die Masse der lyophilisierten Eluate ins Verhältnis, erhält man den prozentualen Anteil an immunologisch aktivem Antigen. Damit kann die Ausbeute an aktivem Antigen bezogen auf g Ausgangsmenge/l Kulturmedium errechnet werden (Tab. 2). So wurden z. B. aus 8,725 g Pellet/l FBK-Medium 0,2 g immunologisch aktive Polysaccharide präpariert. Das entspricht einer Ausbeute von 2,3%.

Diskussion

Für die Entwicklung immunologischer Testsysteme zum Antikörper- und Antigen-Nachweis bei Diagnose einer Candidose ist eine Massenproduktion von geeignetem Antigen unerlässlich. Insbesondere im Zusammenhang mit der Herstellung monoklonaler Antikörper werden größere Mengen Antigen für das Antikörper-Screening und für die Bestimmung von Sensibilität, Spezifität und Affinität der gescreenten Antikörper benötigt.

Voraussetzung dafür sind effektive Kultivierungsverfahren, geeignete Antigen-Präparationstechniken und Reinigungsverfahren.

Das Hauptantigen der äußeren Zellwand von *C. albicans* und anderer Hefen ist D-Mannan. Dieses Antigen wird bei disseminierter Candidose als erstes aus dem Infektionsherd freigesetzt und kann demzufolge im Serum nachgewiesen werden (KAPPE und MÜLLER 1986). D-Mannan liegt zumeist als Peptidopolysaccharid vor und besteht hauptsächlich aus D-Mannose. Die α -(1-6)-glykosidisch verknüpften Mannosemoleküle bilden das Grundgerüst, das an den O₂-Atomen mit Seitenketten substituiert sein kann, die hauptsächlich α -(1-2)- und in geringerem Maße α -(1-3)-glykosidisch verbundene Mannoseeinheiten enthalten (KOGAN *et al.* 1988). Im Vergleich der drei angewendeten Kulturverfahren erwies sich hinsichtlich der Biomasseausbeute erwartungsgemäß die FDK als das geeignetste. Die Steigerungsrate beträgt in bezug auf die SK 1:32 und in bezug auf die FBK 1:1,4 (Tab. 1). Damit wurden die hohen Steigerungsraten durch die FDK bei Bakterien (STARKE *et al.* 1987, 1989) im Ergebnis dieser Fermentationen nicht erreicht. Auffällig ist, daß die Ausbeuten der FDK nur unwesentlich größer sind als bei der FBK (Faktor 1,4). Das deutet darauf hin, daß die Anhäufung niedermolekularer Stoffwechselprodukte sich bei *C. albicans* in geringerem Maße negativ auf das Wachstum auswirkt, als das bei einigen Bakterienarten der Fall ist (STARKE *et al.* 1987, 1989). Es ist auch anzunehmen, daß einzelne Stoffwechselprodukte, wie z. B. Ethanol nach Abbau der Glucose für eine weitere Energiegewinnung genutzt werden, wie das für *Saccharomyces cerevisiae* beschrieben worden ist (SCHEPER *et al.* 1987). Bei Berücksichtigung des vergleichsweise höheren materiellen Aufwandes der FDK (Dialysekreislauf mit 401 Kulturmedium und künstlicher Niere) stellt sich die Frage nach dem effektiveren Verfahren.

Für die Extraktion der immunologisch aktiven Polysaccharide aus der Zellwand von Hefen sind zahlreiche Methoden bekannt, wie z. B. Pufferextraktion (PEAT *et al.* 1961), Laugeextraktion (ELORZA *et al.* 1988), Formamidextraktion (MEISTER *et al.* 1977), Phenol-Wasser-Extraktion (SAKAGUCHI *et al.* 1967), Extraktion mit Kochsalzlösung (KOGAN *et al.* 1988) und andere. Am häufigsten gebräuchlich sind Puffer- und Laugeextraktion sowie Kombinationen beider Verfahren. In den meisten Fällen erfolgt nach Extraktion eine spezifische Mannanfällung mittels Fehlingscher Lösung bzw. eine chromatographische Reinigung. Die angewandte Phenol-Wasser-Extraktion (WESTPHAL *et al.* 1952) brachte im Zusammenhang mit einer nachfolgenden Gelfiltration gute Ergebnisse (Tab. 2). Die Ausbeuten nach Pelletextraktion und Reinigung liegen in der Größenordnung der Ausbeuten nach Peat-Extraktion bei *Saccharomyces cerevisiae*. Während PEAT *et al.* (1961) 0,5% bis 1% Mannan bezogen auf die Ausgangsmenge erhielten, betragen die Ausbeuten an gereinigtem immunologisch aktiven Polysacchariden nach Phenol-Wasser-Extraktion bei FBK-Pellet 2,3% und bei FDK-Pellet 0,5%. Die Ausbeuten aus der Trockenmasse des FBK-Überstandes und die des FDK-Überstandes liegen mit 10,4% bzw. 4,5% deutlich höher. Dies bestätigt die Annahme von DOUGLAS (1988), daß extrazelluläre Polymere nach längerer Inkubation bei 37 °C relativ leicht von der Hefeoberfläche freigesetzt werden.

Die Angaben über die Ausbeuten an immunologisch aktiven Polysacchariden sagen aber noch nichts über die zum Teil sehr unterschiedliche Aktivität der einzelnen Antigenfraktionen mit dem H5-Antikörper bezogen auf die verschiedenen Kultivierungsarten und Antigenpräparationen aus. In Abbildung 6 ist die immunologische Aktivität der einzelnen im Verhältnis 1:100 verdünnten Fraktionen nach Sepharose-4B-Trennung für die drei Kulturüberstandspräparationen dargestellt. Bei einem Vergleich der Kurven wird sichtbar, daß die immunologische Aktivität der Antigenfraktionen aus der SK am geringsten ist und die der Antigenfraktionen aus der FBK und der FDK um das Doppelte bzw. um den Faktor 10 höher sind. Die in Abbildung 6 dargestellten Kurven zeigen einen nahezu identischen Verlauf, wenn die zu ihrer Ermittlung eingesetzten Antigenmengen in den Verhältnissen 1:100, 1:200 bzw. 1:1000 verdünnt werden.

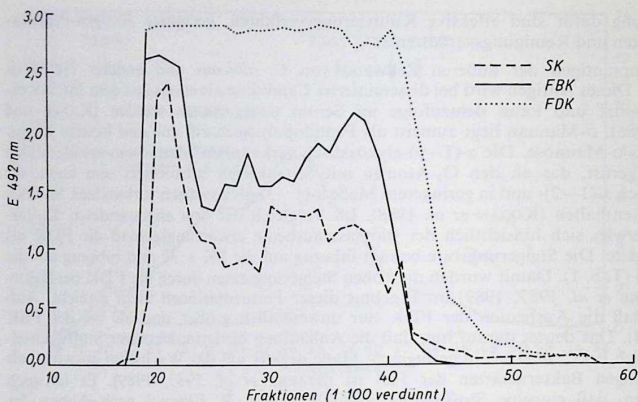


Abb. 6

Sephacrose 4B-Trennung: Vergleich der Immunologischen Aktivität der 1 : 100 verdünnten Antigenfraktionen der drei Kulturüberstandspräparationen

Tabelle 3

Vergleich der unterschiedlichen Kulturverfahren hinsichtlich der immunologischen Aktivität des isolierten und chromatographisch gereinigten Polysaccharid-Antigens

	SK	FBK	FDK
Immunologisch aktives Polysaccharid-Antigen (mg/l)			
Pellet	n.b.	200	59
Überstand	12	74	62
Mittelwert $E^{492} (> 0,5)$			
Pellet	n.b.	2,0	1,0
Überstand	1,2	1,4	1,3
Mittelwert $\mu\text{g Antigen/l Eluat}$			
Pellet	n.b.	108	7,2
Überstand	2,7	3,8	0,7
Quotient $x (E^{492} (> 0,5))$			
$x (\mu\text{g Antigen/l})$			
Pellet	n.b.	0,02	0,14
Überstand	0,44	0,37	1,86

Die immunologische Aktivität der Pelletpräparationen aus FBK und FDK unterscheiden sich etwa um den Faktor 10. Stellt man eine Beziehung zwischen den Mittelwerten der Extinktionen ($>0,5$) aller Antigenfraktionen und den Mittelwerten der entsprechenden eingesetzten Antigenkonzentrationen ($\mu\text{g/l}$) her, können Aussagen über Unterschiede im Gehalt an immunologisch aktiven Antigen determinanten in der Gesamtmasse der einzelnen Eluate gemacht werden. Dabei kommt eindeutig zum Ausdruck, daß das hinsichtlich der immunologischen Aktivität qualitativ beste Antigen aus dem Überstand der FDK isoliert wurde. Das heißt, im Durchschnitt bewirken lediglich $0,7 \mu\text{g}$ Antigen/l eine mittlere Extinktion von 1,3. Für das Erreichen der nahezu gleichen Extinktion (1,2 bzw. 1,4) werden im Durchschnitt $2,7 \mu\text{g}$ Antigen/l aus dem Überstand der SK und $3,8 \mu\text{g}$ Antigen/l aus dem Überstand der FBK benötigt. Der Quotient aus beiden Mittelwerten kann demnach für die Beschreibung der Antigenqualität herangezogen werden (Tab. 3). Das qualitativ schlechteste Antigen entstammt somit dem FBK-Überstand. Das deutet darauf hin, daß sich eine fehlende Dialyse niedermolekularer Stoffwechselprodukte deutlicher auf die Antigenqualität als auf die Quantität auswirkt. Da die höhere Produktivität der FBK gegenüber der SK auch zu einer höheren Konzentration an Abprodukten führt, wäre damit auch eine Erklärung für die bessere Qualität des Antigens aus dem SK-Überstand gegeben. Eine weitere Erklärung resultiert aus der Annahme, daß von Zellen, die sich nicht in der logarithmischen Wachstumsphase befinden das Epitop des H5-Antikörpers verstärkt gebildet wird und der Tatsache, daß die Verzögerungsphase bei der FBK kürzer ist als bei den anderen beiden Verfahren. Ähnliche Aussagen können auch bezüglich der Pelletpräparationen aus FBK und FDK gemacht werden. Vergleicht man die Quotienten der Überstands- und der Pelletpräparationen (Tab. 3) miteinander, zeigt sich ein Qualitätsunterschied etwa um den Faktor 10 zugunsten der aus dem Kulturüberstand isolierten immunologisch aktiven Antigene.

Zusammenfassend können folgende Schlußfolgerungen gezogen werden. Die absoluten Ausbeuten an immunologisch aktiven Polysacchariden sowohl aus Pellet als auch aus Kulturüberstand sind bei der FBK und der FDK deutlich höher als bei der SK. Die größten Ausbeuten liefert die FBK, die aber nur unwesentlich über denen der FDK liegen. Das hinsichtlich seiner immunologischen Aktivität qualitativ beste Antigen entstammt dem FDK-Überstand. Hierbei ist der Unterschied zu den beiden anderen Kulturverfahren, insbesondere zu den Pelletpräparationen klar ersichtlich. Auf Grund dieser Aussagen kann eingeschätzt werden, daß zur Erfüllung unserer Aufgabenstellungen das FDK-Verfahren für die Gewinnung eines qualitativ hochwertigen Antigens besonders geeignet ist und der höhere Arbeits- und Materialaufwand gerechtfertigt werden kann.

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Review

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Cellular role of DNA polymerase I

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Escherichia coli possesses three well-established DNA polymerases, I, II and III. DNA polymerase I (Pol I) is the main repair polymerase in *E. coli* and also has a minor but important role in chromosomal replication. A major advantage of Pol I as an experimental system is its simplicity; unlike other replication enzymes, it is active as a single subunit. To a large extent, mutagenesis appears to be the result of (dis)functions of the DNA replication machinery. It is the purpose of this review to provide an integrated view of this relationship with particular emphasis on the role of Pol I in mutagenic events.

The enzyme and its role in general replication

DNA polymerase I (EC 2.7.7.7.) of *E. coli* (Pol I) is a DNA-binding enzyme involved in the processing of OKAZAKI fragments into high molecular weight DNA and in the repair of damaged duplex DNA. Since the discovery of the Pol I, a great amount of knowledge on its enzymology has accumulated (KORNBERG 1980, 1982). A combination of genetic and biochemical studies has demonstrated that Pol I is a multifunctional, single subunit enzyme possessing three distinct enzymatic activities: i) polymerizing activity; ii) 3'–5' exonuclease, or editing function, and iii) 5'–3' exonuclease function. Mild proteolysis of Pol I produces two enzymatically fully active fragments. The smaller (35,000 daltons), NH₂-terminal fragment contains 5'–3' exonuclease activity. The larger (68,000 daltons) COOH-terminal fragment (the KLENOW fragment) contains both, polymerising and 3'–5' exonuclease activities (BRUTLAG *et al.* 1969, KLENOW and HENNINGSEN 1970). Pol I can promote replication from primed single-strand DNA and gapped duplex DNA. However, a unique feature of Pol I among polymerases is its capacity, unaided by other proteins, to initiate replication at nicks containing a 3' OH terminus. The polymerase domain of the KLENOW fragment catalyzes DNA polymerization in the 5'–3' direction which is dependent on the polarity of the 3' OH primer and the dNTPs. Pol I is a weakly processive enzyme; that is, it does not dissociate from the substrate after each nucleotide addition. Its processivity value averages 15–20 nucleotides, adequate for its physiological roles in DNA synthesis and DNA repair. The 3'–5' exonuclease associated with the KLENOW fragment ensures excision of noncomplementary nucleotides at the 3' end of extended molecules in a rather complex dynamic and topological relation with the polymerizing site (JOYCE *et al.* 1988; reviewed by KUNKEL 1988). Finally, 5'–3' exonuclease degrades DNA and RNA in its path, and its capacity to excise distorted sections of DNA makes it well suited for removing DNA bulky lesions, such as pyrimidine dimers, in repair processes.

The gene for Pol I (*polA*) maps at minute 86 of the *E. coli* K12 map, *PolA* is bordered by an at least 1 kb long blank sequence followed by the ribosomal RNA operon (*rrnA*) on one side, and the *spf* gene for a small RNA on the other (BACHMANN 1983, JOYCE *et al.* 1982). The *polA* gene is constitutively expressed at a rather low level (400 Pol I molecules per cell). Recent results indicate that there may be a

functional relation between the *polA* and *spf* genes or their products (POLAYES *et al.* 1988). Attempts to perform nucleotide sequence analysis of the *polA* gene have been frustrating in the beginning by failures to clone the gene on multicopy plasmids, due to the fact that unregulated expression of the *polA* gene is lethal to *E. coli*. This problem was overcome by cloning the *polA* gene onto a lambda vector (KELLEY *et al.* 1977) which served as a source of material both for nucleotide sequence analysis of the *polA* gene and amino acid sequence characterisation of the DNA polymerase I. Deamination of the primary structure of the *polA* gene revealed that the gene consists of 928 codons starting with an ATG initiation codon downstream from a rather atypical promoter (JOYCE *et al.* 1982). The deduced amino acid sequence was verified by sequence determination of the Pol I enzyme (BROWN *et al.* 1982).

Recently, recombinant DNA approaches have included construction of multicopy plasmid vectors for overproduction of wild-type DNA polymerase I by placing the *polA* gene under the conditional control of the *pl* promoter (MINKLEY *et al.* 1984), gene fusion manipulations for overproduction of the KLENOW fragment (JOYCE and GRINDLEY 1983), and overproduction of the Pol I C-terminal subdomain (FREEMONT *et al.* 1986). *E. coli* clones yielding great quantities of protein in turn opened avenues for the sophisticated crystallographic studies of the KLENOW fragment and its domains. The conformation of the KLENOW fragment shows that this part of the enzyme is folded in two distinct domains with separate functions (OLLIS *et al.* 1985). Several lines of evidence support this notion (reviewed in JOYCE and STEITZ 1987). The larger, C-terminal part of the KLENOW fragment forms a deep cleft whose conformation (OLLIS *et al.* 1985) and overall positive charge (WARWICKER *et al.* 1985) indicate that it might accommodate B-DNA. The close proximity of the primer terminus and dNTP-binding sites has been determined within the cleft by DNAase I foot-printing and affinity-labelling analyses, respectively (JOYCE *et al.* 1986). These findings are nicely complemented by genetic data which show that mutation: *polA5* and *polA6*, which affect the binding of Pol I to DNA, map within the DNA sequence coding for the part of the polypeptide comprising the cleft (JOYCE *et al.* 1985). The subcloned and expressed C-terminal part of the KLENOW fragment exhibits polymerase activity, but no exonuclease activity (FREEMONT *et al.* 1986). On the other hand, binding and competition studies with dNMP's locate a dNMP-binding site within the small, N-terminal domain of the KLENOW fragment (QUE *et al.* 1978, OLLIS *et al.* 1985). This may also mark the position of the phosphodiester bond that is cleaved in the exonuclease proof-reading reaction. Finally, location of the 3'-5' exonuclease site on the small fragment is confirmed by analyses of two mutant proteins lacking this exonuclease activity (DERBYSHIRE *et al.* 1988).

In many *polA* mutants Pol I activities have been analysed without providing a definitive answer as to whether Pol I is essential to cell viability. Difficulties in such studies stem from the leakiness of *polA* mutations and from the multifunctional nature of the enzyme. *PolA* mutants with mutation mapping within the polymerase domain and present in strongly nonsuppressing backgrounds (KELLY and JOYCE 1983) are viable. In contrast, conditional *polA*(Ts) mutations occur within the 5'-3' exonuclease domain (LEHMAN and UYEMURA 1976), indicating the vital role of this latter function. However, these results have been made questionable by recent studies with mutants deleted for the *polA* gene (JOYCE and GRINDLEY 1984). Surprisingly, such mutants grew on minimal medium, but not on rich medium. For growth on rich medium, production of either the KLENOW fragment or a 5'-3' exonuclease fragment of the Pol I was sufficient. Basically, these results indicate the nonessential nature of Pol I, as well as the complicated interplay of the three polymerases under different growth conditions. JOYCE and GRINDLEY (1984) speculate that, under conditions of slow growth, where sequestering of Pol III is not so high at the replication fork, complete lack of PI I may be substituted by Pol III capable of the same enzymatic reactions (KORNBERG 1980). Pol II devoid of 5'-3'

exonuclease functions, would be a less likely candidate, although in theory, it may convert OKAZAKI fragments into high molecular weight DNA in concert with some RNAase, such as RNAase H. That this may be so is supported by additional results indicating the possibility that efficient removal of primers requires enzymes such as RNAase H, in addition to the 5'—3' exonuclease activity of Pol I (OGAWA and OKAZAKI 1980, 1984). Such polymerase replacements under conditions of fast DNA replication would seem unlikely, and as noted the presence of at least one Pol I function is obligatory. It is speculated that during fast growth the 5'—3' exonuclease removes RNA primers, leaving short gaps which are optimal substrate for Pol II (KORNBERG 1980). On the other hand, polymerase activity may displace RNA primers during the synthesis of OKAZAKI fragments, rendering the latter accessible to cellular nucleases. In addition, requirement for at least one Pol I function under fast-growing conditions may also be dictated by the necessity for repair functions of Pol I, due to the presence of DNA-damaging chemicals in rich medium.

Finally, the dispensable nature of Pol I was also shown using ColE-type plasmids, the replication of which is normally dependent on Pol I (KINGSBURY and HELINSKI 1973, STAUDENBAUER 1976). Accordingly, it was demonstrated that initiation of replication in these plasmids by Pol I on the RNA primer, previously precisely processed by RNAase H (Itoh and TOMIZAWA 1980), can be substituted in *polA* mutants by some alternative pathway when RNAase H is absent (KOGOMA 1984).

The complicated scenario of functional relationships among the three *E. coli* DNA polymerases was also demonstrated by MOSES and his group who found that Pol III under certain conditions could be replaced with Pol I. They isolated and described a mutation, *pcbA1*, allowing the by-pass of the conditional lethality of *dnaE*(Ts) strains when the polymerizing function of Pol I was present (BRYAN and MOSES 1984, BRYAN and MOSES 1988, NIWA *et al.* 1981). In a parallel study, they demonstrated the physical presence of DNA polymerase I in the replication holoenzyme complex. They concluded that *E. coli* has two alternate replisome structures: REP-A, in which DNA polymerase I is the functional synthetic subunit; and REP-E, in which the alpha subunit, product of the *dnaE* gene (MCHENRY 1988), is functional (BRYAN *et al.* 1988).

Pol I in recombination

KONRAD was the first to find that recombination of the lactose-fermentation (*lac*) genes on the *E. coli* chromosome was increased in strains lacking polymerizing, 5'—3' exonuclease, or both functions of DNA polymerase I (KONRAD 1977). Quite to the contrary, recombination after transduction or conjugation was found to be much less stimulated in these *polA*-deficient *E. coli* recipients. The *polA107* allele, whose product lacks 5'—3' exonuclease activity (JOYCE *et al.* 1985), exhibited a significant decrease in recombination proficiency (ZIEG *et al.* 1978). Similarly, reduced recombination in *polA* mutants was demonstrated in the lambda inversion assay (ENNIS *et al.* 1987). These contradictory data can, however, be rationalised by a closer examination of the enzymology and mechanics of homologous recombination (reviewed in COX and LEHMAN 1987, SMITH 1987, WEINSTOCK 1987). Namely, "the hyper-Rec" and "hypo-Rec" phenotypes of the *polA* mutants in the given examples possibly reflect different topological requirements imposed on the recombining linear and circular molecules utilizing the RecA RecBCD pathway of recombination. More precisely, the hyper-Rec phenotype, manifested in the Lac duplication system, may be the result of the by-pass of a rate-limiting step in this mode of recombination due to the lack of DNA ends. Namely, nicks and gaps accumulated in the *polA*-deficient strains, as well as double-strand breaks produced by accidental endonucleases where DNA ends are abundant (transduction and conjugation), the rate-limiting step may be in the efficient closure of gaps remaining after the exchange of DNA strands.

The lack of Pol I which fills these gaps may decrease the overall recombination efficiency.

In contrast to the case of the RecBCD pathway, the enzymology of an alternative homologous recombination mode, namely the RecE and RecF pathway, is rather unknown. The possible requirement for DNA polymerase I in these pathways remains to be resolved. A recent study of the *recF*-dependent pathway in the postreplication repair of daughter-strand gaps indicates that Pol I may not play a substantial role in this recombination pathway (SHARMA and SMITH 1987; see Pol I in Excision and Postreplication Repair).

Current thought on transpositions via cointegrate formation is that the entire transposable element is replicated in the course of transposition (SHAPIRO 1980). A transposition-defective mutant, simultaneously deficient in both polymerase and 5'—3' exonuclease activities of Pol I, has been isolated (SYVANEN *et al.* 1982). However, a subclass of intragenic Pol I-proficient revertants do not regain transposition ability, indicating a novel aspect of Pol I in transposition. In addition, same revertants do not regain the ability to support the growth of P1 bacteriophage. This observation indicates that transposition and P1 replication share some common functional aspect of the enzyme. The possible involvement of Pol I in transpositions via simple insertions has, to our knowledge, not been studied.

Pol I and mismatch repair

Mismatch correction embodies a set of enzymatic reactions which ensure recognition and repair of mismatch base pairs. Such mismatches can arise during DNA replication, during recombination of homologous, but nonidentical DNA stretches, or from the deamination of 5-methylcytosine which create T-G mispairing (reviewed by CLAVERYS and LACKS 1986, GLICKMAN 1982, RADMAN and WAGNER 1986, MODRICH 1987). Genetic studies have shown that this excision repair-like process depends on the products of the *dam*, *mutH*, *mutL*, *mutS*, and *uvrD* genes, but does not depend on Pol I. Correction starts with the recognition of the mismatch base pair by the MutS protein and the endonucleolytic incision by the MutH protein at the 5' side of the sequence complementary to the methylated d(GATC) site. Thus, repair is strongly biased towards the unmethylated strand, ensuring removal of base misincorporated during replication. The original incision by the MutH protein is followed by extensive unilateral degradation of the affected DNA strand, with an average excision tract length of approximately 3 kb (WAGNER and MESELSON 1976). This large tract size may possibly reflect the natural spacing of the GA*TC identifying signals spaced along the *E. coli* chromosome. As mentioned above, this methyl-directed correction is independent of DNA polymerase I. Quite possibly, repair synthesis of the created gaps (LU *et al.* 1984) is performed by DNA polymerase III (AU *et al.*, unpublished results). Taking into account both the size of gaps to be filled and the high processivity value of DNA polymerase III (McHENRY 1988), this choice seems quite logical. It might be of interest to study the enzymology of resynthesis at the *E. coli* *oriC* region as this sequence contains 11 GATC sites within a 245 bp stretch (OKA *et al.* 1980).

It should be mentioned in this context that *dam* mutants are inviable in the presence of *recA*, *recB*, *recC*, *lexA* and *polA* mutations (MARINUS and MORRIS 1974, 1975). Such defects can be suppressed by additional mutations affecting mismatch correction, in the *mutH*, *mutL*, or *mutS* genes (GLICKMAN and RADMAN 1980, MCGRAW and MARINUS 1980). Such mutants accumulate double-strand breaks as the result of disorganized mismatch repair, i.e. as the result of the formation of overlapping repair tracts or a single repair tract coupled with a simple cut on the opposite DNA strands (GLICKMAN

et al. 1978, GLICKMAN and RADMAN 1980). The absence of repair of such breaks in the *recA*, *recB(C)*, *lexA* and *polA*-deficient strains is evidence for the involvement of the products of these genes in repair of double-strand breaks (KRASIN and HUTCHINSON 1977, SHARMA and SMITH 1987, WANG and SMITH 1986; see also Pol I in Excision and Postreplication Repair).

Mismatch correction can also work on nonreplicating DNA. This type of repair operates in a very specific, error free mode. Its basic function is to eliminate thymine from a T-G mispair produced by deamination of 5-methylcytosine. In contrast to methyl-directed mismatch correction, this repair involves very short excision tracts never exceeding five nucleotides in length (LIEB 1985, LIEB *et al.* 1986). The enzymology of this type of mismatch correction has not been studied in detail. However, considering the given criteria (size of the gap and polymerase processivity), one could single out Pol I as the first choice polymerase for this task. Recombination studies with such sequences result in a distorted marker distribution among recombinants. Such observations when mapping *cI* lambda mutations has led LIEB to the discovery of very short mismatch repair at CCA/GGT sequences (LIEB 1983). In fact, the whole concept of mismatch correction was born as an explanation to certain unorthodox marker effects associated with crossing over (HOLLIDAY 1964). Mismatches occur during recombination whenever a heteroduplex recombinational bridge encompasses sites of genetic differences between the partner molecules. In contrast to methyl-directed correction and short mismatch repair, this type of correction operates on a democratic basis removing bases from mismatches with equal opportunity. Repair degradation in this case can be rather extensive provoking cocorrection of the nearby genetic markers (AMATI and MESELSON 1965). It is currently thought that repair synthesis of these tracts is the same as during formation of recombinational bridges in DNA stretches of absolute homology, i.e. that it is performed by DNA polymerase I. However, having in mind the possible size of some gaps, one may speculate that DNA polymerase II and DNA polymerase III could also participate, particularly at low intracellular concentration of DNA polymerase I.

Pol I in nucleotide excision and postreplication repair

A large class of chemical and physical agents (exemplified by UV radiation) produce premutagenic alterations in DNA in the form of bulky or helix-distorting lesions. Such lesions call into action a set of strategically different repair system (GANESAN *et al.* 1982, GROSSMAN 1981, HANAWALT *et al.* 1979, KUSHNER 1987, LINDAHL 1982, WALKER *et al.* 1985). In *E. coli*, excision repair of UV-induced pyrimidine dimers and bulky lesions is initiated by a protein complex encoded by *uvrA*, *uvrB*, and *uvrC* genes. Classical models of excision repair had suggested that the incision step by the UvrABC protein complex introduces a *nick* 5' to a lesion. In the next step of this model, DNA polymerase I binds at the formed primer, synthesizes new DNA, simultaneously degrading it with its 5'–3' exonuclease lesion-containing DNA strands (COOPER and HANAWALT 1972a). Repair is completed with dissociation of DNA polymerase I and the ligation of the translated *nick* (GROSSMAN 1981, HANAWALT *et al.* 1979). Thus, both polymerase and 5'–3' exonuclease functions of the Pol I are needed for the efficient completion of the excision repair. However, later studies have thrown a completely different light on the enzymology of the excision repair (reviewed by VAN HOUTEN 1990). First, it was shown that the incision reaction by the UvrABC complex results in endonucleolytic breaks on each side of the lesion (SANCAR and RUPP 1983). Second, Pol I together with UvrABC protein and helicase II (*uvrD* gene product), forms a "repairsome" complex. The repairsome subunits seem to be mutually dependent in monitoring all preligation steps, i.e. bimodal incision (UvrABC), release of oligonucleotide with a lesion (UvrABC with

assistance of helicase II and Pol I), demasking of the 3' OH primer from the UvrABC protein (helicase II), and subsequent polymerisation by Pol I (CARON *et al.* 1985). Therefore, removal of the damaged fragment seems to occur *en bloc* and is not dependent on the nick-translating activity of Pol I; the polymerase only fills the short gap to create a ligatable substrate. Therefore, the role of 5'—3' exonuclease of Pol I, which is essential for excision repair (KONRAD and LEHMAN 1974), seems to be restricted to the terminal stage of the gap filling. More precisely, the exonuclease appears to help efficient closure of gaps to nicks, and may also prevent strand displacement before ligation can occur (WAHL *et al.* 1983).

The overwhelming majority (99%) of repair stretches in excision repair do not exceed 30 nucleotides in length. This indicates that Pol I fills gaps with very little nick translation or exonucleolytic expansion of the gap. In contrast to this "short patch" repair, a much less represented "long patch" type of repair (1%) creates patches at least 1,500 nucleotides in length (COOPER 1982, COOPER and HANAWALT 1972b). Short path repair is constitutive, while long patch repair is inducible and is under control of the SOS regulatory circuit (COOPER and HANAWALT 1972a; reviewed in WALKER 1985, YOUNGS *et al.* 1974). The *raison d'être* of long patch repair is still unknown and is the idea that it occurs at sites where bulky lesions occur in close proximity on opposite DNA strands (BRESLER 1975, SEDGWICK 1976) has been ruled out (COOPER 1982).

Early analyses of DNA polymerase I mutants have shown that these mutants execute their excision repair via long patch repair. This observation suggests that DNA polymerase II and III mediate this type of repair (COOPER and HANAWALT 1972, MASKER *et al.* 1973, TAIT *et al.* 1973, YOUNGS and SMITH 1973). However, a more recent precise study of short- and long-patch distribution in different DNA polymerase mutants has demonstrated that even in wild-type cells DNA polymerase I is responsible for both the short-patch and the long-patch synthesis (COOPER 1982). It has also been deduced from the same results that polymerase II and III are able to substitute for polymerase I in excision repair and probably do so at low frequency in wild-type cells.

Bulky lesions or helix distorting lesions that are not removed by either photoenzymatic repair or excision repair as in the excision defective Uvr⁻ mutants in the absence of light) block the procession of the replication machinery. The replication complex is pictured as moving to the next downstream OKAZAKI fragment origin, creating a gap opposite the lesion. Due to the lack of the proper template this structure can not be dealt with any more by the excision repair. However, such structures can be repaired by the "postreplication repair" process that involves recombination functions (RUPP and HOWARD-FLANDERS 1968, RUPP *et al.* 1971). Recent studies indicate the existence of three *recA*-dependent pathways of postreplication repair (ROTHMAN *et al.* 1975, WANG and SMITH 1981): i) a repair of daughter strand gaps (DSG), with *recF*-dependent (GANESAN and SEAWELL 1975, KATO 1977, ROTHMAN and CLARK 1977, WANG and SMITH 1983), and *recF*-independent (SHARMA and SMITH 1985, WANG and SMITH 1983) branches, and ii) a *recB*-dependent repair of double-strand breaks (DSBs) which may occur by endonucleolytic nicks at unrepaired DSGs (WANG and SMITH 1983).

It has been believed for several years that Pol I is not involved in postreplication repair. This belief was based on experimental data showing that a *uvrA polA1* double mutant exhibits only a slightly higher sensitivity to UV irradiation than a *uvrA* strain (MONK *et al.*, 1971), as well as on observations that *E. coli* mutants deficient for Pol I efficiently complete the repair of DSG (SEDGWICK and BRIDGES 1974, TAIT *et al.* 1974). That this is not so, and that Pol I plays an important role in the postreplication repair of both DSG and DSB, was recently demonstrated. Using strains deleted in the *polA* gene in different combinations with *uvrA*, *recB*, and *recF* mutations, SHARMA and SMITH (1989) reinvestigated the role of Pol I in the postreplication repair. They found, contrary to previous observations, that Pol I plays a major role in the *recF*-independent pathway of

DSG repair, as well as in DSB repair. Furthermore, similar results were obtained with the *polA546* (Ts) mutant defective in 5'—3' exonuclease activity. This demonstrated that the 5'—3' exonuclease activity of Pol I is crucial for carrying out these two branches of postreplication repair. Thus, according to these results, previous misinterpretations stem from the fact that earlier workers (BARKNECHT and SMITH 1978, JOHNSON 1978, SEDGWICK and BRIDGES 1974, TAIT *et al.* 1974) have always used the *polA1* mutation. This mutation brings polymerase I activity down to a 1% level, but leaves 5'—3' exonuclease activity practically intact (KORNBERG 1980). However, the proposed model for DSG repair (SHARMA and SMITH 1987) requires polymerase function of Pol I in filling the gap of the Holliday junction. This is not so with the model for DSB in which the 5—3 exonuclease activity of Pol I in eliminating the lesion by *nick*-translation is required.

Finally, a previous study (BARKNECHT and SMITH 1978), and this study (SHARMA and SMITH 1987), eliminate any substantial role of Pol I in the minor, chloramphenicol-inhibitable pathway of postreplication repair (SEDGWICK 1975, YOUNGS and SMITH 1976).

Pol I in SOS processing

Cessation of DNA replication caused by treatment of *E. coli* cells with a variety of DNA damaging agents triggers a delayed reaction. This cascade of molecular events results not only in a higher cell survival but also in a dramatically increased mutation rate. This "error — prone repair", or "SOS response" (RADMAN 1974, WALKER 1984, 1985, OSANNA *et al.* 1986), is controlled out by a plethora of genes (at least 20 genes). Some of these genes also take part in error-free repair systems such as in methyl-directed mismatch repair, excision repair, and postreplication repair. The products of the *recA* and *lexA* genes are apparently the central regulatory molecules of the SOS system. The LexA protein keeps the whole set of emergency genes at a more all less low expression rate. The distress signal(s) of still unknown nature (possibly long stretches of ssDNA) activate(s) RecA protein. The activated RecA protein in turn either cleaves LexA or stimulates the autocleavage of LexA, thus inactivating its repressing function.

Currently, the central question related to the error-prones of SOS repair is the question of the ability to replicate past noninformational lesions. The existence of an error-prone DNA polymerase capable of replicating opposite the incorrect templates had been postulated some time ago (RADMAN 1974, CAILLET-FAUQUET *et al.* 1977). The idea that SOS-induced mutagenesis must involve the inducible inhibition of the 3'—5' proof-reading exonuclease function of DNA polymerase was supported by findings that pyrimidine dimers and other DNA damages efficiently block DNA elongation (BOITEUX *et al.* 1978, BOITEUX and LAVAL 1982, MOORE *et al.* 1981, 1982, SCHAAPER *et al.* 1983, STRAUSS *et al.* 1982). Today Pol III is generally thought to execute this mutagenic function. Namely, a number of convincing data have been accumulated indicating that RecA and a complex of UmuDC proteins inhibit the proof-reading function of Pol III, enabling it to misincorporate DNA basis and promoting reinitiation of chain elongation using the misincorporated base as a priming end (WALKER 1984, 1985, HAGENSEE 1987, BOCKRATH *et al.* 1987, BOCKRATH 1989).

These data and results which demonstrate that the mutation-prone *umuDC*-dependent pathway is normal in SOS-induced *polA* mutants (KONDO *et al.* 1970, BATES *et al.* 1989), minimize the possible role of Pol I in this type of repair. However, the proof-reading 3'—5' exonuclease may not be the only factor involved in DNA repair synthesis past the lesion. For instance, addition of Mn^{2+} facilitates misincorporation opposite the lesion, not by inhibition of editing function, but through decreased discrimination for dNTPs of the polymerase function of the KLENOW fragment (reviewed in WALKER 1985). In this respect,

results presented by LINN and his group may be of interest for reconsidering a possible role of Pol I in the SOS-induced mutagenesis. They reported the isolation of an altered form of DNA polymerase I (DNA polymerase*) in *E. coli* induced for SOS functions. Pol I* is coded by *polA*, and is composed of Pol I associated with still unidentified subunits called “*-factors”. This altered polymerase exhibits 10–100-fold higher misincorporation during replication of synthetic templates and reduced ability to discriminate against the adenine analogue 2-aminopurine. At the same time, both the 3'–5' (editing) and 5'–3' (nick-translational) exonuclease activities of Pol I and Pol I* are the same (LACKEY *et al.* 1982, 1985). The most recent results demonstrate that Pol I* is a shortened version of Pol I (Pol I stump) which seems to be made from an in-phase internal promoter activated immediately after SOS induction. Examination of the NH₂-proximal part of the *polA* gene region indeed reveals a promoter sequence homologous to the heat shock promoter consensus. This sequence overlaps with a sequence homologous to the *lexA* repressor consensus binding sequence. Finally, only Pol I stump but not normal Pol I can bind the * factor(s) to form the mutagenic Pol I* (MARTIN-MOE and LINN, submitted for publication). Although the appearance of Pol I* is strictly correlated with the induction of SOS functions, there is for the moment no direct evidence implicating it in the SOS processing. As mentioned before, Pol I is required for long-patch repair, another induced function controlled by SOS regulatory circuits (COOPER 1982). One may speculate that pol I* may be responsible for some *unuDC*-independent mutagenesis during long-patch repair synthesis since there is evidence that a portion of the induced mutagenesis occurs in conjunction with excision repair (BRIDGES and MOTTERHEAD 1971, NISHIOKA and DOUDNEY 1970). Whether pol I* has any role in repair synthesis opposite lesions, or mutagenic resynthesis during inducible long-patch repair, remains to be determined.

Spontaneous mutagenesis in Pol I-deficient *E. coli*

COUKELL and YANOFSKY (1970) were the first to demonstrate that *polA*⁻ increases the frequency of deletions in the *trp-tonB* region of *E. coli*. Following this initial study, SIEGEL and VACCARO (1978) and VACCARO and SIEGEL (1975) demonstrated the mutator property of *E. coli* defective in the 5–3 exonuclease and polymerase activities of Pol I; such mutators affected exclusively the reversion rate of plus frameshift mutations. A more recent study, in which *polA1* mutator quality was monitored in the histidine region of *E. coli* by forward mutagenesis, confirmed previous findings about *polA1* propensity to create minus frameshifts and deletions (SAVIC and ROMAC 1982). In contrast to results of SIEGEL and VACCARO (1978), which showed a rather modest mutator property of *polA*⁻ alleles (mutation rate, 5–10 times above the spontaneous background), histidine auxotrophs were detected at a frequency of approximately 10⁻³ in the population, or 10⁻⁴ when a *hisC* frameshift hotspot was subtracted. SAVIC and ROMAC (1982) postulated that the modest mutation frequencies in reversion tests could mean that the used mutations mapped within nucleotide stretches not highly prone to the *polA*-mediated type of slippage.

That this may be true was demonstrated in the most recent sequence analysis of histidine mutations isolated in the *polA1* strain of *E. coli* (JANKOVIC *et al.* 1990). As predicted (SAVIC *et al.* 1988), sequence analyses of frameshift mutations at the *hisC* hotspot revealed that all mutations fall into an extremely monotonous sequence consisting of four consecutive 5'-GTGG-3' quadruplets. All mutations at this site arose by the excision of one of the GTGG quadruplets. Interestingly, none of tested mutations occurred by the excision of two quadruplets. Sequence analysis of the *hisD* deletion showed that a mutational event leading

to a 18 bp-long deletion occurred as a consequence of misalignment of a tandemly repeated pentamer. Similarly, 26 bp-long *hisA* deletions occurred by mispairing of two tandemly repeated decamers. The results presented were fully consistent with the classical proposal that frameshifts and smaller deletions are produced by a mechanism involving misaligned pairing of repeated sequences (STREISINGER *et al.* 1966). It was concluded that the tendency of slippage at these sites is elevated in *polA1* strains because these strains slowly seal OKAZAKI fragments and other single-stranded breaks (JANKOVIC *et al.* 1990).

The most recent study of GLICKMAN and his group with the F'/*lacI* system of *E. coli* provided additional evidence of the mutator property of the *polA1* mutation (reviewed in GLICKMAN *et al.* 1986, FIX *et al.* 1987). Surprisingly, the overall mutation frequency in the *polA1* strain was only 2.3-fold greater, although forward, *lacI*⁻ spontaneous mutations had been scored. In addition, sequence analyses of mutated sites demonstrated quite different mutational spectra than in earlier studies. Besides minus frameshifts and deletions, they also included base substitutions, duplications and some plus frameshifts. Taking into account the very low mutation frequency at F'*lacI*, one may think that the *lacI* mutational profile may have been contaminated by i) spontaneous mutational events unrelated to the Pol I deficiency, and ii) mutational events related to the induction of the SOS system, which may be induced in *polA*⁻ cells (BLANCO and POMES 1977). That this may be possible is indicated by the analysis of *lacI* base substitution mutations (FIX *et al.* 1987) which closely resemble the pattern of *lacI* base substitution mutations found in the *mutD*⁻ (*dnaQ*⁻) strains defective in the proofreading subunit of DNA polymerase III holoenzyme (FOWLER *et al.* 1986). Thus, one can think of an SOS-induced decrease in the proof-reading stringency of DNA polymerase III as a physiological equivalent to the behaviour of *mutD*⁻ (*dnaQ*⁻) strains.

The described incongruities between plasmid and chromosomal systems may also be explained by a difference in DNA replication (and repair) enzymology of these replicons. Namely, detailed functional relations between DNA polymerases may not be the same in the chromosome and in the F plasmid, particularly in situations involving mutual replacement of polymerases in *polA*⁻ strains (JOYCE and GRINDLEY 1984). These differences could ultimately be manifested in different yields and types of mutations. Finally, poor *in trans* supply of the mutagenic 5'-3' exonuclease (BATES *et al.* 1989) can also contribute to the low mutation frequency in the F *lacI* system.

Common to many systems is the use of genetic targets carried on extrachromosomal elements that allow isolation and characterisation of large number of mutant alleles with relative ease. This study shows that these data might not be representative of results with chromosomal targets. Although being rather difficult and less powerful in screening large numbers of mutations, chromosomal systems offer valuable information for attaining a complete picture of mutational events. As suggested (EISENSTADT 1987), information about mutational spectra and mutational yields should be gathered from a variety of systems carrying the same genetic targets. Thus, the described differences could be clarified by studying *polA1*-induced mutagenesis on a F' *his* plasmid and in the chromosomal *lacI* gene.

The most interesting aspect of the analysis of the F' *lac* system was that a 5'-GTGG-3' sequence and its inverted complement 5'-CCAC-3' had been located adjacent to 67% of non-hotspot frameshifts (11 minus frameshifts and only one plus frameshift) and 63% of deletions, indicating an important role of the DNA context in this type of mutagenesis (FIX *et al.* 1987). One may suppose that the given percentages would be even higher provided mutational spectra were indeed hidden by *polA*-unrelated mutational events. GLICKMAN and collaborators speculate that their localisation need not be coincidental, and that this sequence may represent sites where DNA polymerase I plays an important role in DNA replication and/or DNA repair. Similarly to the frequent occurrence of frameshifts adjacent to a 5'-GATC-3' sequence in wild-type strains (SCHAAPER *et al.* 1986), a

sequence at which first incision is produced in mismatch correction, the prevalence of frameshifts and deletions at the sequence 5'-GTGG-3' in the *polA1* strains, may be explained by strand discontinuities that arise at these sites. Evidence for such breaks is nonexistent in the literature, although, interestingly enough, the 5'-GTGG-3' sequence is contained in a number of DNA stretches related to replication, recombination, and regulation of DNA helicity (compiled in FIX *et al.* 1987). In *mutD5* mutants of *E. coli*, particularly when cells are grown in minimal medium, an excess of A-T > T-A transversions in the *lacI* gene arise at the GTGG sites (SCHAAPER 1988). The incidence of the GTGG sequence in such a number of activities requiring specific DNA sequence recognition with the possibility for the creation of the strand breaks gives credence to the suggestion that this specific sequence is a site where DNA polymerase I may perform an important antimitagenic function in DNA replication. In the *PolA*-strain, the sealing of Okazaki fragments is approximately 10 times slower than in the wild-type (KORNBERG 1980). Hence, it is possible that the frequent recovery of frameshift events in the neighbourhood of the GTGG sequence reflects the role that this sequence may play in the replication of DNA. More precisely, the GTGG sequences may represent the site of the termination of OKAZAKI fragments during normal DNA synthesis, and the enhanced frequency with which frameshift events are recovered at these sequences may reflect a delay in strand closure at these sites in a *PolA*⁻ strain. This hypothesis has been put forward before (GLICKMAN *et al.* 1986, FIX *et al.* 1987), and a potential role for OKAZAKI fragments in the origin of frameshifts has also been suggested earlier (RIPLEY and SCHOOMAKER 1983, RIPLEY and GLICKMAN 1984). That GTGG sequence-promoted minus frameshift and deletion mutagenesis is not *lacI*⁻ or plasmid-specific is supported by the most recent sequence analysis of histidine mutations isolated in *polA1* strain of *E. coli* (JANKOVIC *et al.* 1990). As mentioned, histidine mutations were produced by a mechanism involving misaligned pairing of repeated sequences. However, closer inspection of nucleotide sequences at the deletion hotspot in the *hisA* gene, revealed a 5'-GTGG-3' sequence at zero distance from the deletion termini. Similarly, an inverted complement of GTGG (5'-CCAC-3') was found four bases away from the *hisD* deletion. The fact that the 1300 bp-long *hisD* and 720 bp-long *hisA* genes contain only six 5'-CCAC-3' and ten 5'-GTGG-3' sequences, respectively (CARLOMAGNO *et al.* 1988), strongly argues against coincidental positioning of these quadruplets to all the deletion termini. In addition, further inspection of the mutated sites reveals that 5'-CCAC-3' and 5'-GTGG-3' sequences are in both cases practically at the same distance (17 and 16 bases, respectively) in the direction of replication from the nucleotide stretches which template the slippage. This suggests that DNA polymerase III travelling along the lagging strand of the chromosome might sense at this distance a stop signal consisting of the GTGG sequences very possibly embedded in some broader nucleotide context. Such signal would promote polymerase dissociation from the template which in the absence of Pol I would create a situation for the misalignment of the neighbouring homologous sequences. Involvement of both 5'-GTGG-3' or its inverted complement in terminating OKAZAKI fragments is difficult to explain from the steric point of view and remains for the moment obscure. However, one can imagine that *E. coli* has evolved a flexible system for terminating OKAZAKI fragments when replication is advancing from alternative origins and/or for dealing with inverted chromosomal segments.

Interestingly, a direct complement of the 5'-GTGG-3' sequence (5'-CACC-3') is found 16 bp away from the last quadruplet which can template the slippage at the -4 frameshift hotspot in the *hisC* gene (JANKOVIC *et al.* 1990). Given the above figures, the 16 bp distance of 5'-CACC-3' sequence (3 found in the 1100 bp-long *hisC* gene; CARLOMAGNO *et al.* 1988) from the last GTGG quadruplet which can template the slippage at the *hisC* hotspot is also suspect in assisting in deletion formation at this site. Finally, the CACC quadruplet is found in the closest proximity to widely known histidine mutations *hisD3052* (-C) and *hisD3018* (+C) in *Salmonella typhimurium*.

Both mutations are induced in monotonous stretches of cytosines by ICR (HARTMAN *et al.* 1986), a mutagen which may act by stabilising mistemplated structures.

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